UPDATE ON SEX STEROIDS AND BONE DEVELOPMENT IN HEALTHY AND OBESE BOYS

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Op de kaft van dit proefschrift staan er radiografische foto's van linkerhanden die gebruikt worden om de botleeftijd bij kinderen te bepalen. Dit biedt alvast een voorsmaakje van de inhoud van mijn doctoraat.

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Sara
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LIST OF ABBREVIATIONS

3β-HSD	3β-hydroxy-steroid dehydrogenase
17β-HSD	17β-hydroxy-steroid dehydrogenase
A	Androstenedione
aBMD	areal bone mineral density
ACTH	adrenocorticotropic hormone
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
AR	androgen receptor
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
CSA	cross-sectional area
CV	coefficient of variation
DHEA	Dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DHT	Dihydrotestosterone
DXA	dual-energy X-ray absorptiometry
E1	Estrone
E2	Estradiol
ECLIA	chemiluminescence immunoassay
EIA	enzyme immunoassay
ESI	electrospray ionization
ER	estrogen receptor
FE2	free estradiol
FSH	follicle-stimulating hormone
FT	free testosterone
GH	growth hormone
GnRH	gonadotropin-releasing hormone
IGF-1	insulin-like growth factor 1
IVA	instant vertebral assessment
LC-MS-MS	liquid chromatography–tandem mass spectrometry

LH	luteinizing hormone
LOQ	limit of quantification
M1LH	multiple one-legged hopping
pQCT	peripheral quantitative computed tomography
PSA	prostate-specific antigen
RIA	radio-immunoassay
S2LJ	single two-legged jump
SDS	standard deviation score
SHBG	sex-hormone binding globulin
SSIp	strength-strain index
T	Testosterone
TT	total testosterone
vBMD	volumetric bone mineral density

SAMENVATTING

Geslachtshormonen spelen een essentiële rol in de pubertaire ontwikkeling, de botmaturatie, en het verwerven van de piekbotmassa bij opgroeiende kinderen. Verder bepalen zij ook in belangrijke mate de lichaamssamenstelling. Hoewel testosteron (T) steeds naar voren wordt geschoven als het primordiaal geslachtshormoon bij de man, hebben recente ontdekkingen bij mannen met zeldzame syndromen zoals oestrogeenresistentie en aromatasedeficiëntie en onderzoek bij muizen zonder oestrogeenreceptor of aromatase-enzyme het belang van oestrogenen bij de volwassen man aangetoond. Er zijn echter weinig data rond de effecten van oestrogenen op de botontwikkeling, botmaturatie en lichaamssamenstelling bij opgroeiende jongens. Daarenboven werden de meeste studies uitgevoerd met onvoldoende geschikte technieken om deze associaties op een betrouwbare manier te bestuderen. De meeste studies gebruikten immers klassieke immunoassays om concentraties aan geslachtshormonen te bepalen. Deze assays zijn echter onvoldoende nauwkeurig om de lage concentraties in de kinderjaren te meten. Verder gebruikte men meestal de dual-energy x-ray absorptiometry (DXA) techniek om de botmineraaldichtheid te evalueren en de botgrootte te bepalen. DXA heeft als belangrijkste beperking dat de gemeten botmineraaloppervlaktedichtheid of 'areal bone mineral density' (aBMD) sterk afhankelijk is van de botgrootte. Verder levert deze techniek geen informatie over de botgeometrie.

In het eerste deel van ons onderzoek worden de verschillende determinanten van botontwikkeling bij gezonde prepubertaire en pubertaire jongens bestudeerd. Er wordt hierbij specifiek gekeken naar de associaties tussen de geslachtshormonen (adrenale en gonadale geslachtshormonen) en de botmineraaldichtheid (aBMD en volumetrische botmineraaldichtheid (vBMD)), de botgeometrie en de botmaturatie. In ons onderzoek wordt er naast DXA, ook gebruik gemaakt van de peripheral quantitative computed tomography (pQCT) om verschillende botparameters van botgrootte en botmineraaldichtheid te evalueren. Bovendien wordt er gebruik gemaakt van de meer recente techniek van massaspectroscopie om de lage concentraties geslachtshormonen accuraat te bepalen bij deze kinderen. In het tweede deel van ons onderzoek worden de effecten van hoge

oestrogeenspiegels (estradiol (E2) en estrone (E1)) op de botmaturatie, vBMD en botgeometrie bestudeerd. Hiervoor recruteerden we een groep obese adolescente jongens, waarbij we, -op basis van de literatuur bij volwassen mannen-, ook hogere oestrogeenspiegels verwachten door een toegenomen aromatisatie in vetweefsel. De data rond geslachtshormonen bij obese kinderen en adolescenten zijn zeer beperkt en voorts tegenstrijdig. Als eerste werden de pubertaire ontwikkeling en concentraties geslachtshormonen van de obese jongens vergeleken met gezonde controles met een normaal gewicht. Ten tweede werden de determinanten van vBMD, botgrootte en botmaturatie bestudeerd met specifieke aandacht voor de associaties met geslachtshormonen en spiermassa in deze specifieke groep van obese adolescenten met langdurig bestaande obesitas, welke een residentieel vermageringsprogramma gingen aanvatten.

In hoofdstuk 1 wordt een algemene achtergrond voor dit onderzoek geschetst en wordt de wetenschappelijke literatuur rond de seksuele en skeletale ontwikkeling bij gezonde en obese jongens besproken. Verder worden de belangrijkste determinanten voor de botontwikkeling bij kinderen toegelicht. Vervolgens worden de specifieke doelstellingen van dit werk geformuleerd namelijk de studie van de relatieve invloed van androgenen en oestrogenen op botmineraaldichtheid, botgrootte en botmaturatie bij gezonde en obese jongens. Tot slot, bespreken we de studiepopulaties en de materialen en methoden met specifieke aandacht voor de massaspectroscopie en pQCT techniek.

In hoofdstuk 2, rapporteren we de resultaten van onze studies rond de associatie tussen geslachtshormonen (adrenale en gonadale steroïden) en (v)BMD, botgrootte, botmaturatie en lichaamssamenstelling bij gezonde kinderen en adolescenten. Zoals weergegeven in hoofdstuk 2.1, vinden we geen relatie tussen de adrenale steroïden (dehydroepiandrosteronsulfaat (DHEAS), androstenedione (A) en E1) en (v)BMD of botgrootte in een groep van prepubertaire en vroeg pubertaire kinderen. Er is echter wel een positieve associatie tussen DHEAS, A en E1 en de botmaturatie. In hoofdstuk 2.2 tonen we aan dat E2 en vrij E2 (FE2) predictoren zijn voor (v)BMD, de endosteale omtrek en de botmaturatie en dat T en vrij T (FT) positief geassocieerd zijn met de botgrootte in een groep van prepubertaire en pubertaire jongens. E2 en FE2 zijn positief geassocieerd met aBMD van de lumbale wervelzuil en het totaal skelet en de trabeculaire vBMD ter

hoogte van de radius en tibia. De gevonden associaties blijven significant na inclusie van andere mogelijke determinanten van BMD in het statistisch model zoals T, IGF-1, calciuminname of fysieke activiteit. Daarenboven is er een negatieve associatie tussen FE2 en E2 en de endosteale omtrek ter hoogte van de radius en een positieve associatie tussen E2 en FE2 en de botmaturatie. Deze resultaten benadrukken het belang van oestrogenen in de botmaturatie en het verwerven van de piekbotmassa en zijn in overeenstemming met de gegevens van patiënten met oestrogeenresistentie en aromatasedeficiëntie. Daartegenover zijn T en FT spiegels geassocieerd met de botgrootte zowel ter hoogte van axiaal als het appendiculair skelet. Er is een positieve associatie tussen T en FT en de geprojecteerde botoppervlakte ter hoogte van het totaal skelet en de lumbale wervelzuil. Ter hoogte van de radius vinden we een positieve associatie tussen T en FT en de dwarse trabeculaire en corticale botoppervlaktes en de periosteale omtrek. Verder is er een positieve associatie tussen T en FT en de vetvrije massa -als merker voor spiermassa- en de dwarse spieroppervlakte van de onderarm en onderbeen. De gevonden associaties tussen T en FT en de botgrootte zijn niet langer aanwezig na inclusie van de spierparameters in het model. Onze gegevens suggereren dan ook dat T leidt tot een toename in spiermassa en dat deze toegenomen spiermassa meer trekkracht uitoefent op het bot, wat dan weer leidt tot een toegenomen botgrootte. We kunnen echter geen oorzakelijk verband aantonen gezien de transversale opzet van deze studie. Een rechtstreeks effect van T op botgrootte kan echter ook niet volledig uitgesloten worden.

In hoofdstuk 3, rapporteren we de resultaten van onze studies rond de relatie tussen geslachtshormonen en de seksuele en skeletale ontwikkeling (botmaturatie, vBMD en botgrootte) in een groep obese jongens en hun leeftijdsgepaarde gezonde controles. In hoofdstuk 3.1, tonen we aan dat obese jongens tijdens de pubertaire ontwikkeling (meer specifiek in Tanner stadium 3 en 4) lagere totale T (TT) spiegels, hogere E2 concentraties en normale FT spiegels hebben in vergelijking met gezonde controles. Deze afwijkingen verklaren mogelijks de dissociatie tussen de normale pubertaire ontwikkeling (eenzelfde Tanner stadium en serum prostaatspecifiek antigeen (PSA) waarden) en de versnelde botmaturatie (gemiddeld 1 jaar) in deze studiegroep. Onze gegevens tonen aan dat FT een betere indicator is voor androgeenblootstelling dan TT bij obese jongens gezien de

normale pubertaire ontwikkeling en PSA productie. Verder suggereren onze data dat de toegenomen aromatisatie en oestrogeenproductie mogelijks verantwoordelijk is voor de versnelde botmaturatie tijdens de pubertaire ontwikkeling. In hoofdstuk 3.2 tonen we aan dat obese jongens grotere en sterkere botten hebben ter hoogte van het onderbeen en in mindere mate ter hoogte van de voorarm in vergelijking met hun normaal gebouwde leeftijdsgenoten. Obese jongens hebben een toegenomen trabeculaire vBMD ter hoogte van de radius en de tibia in vergelijking met de gezonde controles. Sommige auteurs wijten de verschillen in vBMD aan de versnelde botmaturatie bij obese kinderen. Na correctie voor de versnelde botmaturatie bleven deze verschillen in onze studie echter bestaan. Gezien de positieve associatie tussen (F)E2 en de trabeculaire vBMD, vermoeden we dat de geobserveerde verschillen deels kunnen verklaard worden door de hogere E2 concentraties. Verder hebben obese jongens een significant grotere trabeculaire en corticale botoppervlakte ter hoogte van de radius en de tibia in vergelijking met de controles. Daarenboven weerhouden we een grotere periosteale en endosteale omtrek bij de obese groep. Sommige verschillen kunnen worden verklaard door het verschil in botmaturatie gezien er geen verschil meer was in botgrootte ter hoogte van de radius na correctie voor de versnelde botmaturatie. Ter hoogte van de tibia, weerhouden we echter nog steeds een significant groter botoppervlakte bij de obese jongens. Andere factoren naast een versnelde botmaturatie spelen hier dan ook een rol. De toegenomen spiermassa en kracht bij obese kinderen zoals ook aangetoond kon worden in de door ons bestudeerde populatie, spelen waarschijnlijk een belangrijke rol in de toegenomen botexpansie bij de obese adolescenten.

Tot slot worden in hoofdstuk 4 de belangrijkste bevindingen van deze verschillende studies samengevat en bediscussieerd, aangevuld met het klinisch belang van ons werk en mogelijke toekomstige onderzoeksonderwerpen. Onze gegevens hebben een bijdrage geleverd aan de beschikbare kennis rond het relatief belang van androgenen versus oestrogenen in de pubertaire ontwikkeling, de botmaturatie, de opbouw van het mannelijke skelet en de wijzigingen in lichaamssamenstelling tijdens de lichaamsgroei. In dit proefschrift hebben we aangetoond dat oestrogenen geassocieerd zijn met BMD en essentieel zijn voor de botmaturatie van gezonde en obese jongens. Verder, hebben we aangetoond dat T, spiermassa en spierkracht geassocieerd zijn met botgrootte tijdens de

adolescentie. Hoewel een rechtstreeks effect van T op botgrootte niet volledig kan worden uitgesloten, worden veel van de effecten van T op de botgrootte waarschijnlijk veroorzaakt door het anabool effect van T op de spiermassa bij gezonde en obese jongens.

SUMMARY

Sex steroids play an essential role in pubertal development, skeletal maturation, peak bone mass acquisition and changes in body composition in growing males. Although testosterone (T) has been regarded as the most important sex steroid in males, observations in humans with estrogen resistance or aromatase deficiency and some knock-out mouse models have stressed the importance of estrogens in adult males. Information about the effects of estrogens on skeletal development, skeletal maturation and body composition in the growing-up boy is however scarce. The limited available literature on associations between sex steroids, body composition and bone development in children is hampered by the use of inaccurate immunoassays to determine sex steroids and use of dual-energy X-ray absorptiometry (DXA) to evaluate bone mineral density (BMD) and bone area. An important limitation of DXA, especially in growing children is the size dependence of the areal bone mineral density (aBMD) and the lack of information on bone geometry.

A first part of our research focusses on the determinants of bone development in healthy prepubertal and pubertal boys with a specific interest in the associations between sex steroids (adrenal and gonadal steroids) and bone mineral density (aBMD and volumetric bone mineral density (vBMD)), bone geometry and skeletal maturation using state of the art techniques namely peripheral quantitative computed tomography (pQCT) to evaluate bone parameters and liquid chromatography-tandem mass spectrometry (LC-MS-MS) to determine the low sex steroid concentrations in children. To enhance our understanding of the effects of estrogens on epiphyseal maturation and bone mass acquisition, the second part of our research consists of the study of a group of obese male adolescents, aiming to investigate the associations of high estrogen (estradiol (E2) and estrone (E1)) levels with skeletal maturation, vBMD and bone geometry during adolescence since, based on available information in adult obesity, increased estrogen concentrations were expected in boys with longstanding obesity due to an increased aromatization in fat mass. However, available data on sex steroid (E2 and T) levels in obese children are scarce and contrasting. We therefore first studied pubertal development and sex steroid levels in a sizeable group of obese boys compared to healthy controls. Secondly, we studied vBMD, bone size, and

skeletal maturation in relation to sex steroid levels and muscle mass in this group of obese adolescents entering a residential weight loss program.

In chapter 1, a general background with a review of the available literature on sexual maturation and bone development in healthy and obese boys is provided and discussed. Moreover, the most important determinants of bone development in healthy children are commented. Additionally, we state our research objectives which are to study the relative contribution of androgens and estrogens on bone mineral density, bone size and epiphyseal maturation in healthy and obese boys. Finally, we describe our study populations and the used methodology with special attention for the LC-MS-MS and pQCT techniques.

In Chapter 2, we report our studies which investigated the association between sex steroid levels (adrenal and gonadal steroids) and (v)BMD, bone size, skeletal maturation, and body composition in healthy male children and adolescents. As reported in Chapter 2.1, there is no association between adrenal steroid concentrations (dehydroepiandrosterone sulfate (DHEAS), androstenedione (A) and estrone (E1)) and (v)BMD, or bone size in healthy prepubertal and early pubertal boys. DHEAS, A and E1 are however, positively associated with skeletal maturation. In Chapter 2.2, we show that E2 and free E2 (FE2) are predictors of (v)BMD, endosteal circumference and skeletal maturation and that T and free T (FT) are associated with different parameters of bone size in a group of prepubertal and pubertal boys. E2 and FE2 are positively associated with lumbar spine and whole body aBMD and trabecular vBMD at the radius and the tibia. All associations remain significant after inclusion of other possible determinants of BMD in the statistical model, such as T, IGF-1, calcium intake or physical activity. Moreover, E2 and FE2 are negatively associated with the endosteal circumference at the radius. Additionally, there is a positive association between E2 and FE2 and bone maturation. Our results therefore stress the importance of E2 in epiphyseal maturation and peak bone mass acquisition and are in line with data obtained from men with estrogen resistance or aromatase deficiency. T and FT levels are associated with different parameters of bone size, such as whole body and lumbar spine bone area, trabecular and cortical bone area and periosteal circumference of the radius. Moreover, there is a significant positive association of T and FT with

whole body lean mass and muscle cross-sectional area (CSA) at the forearm and lower leg. After inclusion of whole body lean mass or muscle CSA in the model, the associations of T and FT with bone size were however no longer present. These data suggest that T leads to an increase in muscle mass which causes a larger bone size resulting from an increase in strain exerted on the bone. Due to the cross-sectional design of the study, however, we are not able to draw causative conclusions and a direct effect of T on bone size cannot be definitely excluded.

In chapter 3, we report on our studies of sex steroid levels in relation to sexual and skeletal development (bone maturation, vBMD and bone size) in a well-described group of obese male adolescents and age-matched non-obese healthy controls. In chapter 3.1 we demonstrate that pubertal obese boys have lower total T (TT) levels, higher E2 but normal FT levels, at least during mid-and late puberty. These hormonal differences might be responsible for the observed dissociation between an advanced skeletal maturation (mean advancement around 1 year) and a normal sexual maturation (similar pubertal stage distribution and serum prostate-specific antigen (PSA) concentrations). Our data indicate that FT is a better indicator of androgen exposure than TT, explaining the normal pubertal progression and PSA production in male obese adolescents, and suggest that the increased aromatization and estrogen production might be linked to the advanced skeletal maturation during pubertal progression. As reported in chapter 3.2, obese adolescents have larger and stronger bones at the lower leg and to a lesser degree at the forearm than their normal-weighted peers. Obese boys have a higher trabecular vBMD at the radius and the tibia compared to age-matched controls. Some authors suggest that the differences in trabecular vBMD are due to an advanced skeletal maturation in the obese children, however the differences in trabecular vBMD remain even after correction for the advanced skeletal maturation using a bone-age matched control group. We speculate that the observed differences are due to higher E2 levels since there is a positive association between (F)E2 and trabecular vBMD. Trabecular area, cortical area, periosteal circumference, endosteal circumference at the radius and the tibia are significantly larger in the obese group compared to their age-matched controls. The advanced skeletal maturation might explain at least part of the observed differences in bone expansion, since after matching for bone age, no differences in cortical bone area parameters were present, at least at the radius. However, most

of the geometric differences at the tibia remained, in favor of the obese group. These results indicate that advanced bone maturation is probably not the sole explanation for the observed differences in bone geometry between obese and control boys. The larger muscle size and force in obese boys as described in our population might play an important role in the greater bone expansion in adolescent obesity.

Finally, the main findings of our different studies are summarized and discussed in chapter 4, together with the clinical relevance and the limitations of our research and a perspective on future research topics. Our work has enhanced the understanding of the relative contribution of androgens versus estrogens in the regulation of pubertal development, skeletal maturation, the build-up of the male skeleton and changes in body composition during somatic growth. We showed that estrogens are associated with BMD and are essential in the skeletal maturation of healthy and obese boys. Furthermore, we showed that T, muscle mass and force are associated with parameters of bone size. Although a direct effect of T on bone size can not be definitely excluded, our findings suggest that an important part of the effects of T on bone size are probably due to the anabolic effect of T on muscle mass in healthy and obese boys.

1 INTRODUCTION

1.1 BACKGROUND

Sex steroids play an essential role in pubertal development, skeletal maturation, peak bone mass acquisition and determination of body composition in growing males. Observations from some human 'experiments of nature' and from detailed studies in estrogen receptor knock-out mouse and aromatase transgenic mouse models provided new insights into the critical role of estrogens in adult males[1,2]. Previous research of our group in healthy male adults and men with idiopathic osteoporosis has also provided evidence for a role of estrogens in the acquisition of adult bone mass and maintenance of skeletal integrity in adult life in males[3–5]. Little is known however about the relative contributions of androgens and estrogens and the specific role of estrogens on bone development and epiphyseal maturation in the growing-up boy. Moreover, research on the possible effects of high estrogens levels on bone development and epiphyseal maturation during male childhood and adolescence is very scarce. In order to further unravel the essential role of estrogens on bone growth, epiphyseal maturation and bone mass acquisition in males, this thesis will consist of two parts. Firstly, the associations of adrenal and gonadal steroids with bone mass and skeletal maturation in late male childhood and adolescence will be studied. Secondly, we will study a group of male obese adolescents to investigate the effects of high estrogen levels on skeletal maturation and bone mass acquisition.

A first part of the introduction will consist of a description of the physiology of normal pubertal development, normal bone development and some of the determinants of bone development in healthy boys (section 1.1.1). Thereafter, we will discuss the available data on pubertal development, sex steroid levels and bone development in obese boys (section 1.1.2). Secondly, we will state our main and specific objectives (section 1.2). A third part of this introductory chapter will consist of the description of our study groups (section 1.3) and the used methodology (section 1.4). Special attention will be given to the methodology used to determine sex steroid levels, namely liquid chromatography–tandem mass spectrometry (LC-MS-MS) and to the technique used to determine bone mass, more specifically peripheral quantitative computed tomography (pQCT).

1.1.1 SEXUAL AND SKELETAL DEVELOPMENT IN HEALTHY BOYS

1.1.1.1 PHYSIOLOGY OF ADRENARCHE AND PUBERTY IN HEALTHY BOYS

1.1.1.1.1 PHYSIOLOGY OF NORMAL ADRENARCHE

Adrenarche refers to the sudden rise in adrenal steroid production, primarily dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS), usually about the age of 6-8 years. Histologically, it is associated with the appearance of the zona reticularis in the adrenal gland[6,7]. Recent studies showed that this is not an abrupt process occurring in mid-childhood, but rather a continuous process from birth on[8]. Adrenarche is independent of puberty and can manifest itself clinically as the appearance of axillary and pubic hair[6,9,10], a characteristic adult body odor and increased oiliness of hair and skin[11,12].

The mechanisms underlying the onset of the adrenarche remain unknown. Although adrenocorticotropic hormone (ACTH) is certainly required for adrenarche to occur -as patients with hypopituitarism do not experience adrenarche[13]-, it is probably not the sole factor. Since several studies demonstrated that ACTH and cortisol both remain constant during adrenarche despite the rise in adrenal androgens[14,15], additional factors may be required to initiate adrenarche. Several factors have been proposed in recent years including pro-opiomelanocortin, corticotropin-releasing hormone, prolactin, insulin and insulin-like growth factor (IGF-1)[14–19], although no other "master control" factor than ACTH has been identified up to now.

DHEA and DHEAS, produced by the adrenal gland, are not bioactive androgens themselves. They act as precursors for the production of more potent androgens (e.g. testosterone (T) and dihydrotestosterone (DHT)) or estrogens (e.g. estrone (E1) and estradiol (E2)). In the adrenal gland, DHEA is converted to androstenedione (A) by 3β-hydroxy-steroid dehydrogenase (3β-HSD). Subsequently, A can be further converted to T by 17β-hydroxy-steroid dehydrogenase (17β-HSD) or to estrogens (E1 and E2) by aromatase in other tissues[20] (figure 1). Adrenal steroid secretion (DHEA, DHEAS, A and T) increases significantly with age with a plateau in DHEA secretion at the age of 20 to 30 years[21,22].

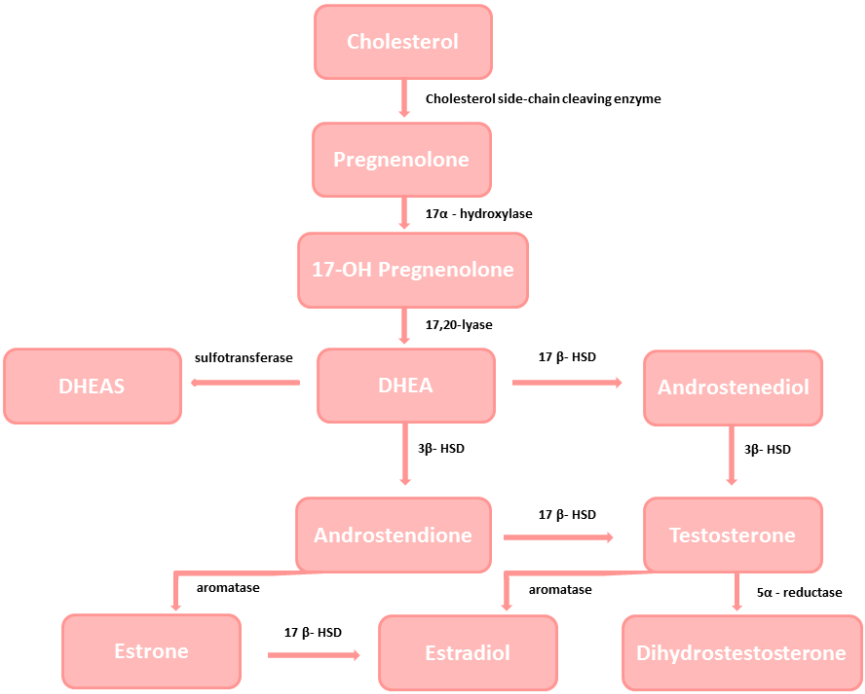

Figure 1: Major pathways in adrenal sex steroid synthesis (adapted from reference [7,23])

Apart from their well-known effects on the pubarche, it is not clear whether adrenal steroids have other effects in the prepubertal period, such as on body composition, bone development or skeletal maturation. Most of the available information comes from conditions with an elevated adrenal secretion such as premature adrenarche and congenital adrenal hyperplasia. These conditions are associated with an advanced skeletal maturation[24–27] and increased areal bone mineral density (aBMD)[28,29]. Some authors report a low aBMD in congenital adrenal hyperplasia, however this is related to the lifelong treatment with glucocorticoids[30,31].

1.1.1.1.2 PHYSIOLOGY OF PUBERTY AND SEX STEROID SECRETION IN HEALTHY BOYS

Puberty is a complex process by which children develop secondary sexual characteristics and reproductive competence. Normal puberty is initiated in the brain. An increase in the pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus triggers gonadotropins secretion namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from the pituitary gland. One to 3 years before the onset of clinical signs of puberty, low serum levels of LH already become evident during sleep. This sleep-entrained LH secretion occurs in a pulsatile fashion and probably reflects endogenous episodic discharge of GnRH. Nocturnal pulses of LH continue to increase in amplitude and to a lesser extent in frequency as clinical puberty approaches. The beginning of puberty is characterized by marked increases of GnRH and gonadotropin secretion, initially only during night[32,33]. As a consequence of increased nocturnal LH output, basal plasma T levels increase in boys, first during the early morning hours[34] before becoming detectable throughout the day. As puberty progresses, pulsatile gonadotropin secretion gradually increases during daytime and an adult pattern of gonadotropin secretion is eventually established. By the end of puberty, day-to-day GnRH and gonadotropin secretion remain fairly constant[32,33]. The physiological mechanisms that trigger activation of the hypothalamic-pituitary-gonadal axis are largely unknown, but attainment of a set point in growth, body composition and energy balance seems important[35]. Furthermore, upstream effects on the hypothalamic-pituitary-gonadal axis may influence pubertal timing. Kisspeptin, a neuropeptide produced in the hypothalamus, plays a central role in GnRH secretion and has been found to affect reproductive function in humans[36].

The increase in gonadotropin secretion subsequently leads to gonadal steroidogenesis, testicular enlargement and spermatogenesis. FSH is mainly involved in stimulating spermatogenesis and the production of inhibin B by the Sertoli cells. LH stimulates the Leydig cells producing T and insulin-like factor 3. As with most hormonal axes, production and secretion of these hormones is regulated by a negative feedback loop. For LH secretion, this negative feedback is exerted by androgen as well as estrogen action both at hypothalamic and pituitary level, whereas for FSH secretion, negative feedback is mainly exerted by inhibin B[37] (figure 2).

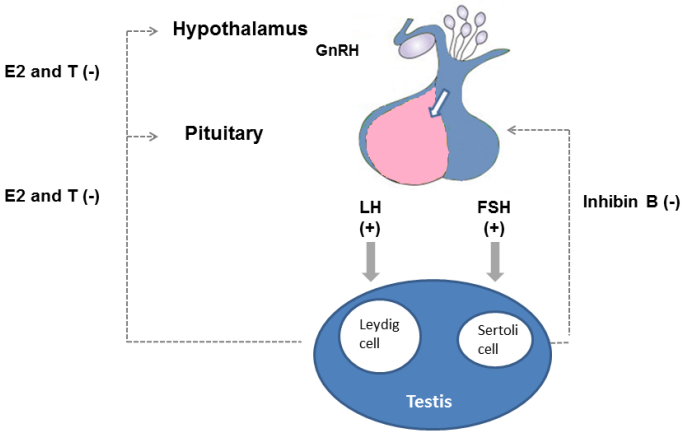

Figure 2: The regulation of the hypothalamic-pituitary-gonadal axis

The major androgen in the male circulation is T. Total T (TT) concentrations increase as puberty progresses. The increase in TT levels is modest from prepuberty (G1) to the early stage of puberty (G2), followed thereafter by a marked increase into mid puberty (G3), followed by a slower increase between mid- and late puberty (G4). No difference was observed between late- and post pubertal (G5) boys[34,38]. T is mainly bound to albumin and sex-hormone binding globulin (SHBG). SHBG concentrations decrease throughout puberty[34]. On average, only 1%-2% of circulating T is free. Free T (FT) is considered to be the hormone that reaches the target organs exerting its function through the androgen receptor (AR). Ankarberg-Lindgren et al. (2004) showed that FT increases –as does TT- from prepuberty, through puberty, to post puberty[34]. T is also in part converted by the aromatase enzyme to E2, which subsequently activates the estrogen receptor (ER)[37]. A study by Ankarberg-Lindgren et al. (2008) showed that E2 levels increase from prepuberty to early puberty but then remain relatively constant until a marked increase between mid puberty and late puberty, followed by a slower increase until post puberty[39]. Furthermore, 6 to 8% of T is converted to 5α-dihydrotestosterone (DHT) by the 5α-reductase enzyme in specific target cells. DHT is a more potent activator of the AR than T[32,37].

Increasing T levels during puberty promote clinical signs of pubertal development (i.e. secondary sex characteristics). In males, growth of the testes (>3 ml; mainly under stimulation by FSH secretion) and thinning of the scrotum are the first signs of puberty, followed by pigmentation of the scrotum and growth of the penis. Pubic hair normally appears a few months later[21]. These first morphological changes of puberty typically begin between the age of 9 and 14 years in boys[32] and are referred to as the gonadarche[35]. A large cross-sectional study on 4219 Flemish boys aged 6-22 years performed from 2002 to 2004, showed that the mean age of pubertal onset (assessed by Tanner genital staging (G2)) was 11.4 years. The mean age at pubic hair stage 2 was some months later namely 11.9 years[40]. Median ages at G2 are similar to those reported for other North-West European countries[41]. A tendency towards a younger age of male pubertal onset during the past decades has been reported. A Danish study of 21612 boys studied in the period 1930 to 1969 showed a decline in age at initiation of pubertal development and age at peak height velocity of 0.4 and 0.3 years respectively during that period[42]. Data from Greece in the period 1968 to 2011 do not support a continuing secular trend[43,44]. However, data from the Copenhagen Puberty study report a 3 months reduction in mean age at pubertal onset during a 15-year period (1991-1993 vs. 2006-2008)[45].

Apart from their effects on the development of secondary sexual characteristics, sex steroids (T and E2) also have marked effects on body composition, bone mass acquisition and skeletal maturation. The effects of sex steroids on bone mass acquisition and skeletal maturation will be discussed more in detail in section 1.1.1.3.1.

1.1.1.2 PHYSIOLOGY OF SKELETAL DEVELOPMENT AND GROWTH IN HEALTHY BOYS

From infancy until young adulthood there is a progressive accrual of bone mass in males. The acquisition of bone mass is however not constant throughout life: it increases rapidly during early childhood, more steadily during late childhood, accelerates during the pubertal growth spurt and ends at early adulthood, when peak bone mass is attained[46]. Peak bone mass can be defined as the maximal amount of bone that is accrued during growth and development plus the subsequent consolidation that continues during early adulthood. The exact age at which peak bone mass is reached depends on gender and skeletal localization[47]. In the axial skeleton, peak bone mass is achieved by the end of the second life decade.

In the appendicular skeleton, the timing of peak bone mass has been estimated to occur from ages as early as 18 years (femoral neck) to as late as 35 years (radius, skull, whole body)[48–51]. Since the attained peak bone mass and the rate of bone loss in later adulthood are important determinants of bone strength at older age, it is essential to maximize bone mass acquisition during growth. Recently, Bonjour et al. (2009) estimated that 1 standard deviation increase in population peak bone mass would reduce fracture risk by as much as 50%[52].

The accrual of bone mass during childhood is mainly the result of an increase in bone size namely bone length and diameter. Although aBMD -as measured by dual-energy X-ray absorptiometry (DXA)- increases during childhood, volumetric bone mineral density (vBMD) does not change during prepuberty[50,53,54]. The increase in aBMD in prepubertal children is thus due to an increase in bone size during growth. Whereas prepubertal boys and girls have a similar vBMD, there are already small differences in bone size between boys and girls during prepuberty[54–57]. In prepubertal boys, bone width is already slightly larger compared to prepubertal girls, no significant sex differences in height or body segment lengths are however observed before puberty[57,58]. The observed differences are already present in newborns[59] and infants 3 months of age[60], suggestive for an intrauterine determination of bone mass. Possible explanations are genetic factors[61] or a higher exposure of male fetuses to androgens in utero. Higher T levels have been measured in the cord blood of newborn males with levels peaking from 1–3 months of age and then decreasing to prepubertal levels by 7 months[62,63]. Determining factors of prepubertal bone mass acquisition are nutrition (calcium and vitamin D)[64–68], muscle mass and physical activity[66,69–71] and the growth hormone-insulin-like growth factor 1 (GH-IGF-1) axis[72]. Some recent studies of Remer et al.[69,73,74] reported a possible beneficial effect of adrenal androgens, specifically DHEAS metabolites, on the accretion of bone strength during prepuberty.

During puberty, there are marked increases in bone mass; it is estimated that 25 to 40% of adult bone mass is acquired in the 2 years surrounding the pubertal growth spurt[75,76]. Peak bone mineral accretion occurs approximately 6 months after the

age of peak height velocity; the age of peak bone accretion in boys is estimated to be around 14.1 years[76].

Lumbar spine and femoral neck aBMD rapidly increase during puberty in both sexes and have shown to plateau at about 15 and 17 years of age in girls and boys, respectively[48–50,77,78]. Areal BMD -as measured by DXA- is however largely dependent on bone size. Therefore, the observed increases in aBMD at the successive pubertal stages do not necessarily mean that the bone becomes denser. Studies using peripheral quantitative computed tomography (pQCT) and quantitative computed tomography give more accurate information on the true vBMD and bone geometry. Volumetric BMD remains independent of age until puberty, during puberty there is however an increase in vertebral trabecular density in both boys and girls[79]. These results are confirmed in the appendicular skeleton where trabecular and cortical vBMD increase by about 10% by the end of puberty in males[54,80]. In line with these data a study with high resolution pQCT showed an increase in trabecular thickness, cortical thickness and cortical vBMD from the age of 15 years onwards in boys[81].

Although there are no large differences in vBMD between men and women in young adulthood[50], men do have a stronger skeleton due to the larger bone size[82–84]. Male puberty is associated with an accelerated periosteal apposition with less endocortical expansion, resulting in enlargement of the bone diameter, cortical thickening and an increase in the medullary diameter[53,55,85]. Most of the cortical bone expansion in males occurs during pubertal growth, whereas periosteal bone expansion after puberty is very limited. When females enter puberty, periosteal apposition is inhibited, probably due to the inhibitory effects of higher estrogen levels on periosteal bone formation, whereas endocortical formation is stimulated, increasing cortical thickness and narrowing the medullary cavity[53] (figure 3). The main determinants of pubertal bone mass are sex steroids, muscle mass and GH-IGF-1 axis. These determinants will be discussed in detail in section 1.1.1.3.

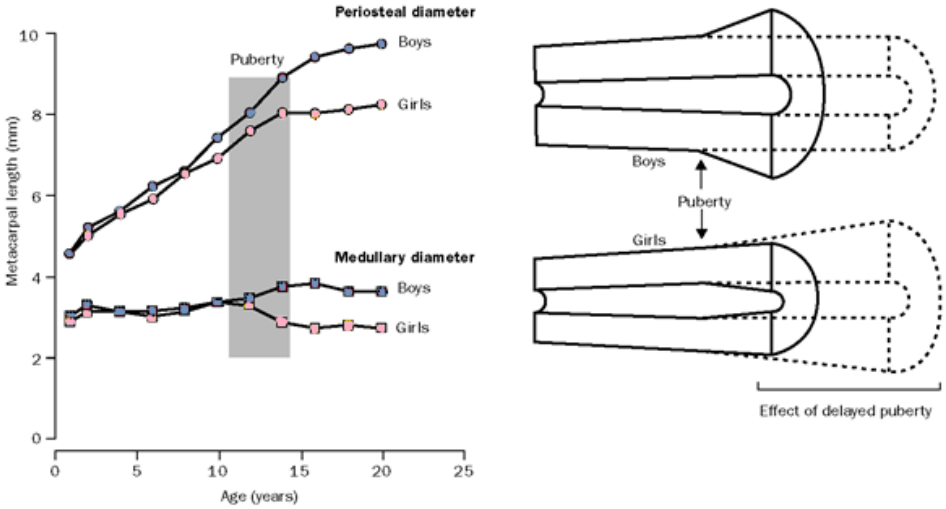

Figure 3: Effects of puberty and delayed puberty in bone development in boys and girls (reprinted from reference [86] with permission of Elsevier)

1.1.1.3 DETERMINANTS OF BONE DEVELOPMENT IN BOYS

Eighty percent of the variance in bone mass is genetically determined[87]. There are however many other factors which influence the accumulation of bone mass during childhood and adolescence namely hormonal influences (e.g. adrenal and gonadal steroids, GH-IGF-1 axis)[69,73,88], nutritional influences (e.g. calcium, vitamin D, protein intake)[65–68,89] and physical activity and muscle mass[66,70,71]. We will focus in the next paragraphs on the main determinants of pubertal bone mass namely sex steroids, the GH–IGF-1 axis and muscle mass.

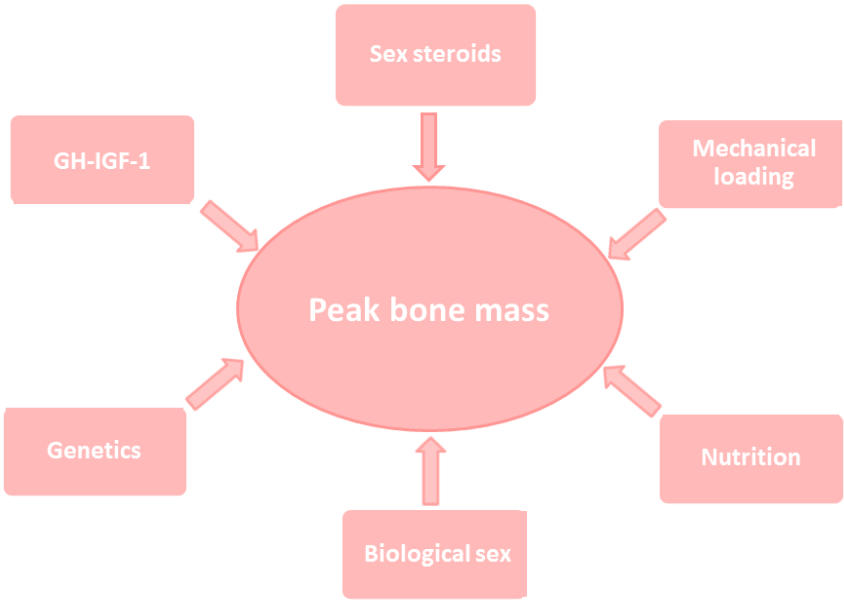

Figure 4: Determinants of peak bone mass (based on reference [65,71,87,88])

1.1.1.3.1 EFFECT OF SEX STEROIDS ON BONE MASS ACQUISITION AND SKELETAL MATURATION

Puberty, characterized by substantial increases in sex steroid levels, is a crucial stage in bone mass acquisition since about 40% of peak bone mass is achieved during pubertal development[46]. Clinical conditions as delayed puberty and primary and secondary hypogonadism stress the importance of sex steroids (T and E2) in bone mass accrual[88,90–92]. Men with a history of delayed puberty have a decreased radial, spinal and femoral aBMD, a smaller bone area and a lower peak bone mass[90,92,93] (figure 3). Moreover, spinal and radial aBMD are also considerably reduced in adolescents suffering from either primary or secondary hypogonadism[94,95].

T is the main circulating androgen in men. Both gonadal and adrenal T can be converted into estrogens by the aromatase enzyme which is present in many peripheral tissues including adipose tissue[96], bone[97], the growth plate[98],... . Bone

cells express AR as well as ER α (ERα) and ß (ERß)[99]. Therefore, T can act directly through the AR or indirectly through aromatization to estrogens and further through ERα and/or ß. The relative contribution of androgens versus estrogens in the regulation of the build-up of the male skeleton and the skeletal maturation is yet to be fully clarified.

Clinical observations in patients with altered secretion or action of sex steroids, studies in sex steroid receptor inactivated transgenic mouse models and very limited data in healthy children already revealed some of the different actions of sex steroids on bone metabolism in males.

An important role of estrogens in bone maturation and achievement of peak bone mass is illustrated by reports on men with impaired estrogen biosynthesis due to hereditary aromatase deficiency[100–103] and a case report of a young man with estrogen insensitivity secondary to a mutation in the ERα gene[104]. These men had a low bone mass as measured by DXA[100–107], a low trabecular and cortical vBMD as measured by pQCT[108,109], a high bone turnover and open epiphyses at the distal radius despite normal to elevated T levels[100–107] (table 1). Moreover, administration of E2 to aromatase deficient men resulted in a gain in bone mass and epiphyseal closure[101–103,105,109,110] (table 1). These case reports stress the importance of estrogens in bone mass acquisition and epiphyseal closure in both girls and boys[101,104,110]. Conversely, conditions of hyperestrogenism as aromatase excess syndrome[111,112] and Peutz-Jeghers syndrome[113,114] are characterized by an advanced bone maturation and aromatase inhibitors have been used to normalize estrogen levels and decrease bone age advancement[113–115].

Table 1: Summary of published studies on skeletal maturation and bone mineral status in male individuals with aromatase deficiency or estrogen resistance

First author	Chronological age	Bone age	Lumbar spine aBMD at start	Lumbar spine aBMD after E2 therapy	vBMD trabecular	vBMD cortical
Aromatase deficiency						
Morishima et al.[100,110]	24	14	↓	↑	N/A	N/A
Carani et al.[101]	31	14.8	↓	↑	N/A	N/A
Herrmann et al.[105]	27	16.5	↓	↑	N/A	N/A
Maffei et al.[107,109]	29	15	↓	↑	N/A	↓
Maffei et al.[106]	25	15.3	↓ (ultradistal forearm)	N/A	N/A	N/A
Bouillon et al.[102]	17	12	↓	↑	no reference values	no reference values
Lanfranco et al.[103]	26.8	15.5	↓	↑	N/A	N/A
Estrogen insensitivity						
Smith et al.[104,108]	28	15	↓	Unchanged	↓	↓

Table adapted from reference[116]. Comparison of lumbar spine areal bone mineral density (aBMD) to reference values; (↓) decreased aBMD, (↑) increase after estrogen substitution, (N/A) no data available.

Individuals with the androgen insensitivity syndrome, caused by a mutation in the AR, offer the opportunity to assess the consequences of a total or near total lack of androgen action. Affected patients experience a normal pubertal growth spurt and achieve epiphyseal closure due to adequate amounts of circulating estrogens, however they present a low bone mass compared to male and female reference values[61,117–125] (table 2). Since the bone mass deficit is most pronounced in gonadectomized patients with a poor compliance to estrogen substitution therapy, one can suspect that the bone mass deficit results at least partly from inadequate estrogen exposure, rather than from lack of androgen effects alone[122]. Furthermore, these patients have a periosteal circumference intermediate between male and female values, supporting an essential role for T as mediator for periosteal bone expansion. T is however probably not the sole stimulus for bone

expansion during growth[61]. A case report of a 16-year old boy with aromatase deficiency suggests that E2 also plays a role in cortical bone expansion as total, cortical and trabecular bone area increased significantly during estrogen treatment[102].

Table 2: Summary of published studies on bone mineral status in individuals with complete androgen insensitivity syndrome

First author	N	Mean Age (y) (range)	Gonadectomy (yes/no)	Hormonal substitution (yes/no)	aBMD		Reference values
					LS	FN	
Soule et al.[117]	6	(13-38)	6/0	4/2	↓	↓	F
Munoz et al.[118]	1	17	no	No	↓	↓	F
Vered et al.[119]	1	39	yes	No	↓	↓	F
Mizunuma et al.[120]	2	19 and 28	no	No	N/↓	N	F
Bertelloni et al.[121]	10	14 (4-20)	7/3	6/4	↓	N/A	F/M
Marcus et al[122]	22	36 (11-65)	20/2	21/1	↓	N	F
Sobel et al.[123]	12	35 (17-62)	10/2	8/4	↓	↓	F/M
Danilovic et al.[124]	5	23 (20-25)	5/0	5/0	↓	N	F/M
Han et al.[125]	46	32 (18-58)	yes	Yes	↓	↓	F
Taes et al.[61]	1	31	yes	No	↓	↓	F

Table adapted from reference[126]. Comparison of lumbar spine (LS) and femoral neck (FN) areal bone mineral density (aBMD) to reference values (F: female reference values; M: male reference values) (↓) lower aBMD, (N) normal aBMD, (N/A) no data available.

Some animal experiments investigated the relative importance of AR-mediated T actions compared to E2 effects. The stimulatory effect of androgens on bone size in males and the inhibitory effect of estrogens in females is suggested by observations of a reduced periosteal perimeter in orchidectomized male growing rats and an increased periosteal circumference in ovariectomized female rats[127]. In line with these results, a study in pubertal mouse models showed that androgen withdrawal, as induced by orchidectomy decreased radial bone growth in male mice during late puberty[128]. Ovariectomy, on the other hand, increased radial bone expansion during early puberty. These observations led to the traditional concept that androgens stimulate male bone size whereas estrogens limit female bone size. The recent finding that estrogen deficiency on top of androgen withdrawal further reduced radial bone expansion in early pubertal male mice, partly challenges this concept[128]. Furthermore, the essential role of estrogens in bone mass acquisition is illustrated by the low bone mass of transgenic mice lacking aromatase or ER α[129,130]. In addition, experimental studies in juvenile ovariectomized rabbits have demonstrated that E2 accelerates the programmed senescence in the proliferation rate and number and size of chondrocytes, leading finally to epiphyseal plate fusion[131].

There are only two reports available on the effects of sex steroids on bone mass in healthy male children and adolescents. Both reports used DXA to evaluate aBMD. Pomerant et al. (2007), found that T was a significant positive predictor of whole body and lumbar spine aBMD in healthy male adolescents. Since they did not study E2 concentrations, they cannot exclude that the positive association between T and aBMD is in fact an E2 effect, related to the aromatization of T[132]. In a study of 83 pubertal boys, Yilmaz et al. (2005) showed a strong positive association of E2 with whole body and lumbar spine aBMD[38].

1.1.1.3.2 Effect of GH-IGF-1 axis on bone mass acquisition

Growth hormone (GH) and insulin-like growth factor 1 (IGF-1) are important hormonal contributors to bone mass accrual during childhood and adolescence[72,133,134]. During puberty GH and IGF-1 levels increase dramatically, augmented by the increasing levels of sex steroids[135,136]. In turn, GH and IGF-1 stimulate gonadal sex steroid secretion and potentiate their effect on bone[137–139].

GH action on bone is mainly mediated through IGF-1 which positively affects bone formation by stimulating osteoblast proliferation and differentiation and collagen synthesis. Moreover, it activates bone modelling and remodelling[140–143].

Several investigators reported low aBMD -measured by DXA- in GH-deficient children, however these differences are probably due to the difference in bone size compared to healthy controls[133,144,145]. A pQCT study in 45 GH-deficient children reported a normal cortical vBMD[146]. Furthermore, a quantitative computed tomography study in 197 healthy children showed a positive association between IGF-1 and both total bone cross-sectional area (CSA) and cortical bone area, there was however no association between IGF-1 and material density[72]. The effects of IGF-1 on bone expansion are supported by GH receptor knock-out and IGF-1 deficient mouse models which present with a smaller bone size due to a limited radial bone expansion during growth[128,147]. The main effects of GH-IGF-1 axis are thus on bone size mainly promoting periosteal apposition and increasing cortical thickness. Furthermore, GH positively influences bone size by stimulating muscle mass[148,149] and strength[150]. The reduced muscle mass and strength in patients with GH deficiency can indeed be improved by replacement therapy[151].

1.1.1.3.3 Effect of muscle mass on bone mass acquisition

In the early nineties, Frost developed the "mechanostat theory" stating that mechanical loading is one of the most important determinants of bone size and mass. Mechanical loading due to gravitational forces, weight-bearing or local muscle contractions causes stress on the bone surface. This leads to strain or deformation of the bone and in order to keep these deformations within safe limits the bones are stimulated to adapt and become stronger[152]. The "mechanostat theory" therefore states that the increasing muscle mass (and thus muscle force) during growth and development stimulates the increase in bone mass (and thus in bone strength). A longitudinal study by Rauch et al. (2004) showed that the bone mass accrual during childhood and puberty seems to follow gain in muscle mass by a few months[153]. It was estimated that mechanical factors account for over 40% of bone strength, while non-mechanical ones e.g. calcium, vitamin D and hormonal factors only account for up to 10%[154,155]. However, increases in muscle mass are also influenced by genetics and hormonal factors as testosterone[156,157] and the GH-IGF-1 axis[150,151,156].

Several cross-sectional studies have reported a strong association between bone mass and parameters reflecting active mechanical loading such as the level of physical activity[158], muscle mass[70,159] and muscle strength[160–162]. Firstly, physical activity during early childhood and adolescence appears to be an important predictor of peak bone mass, since the amount of physical activity may account for up to 17% of the variance in BMD between individuals in their late 20s[158]. Moreover, the type and timing of an exercise intervention during childhood is important. Physical activities, characterized by a considerable loading magnitude applied at a rapid rate have the largest osteogenic effects on the growing skeleton: jumping and running have for example a higher osteogenic effect than walking[163]. In addition, exercise initiated during prepuberty and early puberty appears to be the most beneficial in improving bone mass[71]. Secondly, several studies report a close association between lean body mass -as a surrogate marker for muscle mass- and bone mass (BMC)[70,159]. Moreover, there is a strong positive correlation between muscle and bone CSA at the radius in children and adolescents[164]. Thirdly, several studies report a positive association between muscle strength -as measured by grip strength or peak force using jumping mechanography- and parameters of bone strength at the radius or tibia[160–162]. Furthermore, a functional relationship between mechanical forces and bone development is supported by clinical observations that disease processes interfering with muscle development (e.g. muscle dystrophy, spina bifida, poliomyelitis) have a negative effect on bone development[165–167].

1.1.2 SEXUAL AND SKELETAL DEVELOPMENT IN OBESE BOYS

1.1.2.1 PUBERTAL DEVELOPMENT AND SEX STEROID LEVELS IN OBESE BOYS

A multitude of factors affect the timing and tempo of pubertal development, including environmental influences. Adequate nutrition is a permissive factor for normal pubertal timing and tempo. In the 1970s, Frisch et al.[168,169] developed the critical weight (fat) theory suggesting that a critical body weight of 48 kg or body fatness of 22% is necessary for the onset of menarche. The effects of excess adiposity on various aspects of pubertal development, such as timing of pubertal initiation and sex steroid levels, remain however unclear.

Whereas a large number of studies point to a relationship between obesity and early puberty and menses in girls[170–172], data on the effects of obesity on pubertal development in boys are scarce and contrasting. Some authors describe an advanced sexual maturation in obese boys[170,171,173], whereas others describe a normal[172,174,175] or even a delayed genital development[172,176–178].

Some cohort studies from Sweden[170] and Danemark[171] have suggested that boys with a higher prepubertal body mass index (BMI) experience an earlier onset of puberty. He and Karlberg (2001) followed a large population-based cohort of 3650 Swedish children and found that the higher BMI gain in children (between 2 and 8 years old) was related to an earlier onset of puberty (measured using age at peak height velocity) in both genders[170]. A more recent study exploring the relationship between prepubertal BMI and pubertal onset, assessed by age at onset of pubertal growth spurt and age at peak height velocity confirmed that the heavier boys were at age 7, the earlier they entered puberty[171]. The same research group described a significant association between a higher BMI standard deviation score (SDS) and earlier age at voice break[173].

Denzer et al. (2007), reported however a normal genital development in a group of 582 German boys in comparison with the historical Swiss standard of Largo and Prader[174,179]. Laron et al. (2004) also reported in a short communication no difference in pubertal timing among 136 obese boys and 48 non-obese Israelian boys[175].

A longitudinal study in US boys by Lee et al. (2010) showed that a higher BMI during early and middle childhood was associated with a later onset of puberty as assessed by Tanner genital staging[176]. Similar results were found in a large cross-sectional study on 1520 boys (aged 8 to 14 years). Subjects were classified as early maturers if they reached a Tanner genital stage earlier than the median age for that stage within the cohort; otherwise, they were categorized as late maturers. In this study, boys with a higher BMI were more likely to be classified as late maturers[177]. Kleber et al. (2011) reported that obese boys had a later pubarche and voice break compared to their normal-weighted peers[178]. In the same line, Vignolo et al. (1988) showed that obese boys did not mature earlier than normal, in fact about one fifth had a delayed genital development[172].

These discordant findings in boys can be partly explained by differences in the studied populations and different methodology used in assessing puberty. Most of the cohort studies assessed influences of BMI on sexual maturation in groups of healthy children with limited data on the proportion of truly obese children[170,171,173,176,177], whereas others assessed differences in sexual maturation between obese children and healthy controls[174,175,178]. Furthermore, pubertal development in boys is more difficult to ascertain on a large scale compared to girls. Whereas age of menarche is often used as a reliable marker of pubertal timing in girls[180,181], no such characteristic event in puberty exists in boys and clinical examination of boys is usually required for such an assessment. Few studies have used a direct assessment of pubertal stage using the Tanner method[174–178], most frequently surrogate markers of pubertal onset/progression as age at peak height velocity or voice breaking are used[170,171,173]. This might explain part of the observed differences between studies. Since boys attain peak height velocity only when they reach Tanner stage 3[182], an earlier onset of peak height velocity could represent an earlier progression of puberty rather than an earlier initiation of puberty or could represent simply accelerated growth independent of puberty. Several cross-sectional studies have shown that obese children tend to be taller and to present an acceleration of skeletal maturation compared to normal-weight boys[172,174,178,183]. A longitudinal study by Johnson et al. (2012) showed that during prepuberty obese children already have higher height velocity and accelerated skeletal maturation[184]. The peak differences in height and skeletal maturity were respectively 3 cm and one year, both reached around the age of 13 years. These differences then diminished so that by the age of 18 years overweighted or obese adults were not significantly different in stature compared to their normal-weight peers.

Furthermore, few specific data regarding gonadotropins and sex steroids are available in obese boys. In prepubertal obese boys increased TT concentrations[185,186], as well as normal[187,188] and low TT levels have been described[174]. Reinehr et al. (2005) found higher TT concentrations in group of 81 prepubertal obese boys using a chemiluminescence immunoassay (ECLIA)[185]. These results were confirmed by the same research group on a group of 40 obese boys and girls using LC-MS-MS[186]. A small study on 6 prepubertal obese boys by

Pintor et al. (1984) found no differences in TT levels determined by radio-immunoassay (RIA) compared to lean controls[187]. Similar results were found by Gascon et al. (2000); no difference in TT by an enzyme immunoassay (EIA) was found between 61 boys and girls and their lean controls[188]. Denzer et al. (2007) found lower TT values in prepubertal children using RIA compared to age-related laboratory reference data. No data on pubertal development in the reference population were available[174] (table 3).

In adolescence, normal[185], as well as decreased TT concentrations have been reported[174,189–191]. Reinehr et al. (2005) reported no difference in TT concentrations between 60 early pubertal obese boys and their 18 normal-weighted controls using ECLIA[185]. However, most other studies reported decreased TT concentrations at different pubertal stages. Denzer et al. (2007) found lower TT concentrations in boys aged 8-18 years using RIA[174]. A small study of 20 males aged between 12-19 years (Tanner stage ≥2) found lower TT concentrations using an ECLIA methodology in obese and type 2 diabetic males (DM) as compared to lean males[190]. Similarly, Taneli et al. (2010) found that TT concentrations determined by ECLIA at Tanner stage 2 and 4 were lower in obese boys as compared to lean boys[189] (table 3). Several of these studies also described lower SHBG concentrations in obese boys[174,185,188,189]. As approximately half of TT is bound to SHBG, it is likely that the lower SHBG concentrations can at least partly account for the lower TT concentrations in obese boys.

Table 3: Summary of published studies that have compared total testosterone concentrations in obese and lean boys.

First author	Population	Controls	Age (y)	Used Method	TT (nmol/l)[a]	
Pintor, 1984[187]	6 obese boys (G1) 6 obese boys (G2)	6 lean boys (G1) 6 lean boys (G2)	7-11	RIA	G1: G2:	Obese:0.9±0.2 Lean:1.0±0.1 Obese:1.0±0.2 Lean:1.3±0.1
Gascon, 2000[188]	61 obese children (G1)	61 lean controls (G1)	6-9	EIA	G1:	Obese:0.6±0.03 Lean:0.7±0.03
Reinehr, 2005[185]	81 obese boys (G1) 60 obese boys (G2)	24 lean boys (G1) 18 lean boys (G2)	4-14	ECLIA	G1*: G2:	Obese:0.6(0.1-1.0) Lean:0.1(<0.1-0.4) Obese:3.1(0.9-5.2) Lean:3.0(0.4-5.8)
Denzer, 2007[174]	582 obese boys	Age-related reference values	6-18	RIA	Obese:	12-14y:4.4±4.7 14-16y:9.4±6.0 16-18y:14.8±6.0
Moriarty, 2010[190]	6 obese non-DM boys 7 obese DM boys	7 lean boys	12-19	ECLIA	Obese*: Obese* DM: Lean:	10.4±(N/A) 6.9±(N/A) 17.4±(N/A)
Taneli, 2010[189]	20 obese boys (G2) 20 obese boys (G4)	20 lean boys (G2) 20 lean boys (G4)	11-17	ECLIA	G2*: G4*:	Obese:1.1±0.8 Lean:4.1±4.2 Obese:5.6±4.2 Lean:9.2±5.2
Mogri, 2013[191]	25 obese boys (G4-G5)	25 lean boys (G4-G5)	14-20	LC-MS-MS	G4-G5*:	Obese:10.5±5.2 Lean: 21.4±8.3
Reinehr, 2013[186]	40 obese children (G1)	40 lean controls (G1)	6-10	LC-MS-MS	G1*:	Obese: 0.5(0.4-0.7) Lean: 0.4(0.3-0.5)

[a]Comparison of total testosterone concentrations (TT) expressed as median (P25-P75) or mean±SD between obese and lean children at different pubertal stages (G1: Tanner genital stage 1; G2: Tanner genital stage 2; G4: Tanner genital stage 4; G5: Tanner genital stage 5) * significant difference; DM: diabetes mellitus; N/A: not available.

Almost no data are available on FT levels in obese children. Mogri et al. (2013) showed that obese late pubertal and post pubertal males (aged 14-20 y) had significantly lower FT concentrations compared to their lean counterparts. Taneli et al. (2010) found lower FT in obese boys at Tanner stage 2, but not at Tanner stage 4[189] (table 4). However, in the latter study FT concentrations were measured by direct RIA, an inaccurate method, which underestimates FT concentrations by manifold and is strongly dependent upon SHBG concentrations[192,193].

Table 4: Summary of published studies that have compared free testosterone concentrations in obese and lean boys.

First author	Population	Controls	Age (y)	Used Method	FT (nmol/l)
Taneli, 2010[189]	20 obese boys (G2) 20 obese boys (G4)	20 lean boys (G2) 20 lean boys (G4)	11-17	RIA	G2*: Obese:0.01±0.01 Lean: 0.02±0.02 G4: Obese:0.04±0.03 Lean: 0.03±0.02
Mogri, 2013[191]	25 obese boys (G4-G5)	25 lean boys (G4-G5)	14-20	Equilibrium dialysis	G4-G5* Obese:0.26±0.11 Lean: 0.44±0.18

Comparison of free testosterone concentrations (FT) expressed as mean±SD between obese and lean boys at different pubertal stages (G2: Tanner genital stage 2; G4: Tanner genital stage 4; G5: Tanner genital stage 5)* significant difference.

Whereas high E2 levels are well-described in adult obese populations[194,195], little data is available in obese children. Most studies did not find a difference in E2 levels between obese boys and lean controls[183,187,189,191]. A small study on 6 prepubertal obese boys and 6 pubertal boys found no differences in E2 levels using RIA compared to lean controls[187]. Similar results were found in a study of Klein et al. (1998) on 18 obese boys and girls using a bioassay[183]. Taneli et al. (2010) compared E2 levels, measured with ECLIA, of obese boys at G2 and G4 to lean boys and did not find any difference in E2 levels between both groups[189]. A recent study of Mogri et al. (2013) did not find a significant difference in E2 levels measured by LC-MS-MS between 25 late and post pubertal obese boys and their lean peers[191] (table 5).

Table 5: Summary of published studies that have compared estradiol concentrations in obese and lean boys.

First author	Population	Controls	Age (y)	Used method	E2 (pmol/l)
Pintor, 1984[187]	6 obese boys (G1) 6 obese boys (G2)	6 lean boys (G1) 6 lean boys (G2)	7-11	RIA	G1: Obese:183±29.3 Lean:176±11.0 G2: Obese:146±16.0 Lean:172±18.3
Klein, 1998[183]	18 obese boys and girls (G1 or G2)	30 lean boys and girls (G1 or G2)	6-12	Bioassay	Obese:4.6 ±4.3 Lean:6.7±7.3
Taneli, 2010[189]	20 obese boys (G2) 20 obese boys (G4)	20 lean boys (G2) 20 lean boys (G4)	11-17	ECLIA	G2: Obese:84±16.5 Lean:77±8.4 G4: Obese:94±21.2 Lean:92±30.9
Mogri, 2013[191]	25 obese boys (G4-G5)	25 lean boys (G4-G5)	14-20	LC-MS-MS	G4-G5: Obese:76±36.3 Lean:66±40.7

Comparison of estradiol concentrations (E2) expressed as mean±SD between obese and lean boys at different pubertal stages (G1: Tanner genital stage 1; G2: Tanner genital stage 2; G4: Tanner genital stage 4; G5: Tanner genital stage 5)

Poor assessment of Tanner genital staging, small study groups[183,187,189,191], lack of an age-matched control group[174] and the use of direct immunoassays for TT and E2 determination[183,185,187,189,190] can partly explain these discordant findings between studies.

1.1.2.2 SKELETAL DEVELOPMENT IN OBESE BOYS

Childhood obesity has been linked to an increased risk of skeletal fractures as obese children were overrepresented in studies reporting on fracture rates in children[196–202]. Taylor et al. (2006) showed in their review a significant increase in fracture rate in obese children compared to their non-obese counterparts[196]. These results were confirmed by a recent study of Rana et al. (2009) showing a higher incidence of extremity fractures after trauma in obese children[200]. Goulding et al. showed that overweight boys are overrepresented in groups with single and repeated forearm fractures[197,198]. In addition to these results Manias et al. (2006)

indicated that children with recurrent fractures had a higher BMI than children with only one or no fractures[202]. Furthermore, several cross-sectional studies stated that a higher BMI is also associated with a higher risk for lower extremity fractures[199,201]. The exact mechanisms contributing to this increased fracture risk are however unclear. Possible explanations are an increased impact during falls due to a higher body mass[203], an increased risk of falling due to a poorer balance and/or a decreased protective response[204–206] and a higher fracturing rate caused by a lower bone mass[197,207].

Controversy exists about the effect of obesity on BMD. Some authors report a normal or higher bone mass in overweight children[208–213], whereas others conclude that obesity is linked to a lower bone mass as measured by DXA[197,207,214–216].

Several cross-sectional and longitudinal studies have reported a negative association of fat mass with BMC[214–216], BMD[214,216] and bone area[214,215] measured by DXA in healthy children (table 6). Dimitri et al. (2010) reported a negative association between fat mass and lumbar spine and whole body aBMD and, between fat mass and lumbar spine area[214]. In line with these results, fat mass measured at the age of 3.4 y was inversely related to bone area and BMC at the age of 7 years[215]. Weiler et al. (2000) showed that increased body fat had a negative effect on attaining peak bone mass and BMC in adolescents[216]. Foley et al. (2009) assessed potential factors that may lead to deviation in bone mass tracking during skeletal growth and maturation. They found that an increase in fat mass resulted in a negative deviation from normal bone mass tracking in both sexes. Furthermore, there are several case-control studies reporting a low aBMD in obese and overweight children compared to lean controls[207,217]. Goulding et al. (2002) reported that overweight and obese children have a low vertebral BMC for their bone area, body height, body weight and pubertal development[217]. In another study, the same group reported that total body BMC was low relative to body weight in the overweight and obese children[207] (table 7).

Table 6: Summary of published studies evaluating the associations between fat mass and lumbar spine and whole body areal bone mineral density, bone mineral content and bone area.

First author	Population	Age (y)	Association fat mass–aBMD	Association fat mass–BMC	Association fat mass–area
Weiler, 2000[216]	61 children	10-19	WB aBMD:-	WB BMC:-	N/A
Clark, 2006[211]	3503 children	10	N/A	WB BMC:+	WB area:+
Wosje, 2009[215]	215 children	3.5-7	N/A	WB BMC:-	WB area:-
Dimitri, 2010[214]	52 obese and 51 lean children	7-14	WB aBMD:- LS aBMD:-	WB BMC:- LS BMC:-	WB area: no association LS area:-

Associations between fat mass and lumbar spine (LS) and whole body (WB) areal bone mineral density (aBMD), bone mineral content (BMC) and bone area: (+) positive association, (-) negative association, (N/A) no data available.

Table 7: Summary of published studies that have compared lumbar spine and whole body areal bone mineral density, bone mineral content and bone area using DXA in obese and lean boys.

First author	Population	Controls	Age (y)	aBMD obese vs. lean	BMC obese vs. lean	Bone area obese vs. lean
De Schepper, 1995[209]	59 obese children	59 lean children	6-15.6	LS aBMD=	N/A	N/A
Fischer, 2000[208]	16 obese children	16 lean children	5-13	WB aBMD* ↑ Hip aBMD= LS aBMD=	WB BMC* ↑	N/A
Hasanoglu, 2000[210]	37 obese children	37 lean children	5-15	LS aBMD =	N/A	N/A
Goulding, 2000[207]	39 overweight 21 obese children	276 lean children	3-19	N/A	WB BMC* ↓	WB area* ↓
Goulding, 2002[217]	45 overweight 18 obese children	299 lean children	3-19	N/A	LS BMC* ↓	LS area girls* ↓ LS area boys=
Leonard, 2004[213]	103 obese children	132 lean children	4-20	WB aBMD* ↑ LS aBMD* ↑	WB BMC* ↑ LS BMC* ↑	WB area* ↑ LS area* ↑
Rocher, 2008[212]	20 obese prepubertal children	23 lean controls	9-12	WB aBMD= LS aBMD* ↑	WB BMC* ↑ LS BMC* ↑	WB area* ↑ LS area=

Comparison of mean lumbar spine (LS) and whole body (WB) areal bone mineral density (aBMD), bone mineral content (BMC) and bone area between obese children and lean controls. (↑) higher aBMD, BMC or bone area in the obese boys compared to healthy controls, (↓) lower aBMD, BMC or bone area in the obese boys compared to healthy controls, (=) similar aBMD, BMC or bone area in the obese boys compared to healthy controls, (N/A) no data available. *significant difference

In contrast, several other studies conclude that obesity is linked to a normal[208–210,212] or a higher aBMD[208,211,213] as measured by DXA (table 6 and table 7). Cross-sectional analysis of a large cohort of British prepubertal children demonstrated a strong positive relationship between whole body fat mass and whole body BMC and area, before and after adjustment for height and lean mass[211]. Several case-control studies showed a normal[208–210,212] or increased aBMD[208,212,213] at the lumbar spine and the whole body. A study by Leonard et al. (2004) showed that obese children and adolescents had increased vertebral and whole body aBMD and bone area compared to lean controls. The observed differences remained after adjustments for height and pubertal stage, except for the difference in lumbar bone area[213]. Rocher et al. (2008), found similar results, however after adjustment for body weight or lean mass obese children had a lower whole body aBMD than their controls[212].

The observed differences between studies can partly be explained by differences within the studied populations (groups of healthy children versus case-control studies of obese boys and lean controls), and the use of DXA technique to evaluate bone strength accompanied by the use of different adjusting factors in the different studies. An important limitation of DXA is the two-dimensional projection of a three-dimensional structure, so that the third dimension of the bone i.e. the depth is not taken into account. Bones in taller persons are longer, wider and deeper; however, DXA bone area only captures the larger bone length and width and not the greater depth. Therefore, aBMD overestimates vBMD of tall persons. Consequently, assessment of aBMD for age is biased by the increased stature in obese children. It is therefore important to correct for differences in body size namely height. On the other hand, some studies also additionally correct for body weight in their obese groups[207,212,217], thereby using an overadjusted model. Furthermore, DXA gives no information about bone geometry. Prediction of bone strength requires knowledge of both the material (e.g. vBMD) and geometric properties of bone (e.g. size and shape)[218]. Therefore, pQCT is a more useful approach in bone strength analysis since it can provide three-dimensional information about BMD, size and shape overcoming some of the problems intrinsic to the DXA technique[219].

Literature on the effects of adiposity and obesity on vBMD and bone size in children is scarce and contrasting[220–222]. Some cross-sectional and longitudinal studies have reported a negative association between fat mass and vBMD[223,224] and bone area[223], however others have described a positive association between fat mass and bone area[224,225] in healthy children (table 8). In contrast, the two available case-control studies comparing obese prepubertal children and lean controls reported a higher vBMD and bone area in the obese group[220,221]. Wetzsteon et al. (2008) described a larger total vBMD, bone area and bone strength parameters at the tibia in overweight children[220]. These results were confirmed by Ducher et al. (2009) who found a significantly larger bone size and trabecular density at both the forearm and the lower leg in the overweight group[221]. No difference in cortical density of the long bones could be found in either study[220,221]. Only one study was performed in late childhood and adolescence. Ehehalt et al. (2011) found in a group of 84 overweight children (mean age 12 years) an altered bone structure at the radius: cortical vBMD was decreased, bone circumferences were larger, whereas the cortex was thinner compared to normal-weight peers[222] (table 9).

Table 8: Summary of published studies evaluating the associations between fat mass and vBMD and bone area

First author	Population	Age (y)	Measure site	Association fat mass–vBMD	Association fat mass–bone area
Wey, 2011[223]	138 boys	8-18	radius	-	-
Cole, 2012[224]	132 children	6	tibia	-	+
Uusi-Rasi, 2012[225]	34 adult men with childhood obesity	36	radius tibia	no association	+

Associations between fat mass and volumetric bone mineral density (vBMD) and bone area at the radius and tibia: (+) positive association, (-) negative association.

Table 9: Summary of published studies that have compared vBMD and bone size using pQCT in obese and lean boys

First author	Population	Controls	Age (y)	Measure site	vBMD obese vs. controls	Bone size obese vs. controls
Wetzsteon, 2008[220]	143 overweight children	302 lean children	8-12	tibia 8% 66%	total vBMD* ↑ cortical vBMD=	total area* ↑ cortical area* ↑
Ducher, 2009[221]	93 overweight children	334 lean children	7-10	radius tibia	trabecular vBMD* ↑ cortical vBMD=	total area* ↑ cortical area* ↑
Ehehalt, 2011[222]	84 obese children	reference values	5-19	radius	cortical vBMD↓	marrow area↑ cortical thickness↓

Comparison of mean volumetric bone mineral density (vBMD) and bone size at the radius and tibia between obese children and lean controls. (↑) higher vBMD or larger bone area in the obese boys compared to healthy controls, (↓) lower vBMD or smaller bone area in the obese boys compared to healthy controls, (=) similar vBMD or bone area in the obese boys compared to healthy controls *significant difference

1.2 RESEARCH OBJECTIVES

1.2.1 GENERAL AIMS

From infancy until young adulthood there is a progressive accumulation of bone mass in males with a rapid increase of bone mass during puberty. Sex steroids, the GH–IGF-1 axis and muscle mass are considered to be the main determinants of pubertal bone mass. However, little data is available on the relative contribution of androgens versus estrogens in the regulation of the build-up of the male skeleton during adolescence. Moreover, data on the possible role of adrenal steroids during prepuberty and early puberty are scarce. Most of the available information is based on some human experiments of nature and bone studies in sex steroid receptor inactivated transgenic mouse models, indicating that E2 plays an essential role in skeletal maturation and peak bone mass acquisition.

Moreover, the limited available literature on associations between adrenal and gonadal steroids and bone development is hampered by the use of inaccurate immunoassays to determine sex steroids and the use of the DXA technique to evaluate BMD and bone area. Commercial immunoassays are unable to measure low estrogen and androgen levels accurately in children and adolescents. DXA studies are limited by the size dependence of aBMD, which is especially important in growing children. Moreover, this technique gives no information about bone geometry.

In a first part of our research we study determinants of epiphyseal maturation and bone development in healthy prepubertal and pubertal boys with a specific interest in the associations between sex steroids and skeletal maturation, vBMD and bone geometry using state of the art techniques namely pQCT to evaluate vBMD and bone geometry and LC-MS-MS to determine the sex steroid concentrations as required when studying low androgen and estrogen serum levels in children (chapter 2).

To further unravel the essential role of sex steroids, more specifically estrogens, on epiphyseal maturation and bone mass acquisition, a second part of our research consists of the study of bone parameters and sex steroids in a well-defined group of children and adolescents with longstanding obesity. By using childhood obesity

as a model of increased estrogen production during childhood and adolescence, we aim to investigate the effects of high estrogen (E1 and E2) levels on skeletal maturation, vBMD and bone geometry. While it is well-known that adult obesity is associated with high circulating E2 levels due to an increased aromatization in fat mass, data on serum E2 levels in obese children are scarce. Moreover, only very limited and contrasting data have been published concerning pubertal development and circulating TT and FT levels in obese adolescents. Firstly, we describe the pubertal development and the sex steroid levels (E2 and T) in a sizeable well-described group of obese adolescents in comparison to age-matched controls. Sex steroid levels (E2 and T) are measured by LC-MS-MS and Tanner genital staging is determined by trained pediatricians. Secondly, we study the possible effects of disturbed sex steroid levels on vBMD, bone size and skeletal maturation using pQCT (chapter 3).

1.2.2 SPECIFIC AIMS

The first part of our research (chapter 2) focusses on the associations between sex steroids and parameters of bone strength and skeletal maturation in healthy boys.

Our first objective is to investigate the determinants of bone strength in healthy children and adolescents with a specific interest in the associations of adrenal and gonadal steroids with bone size and (v)BMD. In chapter 2.1, we study the associations of adrenal steroids (DHEAS, A and E1) with (v)BMD and bone geometry in prepubertal and early pubertal children. Only prepubertal and early pubertal boys were selected, to investigate if adrenal steroids might impact on (v)BMD and bone size before pubertal development. Chapter 2.2 reports on the associations of sex steroids, namely E2 and T, with (v)BMD and bone size during late childhood and adolescence. Based on the available results from bone studies in sex steroid receptor inactivated transgenic mouse models and some case reports of men with estrogen resistance or aromatase deficiency, we hypothesize that during adolescence circulating E2 levels will be positively associated with (v)BMD and circulating T levels will be positively associated with bone size. We also hypothesize that similar associations between adrenal steroids and (v)BMD and bone size might be found during pre- and early puberty.

Our second objective is to study the associations between adrenal steroids (DHEAS, A, E1) and gonadal steroids (FE2, E2, FT and TT) and bone maturation in healthy children and adolescents. The results of these studies are discussed in chapter 2.1 and chapter 2.2. Based on literature on estrogen resistant and aromatase deficient men, we hypothesize that estrogens levels (E1 and E2) will be positively associated with bone maturation.

In the second part of our research (chapter 3), we studied male adolescents with primary obesity to investigate the effects of high estrogen levels on skeletal maturation, vBMD and bone geometry. In chapter 3.1 the differences in sex steroid levels (TT, FT, E2) in parallel with skeletal and sexual maturation in obese boys compared to healthy controls are given.

Our first objective is to clarify if sex steroid levels (TT, FT, E2) are indeed disturbed in obese adolescents. Secondly, we want to investigate if these possible disturbances have an effect on sexual maturation (evaluated by genital development and serum PSA levels) and skeletal maturation (evaluated by X-ray of the left hand) in obese boys. We hypothesize a normal genital development in association with normal FT concentrations and a more rapid skeletal maturation in relation to increased E2 levels. Our third objective is to analyze whether the observed hormonal differences in adolescent obesity might result in a different bone size or vBMD. Previous studies on vBMD and bone size in obese adolescents are scarce and their results are contrasting. Therefore, vBMD and bone size of a group of obese adolescent boys and age-matched controls are studied in parallel with sex steroids and muscle strength in chapter 3.2. We hypothesize that the higher estrogen levels will be associated with a higher vBMD.

1.3 STUDY POPULATIONS

1.3.1 HEALTHY BOYS AGED 6-19 Y

All analyses presented in chapter 2.2 were performed on a cohort of one hundred and ninety-nine healthy male children and adolescents aged between 6 and 19 years (mean age: 12.5 years). Participants were recruited by letters distributed in primary and secondary schools within the Ghent area. Two hundred and six children participated in our study, however 7 were excluded. Children were excluded if they were taking medication known to influence bone or mineral metabolism in the past year or if they had a metabolic bone disease, thyroid disorder or diabetes, if their height standard deviation score (SDS) was <-2.5 or >2.5 or if their BMI SDS was <-2 or >2. The study protocol was approved by the Ghent University Hospital Ethical Committee. Informed consent was obtained from the parents and all participants gave their assent. Blood sampling, determination of anthropometry and Tanner genital staging, an X-ray of the left hand, and measurements of bone strength ((v)BMD and bone size) and muscle mass (lean mass and muscle CSA) were performed in all participants. This methodology is discussed more in detail in chapter 1.4.

All analyses presented in chapter 2.1 were performed on a subgroup of the previous cohort by selecting only prepubertal (Tanner genital stage 1 n=65) and early pubertal boys (Tanner genital stage 2 n=33). Ninety-eight healthy male children and adolescents aged between 6-14.5 years (mean age: 10.2 years) were included in this subgroup; 81 children were pre-pubarchal and 17 children had pubic hair stage 2.

Information about medical history, lifestyle and socio-economic background was collected through a questionnaire, which all participants (>12 y) or their parents (<12 y) completed. Calcium intake was estimated by a food questionnaire on dairy products accounting for the number of standard portions per week which was previously validated in adults[226]. Physical activity was assessed using the Flemish Physical Activity Questionnaire[227,228].

1.3.2 OBESE BOYS AGED 10-19 Y

All analyses presented in chapter 3.1 were performed on a case-control study. Ninety male obese (BMI SDS >+2) adolescents, aged between 10 and 19 years, were recruited at the entry of a residential weight-loss program at the Zeepreventorium in De Haan in 2011 (n=51 obese boys) and 2013 (n=39 obese boys). Ninety age-matched normal-weighted controls were randomly selected from an ongoing longitudinal study evaluating changes in bone geometry, bone maturation and muscle strength in relation to sex steroids in childhood and adolescence (study population described in section 1.3.1). Subjects with a history of hypogonadism, panhypopituitarism, diabetes, previous or ongoing treatment with T or oral steroids were excluded. Blood sampling, determination of anthropometry, Tanner genital staging and an X-ray of the left hand were performed in all participants.

The analyses presented in chapter 3.2 were performed on a subgroup of the obese study population, namely the obese boys recruited in 2011. These 51 male obese (BMI SDS >2) adolescents received in addition to the previously described examinations, some additional measurements of bone strength (vBMD and bone geometry) and muscle mass using pQCT and muscle strength using jumping mechanography. Their results were compared to 51 age- and body height matched healthy normal-weighted controls as well as to 51 bone age- and body height matched healthy normal-weighted controls, who were randomly selected from an ongoing longitudinal study evaluating changes in bone geometry and muscle strength in relation to sex steroids in childhood and adolescence (study population described in section 1.3.1). Obese and control children were excluded if they were taking medication known to influence bone or mineral metabolism in the past year or if they had a metabolic bone disease, thyroid disorders or diabetes. Both study protocols were approved by the Ethical Committee of the Ghent University Hospital. Informed consent was obtained from the parents and all participants gave their assent.

1.4 METHODS

1.4.1 ANTHROPOMETRY AND TANNER GENITAL STAGING

Body weight was measured in light indoor clothing without shoes to the nearest 0.5 kg. Standing and sitting height were measured to the nearest 0.1 cm using a wall-mounted Harpenden stadiometer (Holtain Ltd., Crymuch, UK). Length of the forearm (from the olecranon to the processus ulnaris) and the tibia (from the medial knee joint line to the tip of the medial malleolus) was measured to the nearest 0.1 cm. BMI was calculated as the body weight in kilograms divided by the square of the body height in meter. As described in the review of Lobstein et al. (2004), BMI is widely used as an index of relative adiposity among children, adolescents and adults[229]. As confirmed by Lazarus et al. (1996)[230] and Sardinha et al. (1999)[231], BMI can be used as reliable index of relative adiposity compared with DXA. Lazarus et al. (1996) showed that BMI had a true positive rate of 0.67 and a false positive rate of 0.06 for predicting high fat percentage in children 4 to 20 years old. In a study of Sardinha et al. (1999) BMI had a true positive rate of 0.96 for 10-11 year old, 0.86 for 12-13 year old and 0.50 for 14-15 year old boys for predicting a high percentage of total body fat as assessed by DXA[231]. Waist circumference, defined as the smallest abdominal circumference if present or otherwise measured halfway between the iliac crest and the rib cage, was determined to the nearest 0.1 cm. Waist circumference is regarded as one of the most reproducible anthropometric measures of girth and is also the best simple indicator of intra-abdominal fat mass in children[232]. All anthropometric measurements were performed by the same trained physician. The SDS for body height, weight, and BMI was computed using the reference data of the 2004 Flemish growth study[40]. The SDS for waist circumference was also computed using the reference data of the 2004 Flemish growth study (Dr. M. Roelants; unpublished).

Pubertal status of the subjects was assessed by trained pediatricians according to the method established by Tanner (Tanner Genital Staging: stage 1: prepuberty;

stage 5: post puberty). Testicular volume was determined with use of a Prader orchidometer.

1.4.2 BLOOD SAMPLING AND BIOCHEMICAL ANALYSES

Blood samples in the healthy children were collected after a small breakfast between 0800 and 1000 h to avoid diurnal variation of T and E levels. Venous blood samples in the obese group were also obtained between 0800 and 1000 h, but after overnight fasting. Cream with 2.5% lidocaine and 2.5% prilocaine (EMLA ®; Astra Zeneca; UK) was applied to reduce the pain of the puncture, and unusually anxious or unwilling children were excluded from blood sampling. A maximum of 15 ml of blood was drawn (both EDTA-plasma and serum). This was less than 1% of the total blood volume in all children which is regarded as an acceptable amount in research setting[233]. The obtained plasma and serum was divided in aliquots in adequately sealed tubes and stored at -80°C until batch analysis.

Serum E2, E1, TT, A and cortisol were determined by LC-MS-MS (AB Sciex 5500 triple-quadrupole mass spectrometer; AB 173 Sciex, Toronto Canada). Serum limit of quantification (LOQ) was <0.5 pg/mL (1.9 pmol/L) for E2 and E1 and the interassay coefficients of variation (CV's) were 4.0% at 21 pg/mL (77 pmol/L) for E2, 7.6% at 25 pg/mL (93 pmol/L) for E1[234]. Serum LOQ was 1.2 ng/dl for TT and the interassay coefficient of variation (CV) was 8.3% at 36.7 ng/dl and 3.1% at 307.8 ng/dl. Serum LOQ was 4.25 ng/dl for A and the interassay CV was 2.9% at 59.8 ng/dl. Serum LOQ was 0.05 µg/dl for cortisol and the interassay CV 's were 2.3% at 7.43 µg/dl and 3.1% at 24.7 µg/dl.

LC-MS-MS is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (LC) or high performance liquid chromatography with the mass analysis capabilities of mass spectrometry (MS). LC is a separation technique by which components of a sample are separated according to their polarity, electrical charge or molecular size. In general, a liquid mobile phase consisting of a mixture of solvents with different polarity is passed under high pressure through a column coated with adsorbent material. This adsorbent material interacts with the different components of the sample with a slightly different strength, resulting in specific retarding of the flow of each component[235]. This sample preparation increases the sensitivity of the subsequent

analysis by mass spectrometry. After separation, the eluate of the chromatography column is coupled to a MS detector. MS is an analytical technique that measures the mass-to-charge ratio of charged particles. Briefly, the loaded sample undergoes vaporization and is most often ionized and subsequently fragmentized into charged molecules. There are three different ionization modes namely electrospray ionization (ESI), heated nebulizer atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI). The ESI ionization source is considered more sensitive than the APCI source for polar compounds. However, for the nonpolar or lower polar compounds, which comprises most steroid molecules, APCI provides a better sensitivity[236]. These ions are then accelerated in a magnetic or electric field and are separated according their mass-to-charge ratio by filtering on an analyzer. A fragmentation pattern of the molecule is generated and the obtained spectrum allows identifications of the fragment ions. The tandem MS in LC-MS-MS uses two sequential mass-filtering devices[237,238]. The advantages of mass-spectrometry are the high sensitivity and specificity. The limits of quantification are very low. Unlike immunoassays, LC-MS-MS allows for measuring whole steroid profiles in one single run from one sample. In addition the technique requires only small sample volumes (50 to 200 µl) for a complete profile. However, using LC-MS-MS requires more technical experience than immunoassays and although running costs are affordable the sensitive equipment is expensive[239].

Free testosterone (FT) was determined by equilibrium dialysis[240], CV of the method calculated from duplicate measurements is 11.7%. Free estradiol (FE2) was calculated from total E2, SHBG and albumin concentrations using a previously validated equation derived from the mass action law[241].

Commercial immunoassays were used to measure serum IGF-1 (Diagnostic Systems Laboratories, Webster, TX), leptin (Linco Research Inc., Missouri, USA), SHBG, LH, FSH and DHEAS (Modular, Roche Diagnostics, Mannheim, Germany). The intra- and interassay CV's for all assays were less than 10%. PSA was measured by a commercial immunoassay (Elecsys, Roche Diagnostics, Mannheim, Germany). The lower detection limit for PSA was 0.003 ng/ml and the intra-assay and interassay CV's were respectively 1.2% and 3.5%.

1.4.3 TECHNICAL EXAMINATIONS

1.4.3.1 DUAL-ENERGY X-RAY ABSORPTIOMETRY (DXA)

In the group of healthy boys, areal bone parameters (aBMD and bone area) at the lumbar spine and whole body, as well as whole body fat and lean mass were measured using a commercial DXA densitometer (Hologic QDR 4500, software version 11.2.1; Hologic Inc, Bedford, MA). The CV for both spine and whole-body calibration phantoms was less than 1%, as calculated from daily and weekly measurements, respectively. DXA measurements in the obese population, which were performed with a different device, are not reported in this thesis.

DXA is presently the most used method to assess aBMD as well as whole body soft tissue composition. It was first introduced for osteoporosis diagnosis and screening in postmenopausal women in the late 1980's[242]. Since the 1990's DXA measurements are also used in children. However, these measurements have some potential problems especially in growing children. Apart from the fact that bones change in size, shape and mass, the tempo of change varies by skeletal site and individual. An important pitfall of DXA measurements is the fact that it is a projection technique, making a two-dimensional image and measurement of a three-dimensional structure. The third dimension of the bone i.e. the depth is not taken into account. Larger bones with more volume will attenuate more photons and will be reported as more dense because of lack of correction for the third dimension. Therefore, children with smaller bones may have a lower aBMD than children with larger bones despite a similar vBMD. Changes in bone mass during longitudinal follow-up due to increased bone size may be misconstrued for increased bone density. Additionally bone growth of an individual bone is not uniform in the three dimensions making interpretation more challenging[243]. Moreover, bone development and thus DXA measurements in children are strongly influenced by pubertal stage and bone age, which represents a major challenge as to the establishment of relevant reference databases[244,245].

DXA has some important advantages; these include the high accessibility, the relatively low cost, the rapid scan time, low ionizing radiation dose (effective dose whole body: 1.8 µSV; effective dose lumbar spine: 2.2 µSV[246]) and the high reproducibility. The major disadvantages of DXA are the inability to obtain

separate measurements on cortical and trabecular bone, the lack of information on bone geometry and the influence of bone size on aBMD measurements as described above[247].

1.4.3.2 PERIPHERAL QUANTITATIVE COMPUTED TOMOGRAPHY (PQCT)

In both study groups, bone variables, estimates of bone strength and regional body composition of the forearm and the lower leg were measured using pQCT (Stratec XCT-2000, Stratec Medizintechnik). The scanner was positioned on the non-dominant forearm (radius) and lower leg (tibia). Two 2.0 mm slices (voxel size 0.5 mm) were measured at the sites respectively 4 and 66% of radius length proximally from the distal end of the radius and two slices at the sites 4 and 38% of tibia length proximally from the end of the tibia. The CSA of the radius/tibia was determined after detecting the outer bone contour at a threshold of 280 mg/cm^3. For determining cortical vBMD, the threshold was set at 710 mg/cm^3, whereas for trabecular bone, it was set at 180 mg/cm^3. The cortical vBMD (mg/cm^3), cortical CSA (mm^2), muscle and fat CSA (cm^2), endosteal and periosteal circumferences (mm), and cortical thickness (mm) were measured at the mid-radius (66% of bone length from the distal end) and mid-shaft tibia (38% of bone length from the distal end). The combined CSA of muscle and bone (fibula and tibia or radius and ulna) was determined at a threshold of 40 mg/cm^3 and the bone CSA was determined with the threshold set at 280 mg/cm^3. Muscle CSA was calculated by subtracting the bone CSA from the combined muscle and bone CSA. Fat CSA was calculated by subtracting the combined muscle and bone CSA from the total CSA. The strength-strain index (SSIp) of the radius 66% and the tibia 38% was calculated using the formula of Schiessl et al. (1996)[248,249]. To assess the SSIp, a threshold of 480 mg/cm^3 was used. Trabecular vBMD (mg/cm^3) and area were measured using a scan through the distal metaphysis at the radius and the tibia (at 4% of bone length). The CSA of radius and tibia was determined after detecting the outer margin; 55% of this cross-sectional bone area was peeled off to separate trabecular bone from the cortical shell. The CV for the calibration phantom was <1% as calculated form daily phantom measurements.

Peripheral QCT is a non-invasive cross-sectional imaging tool used to assess parameters of bone quality of the peripheral skeleton (forearm and lower leg). In

addition to providing a measure of tissue mineral density by calculating the three-dimensional vBMD, pQCT can make a differential assessment between cortical and trabecular bone. This is particularly interesting in children since the two types of bone tissue respond differently to stimuli such as pubertal changes, mechanical forces and disease related stresses. Moreover, it gives additional information about bone geometry, estimates of bone strength and estimates of CSA 's of muscle and fat tissue[250]. Furthermore, the radiation dose is very low (effective radiation dose at the radius and tibia is 0.1 μSV). A disadvantage of the pQCT is the difficulty to obtain repeated measurements at the same bone site in pediatric longitudinal studies due to variations in longitudinal bone growth rates. In addition movement can cause errors in locating the measurement site.

1.4.3.3 X-RAY OF THE LEFT HAND

Skeletal maturation was evaluated by the Greulich and Pyle method, developed from films taken in 1930 and 1940's[251]. This technique is still the most frequently used standard for evaluation of skeletal maturation for children older than 2 years and was found to be still applicable in a contemporary pediatric and adolescent population in the Netherlands[252]. Skeletal age reading of an X-ray of the left hand and wrist was done by two independent readers (two pediatric radiologists), both blinded for the chronological age and the mean of both readings was taken. If the difference was more than one year a third independent reading (by a trained pediatrician) was performed and the two closest estimates were retained. Skeletal age differences were calculated by subtracting the chronological age from the skeletal age: positive differences reflecting an accelerated skeletal maturation and negative differences a delayed bone maturation. The radiation dose of this examination was minimal (X-ray left hand: 0.1 μSV)[253].

1.4.3.4 MEASUREMENT OF MUSCLE STRENGTH

Muscle strength in lower limbs in the obese boys and their respective controls was evaluated by jumping mechanography, designed to measure muscle force and power by deriving measurements from an individual's ground reaction forces[254,255]. All measurements were recorded with the Leonardo Mechanography Ground Reaction Force Platform (Novotec Medical GmbH, Pforzheim, Germany). Both the multiple one-legged hopping (M1LH) and the single two-legged jump (S2LJ) were

analyzed using the Leonardo Mechanography GRFP Research Edition software version 4.2-b05.46d. M1LH represents one-legged hopping on the forefoot with the aim to achieve a maximal ground reaction force. It evaluates the maximal force to which the tibia is exposed, and thus can serve to evaluate the muscle-bone unit. The maximal force and the maximal force relative to body mass of the left and the right leg were analyzed for this hop. The S2LJ is a vertical counter-movement jump to achieve maximum jump height. Parameters of this particular analysis were jump height, peak velocity, maximal force, maximal force/body mass, maximal peak power, and maximal peak power/body mass[254]. Each subject performed three S2LJ's and the recording with the highest jump height was selected. For the M1LH a minimum of 10 accurate jumps had to be performed on each leg. All tests were performed between 10 am and 3 pm by the same observer using the same device. All subjects were fed and had exerted normal daily activity before the test. The CV for the S2LJ and the M1LH was respectively 12.7% and 5.3%[254].

1.4.4 STATISTICAL ANALYSES

For all considered parameters, normality was checked using quantile-quantile-plots (QQ-plots) and Shapiro-Wilk tests. Data are presented as mean±standard deviation or as median (25th–75th percentile) in case of a non-normal distribution. To evaluate between-group differences (obese versus normal-weighted boys), independent Student t-tests or Mann-Whitney-U tests in case of non-Gaussian distribution were used. Between-group differences of categorical variables were calculated with χ^2 tests. To study difference between pubertal stages within the healthy controls ANOVA test or Kruskal-Wallis test in case of a non-normal distribution was used. Multiple linear regression was used to assess relationships between variables taking into account the effect of possible confounding factors. When necessary, the analysis was done on Box-Cox transformed data to meet the required model assumptions. Box-Cox transformations were performed using MedCalc for Windows, version 12.5 (MedCalc Software, Ostend, Belgium). For all statistical analyses, p-values <0.05 were considered to indicate statistical significance. All descriptive statistical analyses, tests between groups and multiple linear regression analyses were performed using SPSS software version 19.0 (SPSS Inc., Chicago, IL, USA).

2 ASSOCIATIONS OF SEX STEROIDS WITH BONE MASS ACQUISITION AND SKELETAL MATURATION IN HEALTHY MALE CHILDREN AND ADOLESCENTS

2.1 RELATION OF ADRENAL-DERIVED STEROIDS WITH BONE MATURATION, MINERAL DENSITY AND GEOMETRY IN HEALTHY PREPUBERTAL AND EARLY PUBERTAL BOYS

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ABSTRACT

Background: Little is known about the effects of adrenal steroids on skeletal maturation and bone mass acquisition in healthy prepubertal boys.

Objective: To study whether adrenal-derived steroids within the physiological range are associated with skeletal maturation, areal and volumetric bone mineral density (aBMD and vBMD) and bone geometry in healthy prepubertal and early pubertal boys.

Methods: 98 healthy prepubertal and early pubertal boys (aged 6-14 y) were studied cross-sectionally. Androstenedione (A) and estrone (E1) were determined by liquid chromatography-tandem mass spectrometry and DHEAS was determined by immunoassay. Whole body and lumbar spine aBMD and bone area were determined by dual-energy X-ray absorptiometry. Trabecular (distal site) and cortical (proximal site) vBMD and bone geometry were assessed at the non-dominant forearm and leg using peripheral QCT. Skeletal age was determined by X-ray of the left hand.

Results: Adrenal-derived steroids (DHEAS, A and E1) are positively associated with bone age in prepubertal and early pubertal children, independently of age. There are no associations between the adrenal steroids and the studied parameters of bone size (lumbar spine and whole body bone area, trabecular or cortical area at the radius or tibia, periosteal circumference and cortical thickness at the radius or tibia) or BMD (aBMD or vBMD).

Conclusion: In healthy prepubertal and early pubertal boys, serum adrenal-derived steroid levels, are associated with skeletal maturation, independently of age, but not with bone size or (v)BMD. Our data suggest that adrenal-derived steroids are not implicated in the accretion of bone mass before puberty in boys.

INTRODUCTION

In boys, estradiol (E2) and testosterone (T), produced in increasing amounts during puberty, play an important role in the regulation of bone growth, bone mass acquisition and bone maturation[1,2]. The contribution of the adrenal-derived steroids, which are secreted in increasing amounts from the age of 5-6 years, has not been well studied[3,4,5]. In animal studies adrenal androgens have been shown to accelerate bone maturation and bone growth[6] and several conditions with an elevated adrenal secretion such as premature adrenarche and congenital adrenal hyperplasia are associated with an advanced skeletal maturation[7,8,9,10] and increased areal bone mineral density (aBMD)[11,12]. There is, however, little data on the possible role of adrenal-derived steroids on bone maturation, areal and volumetric bone mineral density (aBMD and vBMD) or bone geometry in prepubertal and early pubertal boys. DHEAS and androstenedione (A) can be converted to the potent androgens T and dihydrotestosterone (DHT) in target tissues, whereas A is aromatized to estrone (E1). The effects of E1, a weaker estrogen compared to E2, on bone mass accretion have not been evaluated in prepubertal boys.

Therefore, this study aims to describe for different age groups of healthy prepubertal and early pubertal boys, serum levels of adrenal-derived steroids and bone maturation, -mineral density (aBMD and vBMD) and -geometry, as well as their association. Our working hypothesis is that the rising production of adrenal-derived steroids from adrenarche might have an impact on bone maturation and accrual of bone mass and size in prepubertal and early pubertal healthy boys.

METHODS

SUBJECTS

Ninety-eight healthy male children and adolescents aged 6-14.5 years (mean age: 10.2 years) were included in this cross-sectional study. In total 65 were prepubertal Tanner genital stage 1 and 33 boys had Tanner stage 2. Eighty-one children were pre-pubarchal and 17 boys had pubic hair stage 2. Children were excluded if they were taking medication known to influence bone or mineral metabolism in the past year or if they had a metabolic bone disease, thyroid disorder or diabetes, if their height standard deviation score (SDS) was <-2.5 or >2.5 or if their BMI SDS was <-2 or >2. The study protocol was approved by the Ethics Committee of the Ghent University Hospital. Informed consent was obtained from the parents and all participants gave their assent. Participants were recruited by letters distributed in schools within the Ghent area.

METHODS

ANTHROPOMETRY AND WHOLE BODY COMPOSITION AND WHOLE BODY AND LUMBAR SPINE BONE PARAMETERS BY DXA

Information on medical history, lifestyle and socio-economic background was collected through a questionnaire. Standing height was measured to the nearest 0.1 cm using a Harpenden stadiometer (Holtain Ltd, Crymuch, UK). Body weight was measured in light indoor clothing without shoes to the nearest 0.5 kg. The length of the forearm (from the olecranon to the processus ulnaris) and the tibia (from the medial knee joint line to the tip of the medial malleolus) were measured with a ruler to the nearest 0.1 cm. All anthropometric measurements were performed by the same trained physician (SV). Pubertal status was determined by the same trained physician (SV) according to the Tanner staging method (Tanner Genital Staging: stage 1: prepuberty; stage 2: early puberty). Standard bone parameters at the lumbar spine (LS) and whole body (WB) namely LS and WB bone area and LS and WB areal BMD, as well as WB fat and lean mass were measured using DXA (Hologic QDR 4500, software version 11.2.1; Hologic Inc, Bedford, MA). Areal BMD (aBMD) is obtained by dividing bone mineral content by bone area. Since the third dimension of the bone i.e the depth is not taken into account, aBMD is strongly

bone size dependent. The coefficient of variation (CV) for both LS and WB calibration phantoms was less than 1%, as calculated from daily and weekly measurements, respectively.

BONE AGE DETERMINATION

Bone age reading of an X-ray of the left hand and wrist was done by two independent readers (a pediatric radiologist and a pediatrician), both blinded for the chronological age, using the Greulich and Pyle method[13]. The mean of both readings was taken as variable for analysis.

REGIONAL BODY COMPOSITION AND vBMD AND BONE GEOMETRY PARAMETERS BY pQCT

Standard bone parameters, estimates of bone strength[14] and regional body composition of the non-dominant forearm (radius) and the lower leg (tibia) were measured by pQCT (Stratec XCT-2000, Stratec Medizintechnik, Germany, version 6.0) which can provide three-dimensional information about bone mineral density (BMD), size and shape. A 2.0 mm slice (voxel size 0.5 mm) was performed at the 4 and 66% sites proximally from the distal end of the radius and at the 4 and 38% site proximally from the distal end of the tibia. Due to movement artefacts, radius 66% measurements of only 76 boys could be analyzed. The cross-sectional area (CSA) of the radius/tibia was determined after detecting the outer bone contour at a threshold of 280 mg/cm^3. For determining cortical vBMD, the threshold was set at 710 mg/cm^3, whereas for trabecular bone, it was set at 180 mg/cm^3. The cortical vBMD (mg/cm^3), cortical CSA (mm^2), muscle and fat CSA, endosteal and periosteal circumferences (mm), and cortical thickness (mm) were measured at the mid-radius (66% of bone length from the distal end) and mid-shaft tibia (38% of bone length from the distal end). The combined CSA of muscle and bone (fibula and tibia or radius and ulna) was determined at a threshold of 40 mg/cm^3 and the bone CSA was determined with the threshold set at 280 mg/cm^3. Muscle CSA was calculated by subtracting the bone CSA from the combined muscle and bone CSA. Fat CSA was calculated by subtracting the combined muscle and bone CSA from the total CSA. The strength-strain index (SSIp) of the radius 66% and the tibia 38% was calculated[14]. To assess the SSIp, a threshold of 480 mg/cm^3 was used. Trabecular

vBMD (mg/cm^3) and area were measured using a scan through the distal metaphysis at the radius and the tibia (at 4% of bone length). The CSA of the radius/tibia was determined after detecting the outer margin; 55% of this cross-sectional bone area was peeled off to separate trabecular bone from the cortical shell. The CV for the calibration phantom was <1% as calculated form daily phantom measurements.

HORMONAL MEASUREMENTS

Venous blood samples were collected between 0800 and 1000 h after a small breakfast. Serum samples were stored at –80°C until batch analysis. Commercial immunoassays were used to measure serum DHEAS, SHBG, LH and FSH (Modular, Roche Diagnostics, Mannheim, Germany). The intra- and interassay CV's for these assays were less than 10%. The lower detection limit for DHEAS was 5 µg/dl and the interassay CV was 2.7% at 157.3 µg/dl. E1, E2, A, T and cortisol were determined by liquid chromatography-tandem mass spectrometry (AB Sciex 5500 triple-quadrupole mass spectrometer; AB Sciex, Toronto Canada). Serum limit of quantification (LOQ) was <0.5 pg/mL (1.9 pmol/L) for E2 and E1 and the interassay CV was 4.0% at 21 pg/mL (77 pmol/L) for E2 and 7.6% at 25 pg/mL (93 pmol/L) for E1 [15]. Serum LOQ was 1.2 ng/dl for T and the interassay CV was 8.3% at 36.7 ng/dl and 3.1% at 307.8 ng/dl. Serum LOQ was 4.25 ng/dl for A and the interassay CV was 2.9% at 59.8 ng/dl. Serum LOQ was 0.05 µg/dl for cortisol and the interassay CV's were 2.3% at 7.43 µg/dl and 3.1% at 24.7 µg/dl.

STATISTICS

Normality was checked using quantile-quantile plots. Data are presented as mean±standard deviation or as medians (25th–75th percentile) in case of a non-normal distribution. Differences between the age categories were evaluated using ANOVA, when criteria for normality were met. We used LSD test as post-hoc test. In case of a non-normal distribution, Kruskal-Wallis tests were performed. The independent predictors of the various bone geometry, -density and -maturation parameters were tested using linear regression analysis including age, height for the analyses of the DXA parameters and bone length for the analyses of the pQCT parameters, body weight and serum E1, A or DHEAS levels. The difference was

considered statistically significant at $p<0.05$. Data were analyzed using SPSS software version 19.0.

Based on the available literature[16,17,18], sample size calculations were performed using G*power (version 3.1.5) (α: 0.05; ß: 0.20). We calculated a necessary sample size of 10 to 14 children in each age-group to discern the published differences in bone size between the different age groups. To detect the effects of adrenal steroids on bone density and bone size, a sample size between 73 and 125 children was needed depending on the studied parameter.

RESULTS

ANTHROPOMETRIC CHARACTERISTICS, BODY COMPOSITION AND HORMONAL PARAMETERS IN HEALTHY PRE-AND EARLY PUBERTAL BOYS ACCORDING TO AGE.

Growth, body composition and hormonal parameters of the study population classified according to age are shown in table 1 and 2. As expected, body height, body weight and body composition differ significantly between the age groups ($p<0.001$) (table 1).

Table 1: Anthropometric data and measures of body composition in prepubertal (G1) and early pubertal healthy boys (G2) (n=98) at different age groups.

	6-7 y (n=15) Mean±SD	8-9 y (n=24) Mean±SD	10-11y (n=42) Mean±SD	12-14 y (n=17) Mean±SD
Anthropometry				
Age (y)[a]	6.8±0.6	9.0±0.5	10.9±0.5	12.9±0.7
Bone age (y)[a]	6.7±0.8	8.9±1.2	10.9±1.0	12.0±1.0
Height (cm)[a]	124±4.3	137±6.9	145±6.6	151±7.2
Weight (kg)[a]	23.7±2.6	31.6±5.7	35.8±4.7	39.2±6.1
BMI (kg/m²)[b]	15.5±1.1	16.6±1.9	16.9±1.5	17.0±1.5
Body composition				
Whole body				
Lean mass (kg)[a]	18.6±2.1	24.0±3.0	27.7±3.4	30.1±4.5
Fat mass (kg)[c]	4.0±1.0	5.9±2.8	6.2±2.5	6.5±2.6
Fat percentage (%)[d]	17.1±3.8	18.3±5.5	17.4±5.6	16.5±5.2
Radius 66%				
Muscle area (cm²)[a]	1427±225	1551±170	1721±201	1904±282
Fat area (cm²)[d]	598±141	836±291	778±275	744±304
Tibia 38%				
Muscle area (cm²)[a]	1923±312	2089±294	2358±359	2468±461
Fat area (cm²)[d]	1218±267	1540±493	1472±411	1551±551

Differences between the age groups were evaluated using ANOVA. [a] $p<0.001$; [b] $p<0.01$; [c] $p<0.05$; [d] non-significant

A significant difference in DHEAS, A, E1, T, SHBG, LH, and FSH levels is present between the different age groups ($p<0.01$). Cortisol levels remain however stable during pre- and early puberty (table 2). As shown in table 2, DHEAS (by 7 fold), A (by 4 fold), and E1 (by 4 fold) levels increase significantly from age group 6-7y to age group 12-14y ($p<0.001$). After an initial decline in SHBG levels between age group 6-7 y and 8-9 y, SHBG levels remain stable. For the age-groups 6-7 y until 10-11 y E2 levels are stable. The increases in T, E2, FSH and LH levels only become apparent at the onset of puberty (age group 10-11 and 12-14 y).

Table 2: Hormonal parameters in prepubertal (G1) and early pubertal healthy boys (G2) (n=98) at different age groups.

Hormonal parameters	Age groups	n	n(G1)/n(G2)	Median (P25-P75)	Significance level (p)
DHEAS (µg/dl)	6-7 y	15	15/-	15.2 (8.1-48.4)	<0.001
	8-9 y	24	24/-	65.7 (25.0-83.9)	
	10-11 y	42	25/17	77.2 (49.3-116)	
	12-14 y	17	1/16	105 (73.9-150)	
Androstenedione (ng/dl)	6-7 y	15	15/-	5.2 (4.0-9.5)	<0.001
	8-9 y	24	24/-	10.3 (4.9-14.5)	
	10-11 y	42	25/17	15.9 (10.8-22.4)	
	12-14 y	17	1/16	20.5 (15.5-25.0)	
Estrone (ng/l)	6-7 y	15	15/-	1.4 (0.9-2.2)	<0.001
	8-9 y	24	24/-	2.8 (1.6-3.7)	
	10-11 y	42	25/17	4.7 (3.2-6.3)	
	12-14 y	17	1/16	5.9 (4.6-8.4)	
Estradiol (ng/l)	6-7 y	15	15/-	0.86 (0.5-1.2)	<0.05
	8-9 y	24	24/-	0.50 (0.5-0.6)	
	10-11 y	42	25/17	0.61 (0.5-1.0)	
	12-14 y	17	1/16	1.0 (0.5-1.9)	
Testosterone (ng/dl)	6-7 y	15	15/-	1.8 (1.1-2.7)	<0.001
	8-9 y	24	24/-	2.7 (1.9-3.9)	
	10-11 y	42	25/17	7.1 (4.7-10.8)	
	12-14 y	17	1/16	24.0 (8.3-53.3)	
Cortisol (µg/dl)	6-7 y	15	15/-	7.3 (3.9-9.5)	ns
	8-9 y	24	24/-	5.8 (4.3-7.2)	
	10-11 y	42	25/17	6.7 (5.3-9.2)	
	12-14 y	17	1/16	7.6 (6.3-8.3)	

Table 2 continued

Hormonal parameters	Age groups	n	n(G1)/n(G2)	Median (P25-P75)	Significance level (p)
SHBG (nmol/l)	6-7 y	15	15/-	171 (135-190)	<0.01
	8-9 y	24	24/-	119 (95.1-145)	
	10-11 y	42	25/17	115 (88.1-147)	
	12-14 y	17	1/16	118 (78.6-147)	
LH (U/L)	6-7 y	15	15/-	0.1 (0.1-0.1)	<0.001
	8-9 y	24	24/-	0.1 (0.1-0.1)	
	10-11 y	42	25/17	0.3 (0.1-0.8)	
	12-14 y	17	1/16	1.3 (0.3-2.0)	
FSH (U/L)	6-7 y	15	15/-	0.5 (0.4-0.9)	<0.001
	8-9 y	24	24/-	0.8 (0.4-1.1)	
	10-11 y	42	25/17	1.7 (1.2-2.1)	
	12-14 y	17	1/16	1.9 (1.3-2.5)	

Non-Gaussian distribution: data presented as median (25th-75th percentile (P25-P75)). Comparison between age groups were performed using Kruskal-Wallis tests. Conversion factor to SI-units for DHEAS from µg/dl to nmol/l is 2.714, for A from ng/dl to nmol/l is 0.0349, for E1 from ng/l to pmol/l is 3.698, for T from ng/dl to nmol/l is 0.0347, for E2 from ng/l to pmol/l is 3.671, for cortisol from µg/dl to nmol/l is 27.59.

AREAL BMD AND BONE AREA AT THE LUMBAR SPINE AND WHOLE BODY, VBMD AND BONE GEOMETRY AT THE RADIUS AND TIBIA IN PREPUBERTAL AND EARLY PUBERTAL BOYS ACCORDING TO AGE.

Figure 1 shows significantly higher LS and WB aBMD and bone area values in the successive age groups (p<0.001).

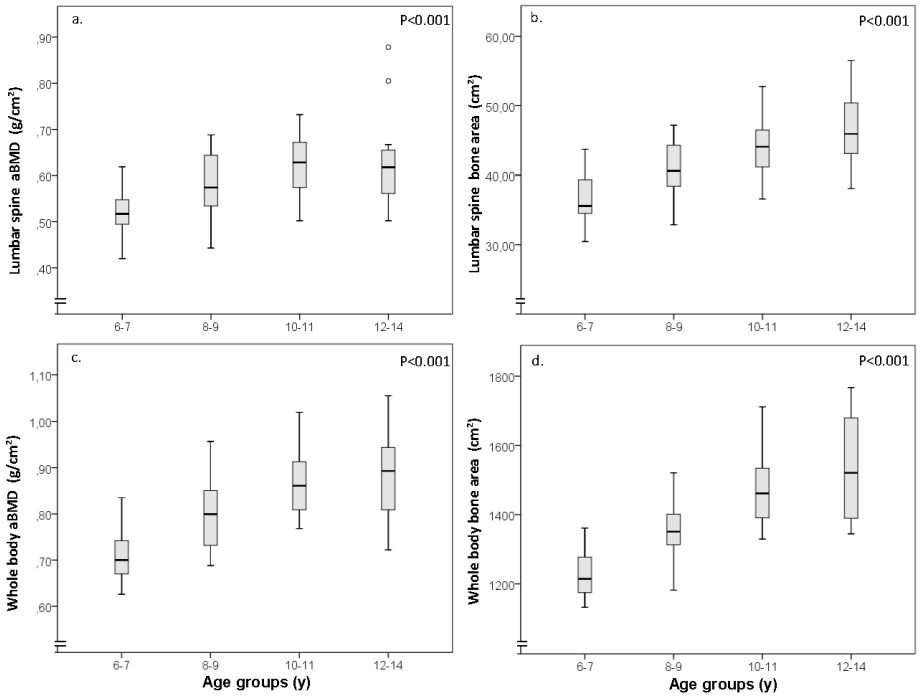

Figure 1 (a-d): Whole body and lumbar spine aBMD and bone area at different age groups. The box and whisker plots present (a) lumbar spine aBMD, (b) lumbar spine bone area, (c) whole body aBMD and (d) whole body bone area for different age groups.

As shown in figure 2 and 3, cross-sectional bone size (trabecular and cortical bone cross-sectional area (CSA), periosteal circumference and cortical thickness) at the radius and tibia are significantly larger in the successive age groups ($p<0.001$). At the distal radius and tibia, the increase in trabecular bone area between age groups 6-7 y and 12-14 y is respectively 33% ($p<0.001$) and 39% ($p<0.001$). Cortical CSA, periosteal circumference and cortical thickness at the proximal radius are respectively 35% ($p<0.001$), 8% ($p<0.05$), and 30% ($p<0.01$) larger in the age group 12-14y compared to the age group 6-7y. Cortical CSA, periosteal circumference, endosteal circumference and cortical thickness at the tibia are respectively 39% ($p<0.001$), 20% ($p<0.001$), and 25% ($p<0.001$) larger in the age group 12-14y compared to the age group 6-7y. As a consequence of these changes, there is a significant increase in estimated bone strength as measured by the strength-strain index (SSIp) between age group 6-7 y and age group 12-14 y (SSIp : +50% at tibia and +37% at radius). Trabecular and cortical vBMD at the radius and tibia remain stable during prepuberty and early puberty.

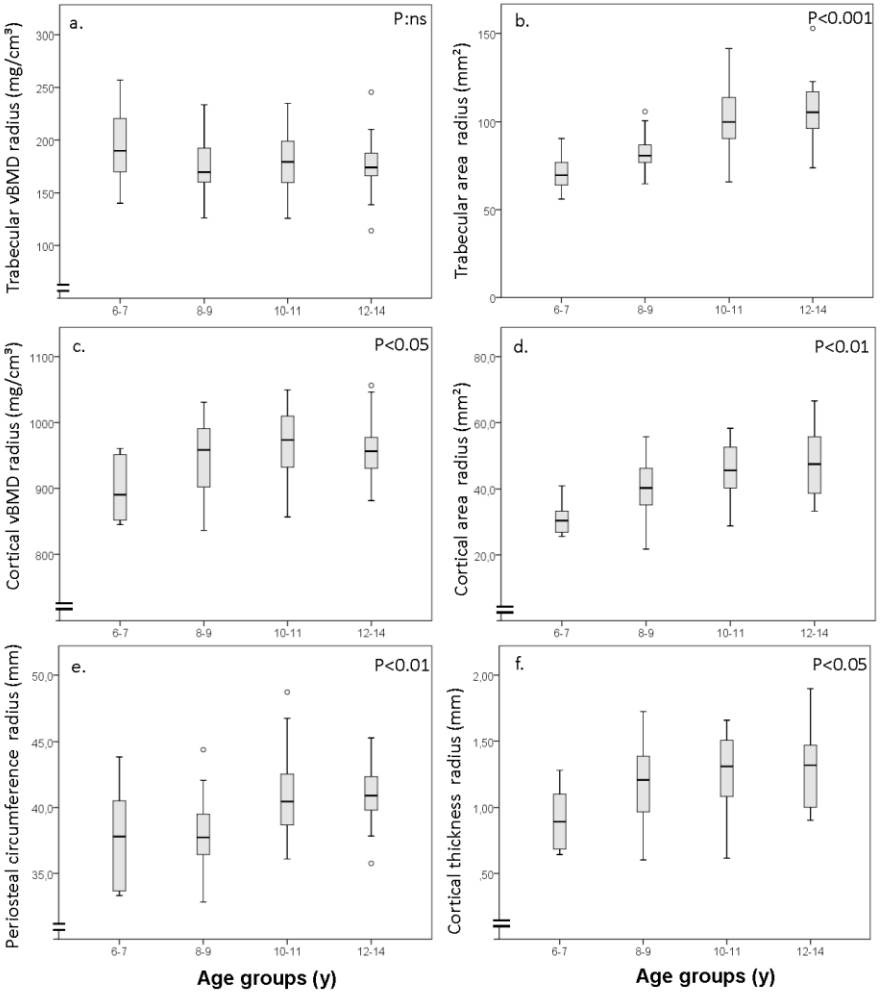

Figure 2 (a-f): Trabecular and cortical bone parameters at the radius at different age groups. The box and whisker plots present (a) trabecular vBMD, (b) trabecular area, (c) cortical vBMD, (d) cortical area, (e) periosteal circumference and (f) cortical thickness at the radius at different age groups.

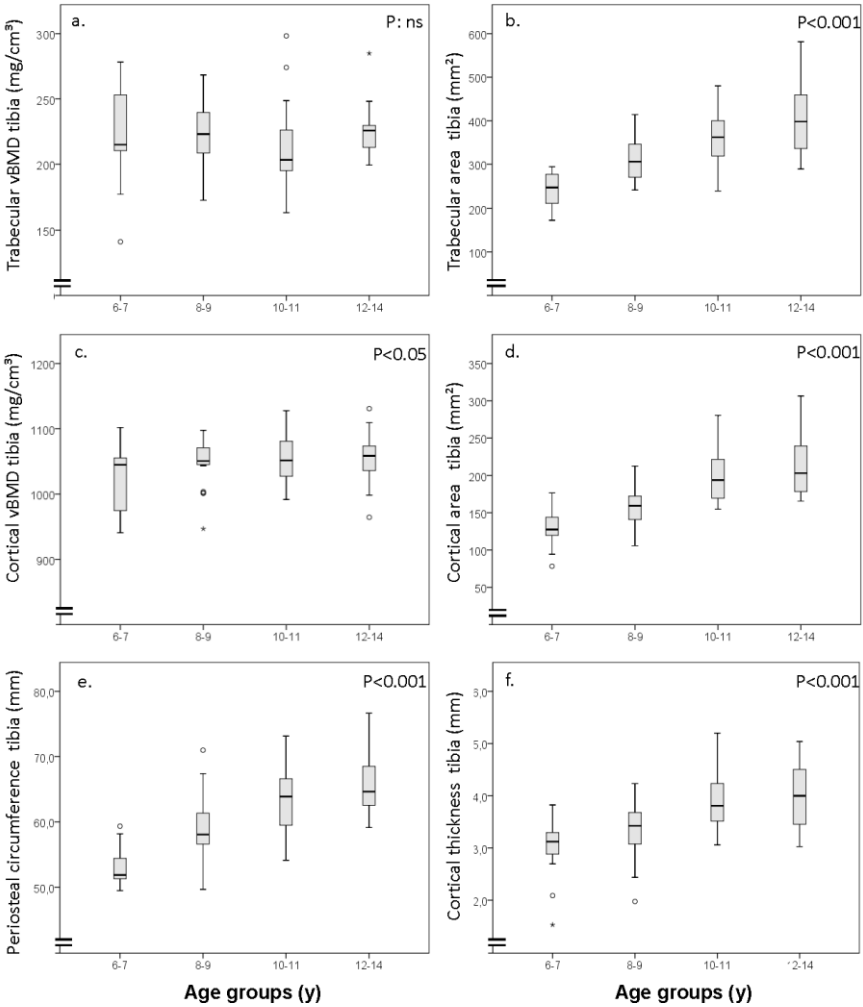

Figure 3 (a-f): Trabecular and cortical bone parameters at the tibia at different age groups. The box and whisker plots present (a) trabecular vBMD, (b) trabecular area, (c) cortical vBMD, (d) cortical area, (e) periosteal circumference and (f) cortical thickness at the radius at different age groups.

ADRENAL STEROIDS AS INDEPENDENT PREDICTORS OF BONE MATURATION.

DHEAS, A and E1 are highly correlated (spearman correlation coefficient (r) r(DHEAS-A)=0.80 p<0.001; r(DHEAS-E1)=0.77 p<0.001; r(A-E1)=0.86 p<0.001). In multivariable-adjusted analysis (including age, body weight and height) adrenal-derived steroids (DHEAS, A, E1) are found to be independent positive predictors of bone age (DHEAS: ß=0.24 p<0.001; A: ß=0.23 p<0.001; E1: ß=0.23 p<0.001). The positive association between E1 and bone age remains after inclusion of E2 in the model (E1: ß=0.23 p<0.001) and the positive association between A and bone age remains after inclusion of T in the model (A: ß=0.23 p<0.001). Similar results are found when studying only the prepubertal group (DHEAS: ß=0.31 p<0.001; A: ß=0.29 p<0.001; E1: ß=0.31 p<0.001). The positive association between DHEAS and bone age remains after inclusion of E1 (DHEAS: ß=0.19 p<0.01) and A (DHEAS: ß=0.18 p<0.01) in the model. The positive association between A and bone age remains after inclusion of E1 in the model (A: ß=0.16 p<0.05), but disappear after inclusion of DHEAS. The positive association between E1 and bone age disappears after inclusion of A or DHEAS in the model.

ADRENAL STEROIDS AS INDEPENDENT PREDICTORS OF aBMD AND BONE AREA OF THE LUMBAR SPINE AND WHOLE BODY AND OF THE vBMD AND BONE GEOMETRY PARAMETERS AT THE RADIUS AND TIBIA.

All multivariable adjusted analyses are corrected for age, body weight, height (for the DXA parameters) and bone length (for pQCT analysis). There is no association between DHEAS and either parameters of bone size (WB and LS bone area, trabecular area at the radius and tibia, cortical area at the radius and tibia, periosteal circumference at the radius and the tibia, cortical thickness at the radius and tibia; p=ns), BMD (WB and LS aBMD, trabecular vBMD at the radius and tibia, cortical vBMD at the radius and tibia; p=ns), or parameters of bone strength (SSIp at radius or tibia; p=ns). A is not associated with parameters of bone size (WB and LS bone area, trabecular area at the radius and tibia, cortical area at the radius and tibia, periosteal circumference at the radius and the tibia, cortical thickness at the radius and tibia; p=ns) or bone strength (SSIp at radius or tibia; p=ns). Furthermore, there is no association between A and BMD (WB aBMD, trabecular vBMD at the radius and tibia, cortical vBMD at the radius and tibia; p=ns), except

for lumbar spine aBMD (A: ß= 0.22 p<0.05). E1 is not associated with parameters of bone size (WB and LS bone area, trabecular area at the radius and tibia, cortical area at the radius and tibia, periosteal circumference at the radius and the tibia, cortical thickness at the radius and tibia; p=ns), bone strength (SSIp at the radius and tibia p=ns) or BMD (WB and LS aBMD, trabecular vBMD at the radius and tibia, cortical vBMD at the radius and tibia; p=ns).

DISCUSSION

This cross-sectional study presents data on the age-related differences in adrenal steroids levels, body composition, (v)BMD and bone geometry in healthy pre- and early pubertal boys, enabling the study of the associations of circulating adrenal-derived steroids levels (DHEAS, A, E1) with bone maturation, growth and mineral density in childhood and early puberty. To the best of our knowledge, this is the first study to investigate the role of circulating adrenal-derived sex steroids in bone mass accretion and bone maturation in healthy prepubertal and early pubertal boys.

Firstly, we found –as previously described by others- a significant increase in adrenal androgens namely DHEAS and A from the age of 6 onwards[18,19,20,21]. While no significant increase in E2 was observed before puberty in our male population, E1 increased 4 fold between 6 and 14 years. In contrast with the increase in A and DHEAS, we observed no age-related difference in cortisol levels. This is in line with the results of other studies showing stable cortisol levels during adrenarche[22,23].

Secondly, there was a significant increase in LS and WB aBMD and bone area during pre-and early puberty. Furthermore, trabecular and cortical CSA, periosteal circumference and cortical thickness at the radius and tibia increased significantly at the successive age groups, as described previously[16,24,25]. Since trabecular and cortical vBMD at the radius and tibia remained stable during the prepubertal period, the observed increases in WB and LS aBMD in prepubertal boys reflect the increase in bone size rather than in true vBMD.

Thirdly, the associations between adrenal-derived steroids and (v)BMD and bone geometry were studied. Since it is well-known that bone mineral content and cross-sectional bone size increase with age, body height and weight[26], all

multivariate analyses were adjusted for age, weight and height or bone length depending on the analysis. No association between the adrenal-derived steroids (A, DHEAS and E1) and bone size or (v)BMD was found. Whether adrenal androgens within physiological range may contribute to changes in bone geometry has been previously studied by Remer et al. in 59 healthy prepubertal boys using the urinary excretion of the major urinary androgen (C19) metabolites[3,4]. While in this study positive effects of DHEA and its 16-hydroxylated downstream metabolites on cortical vBMD and bone mineral content at the proximal radial diaphyseal bone were observed in prepubarchal children, no effect on the metaphyseal site was observed[3,4]. Their finding that of all adrenal androgen metabolites studied, only androstenediol, which acts as an estrogen and androgen receptor agonist, showed a long term prediction of bone strength suggests that adrenal DHEA increases are not bone anabolic per se[17]. In a study of 118 boys, 7 to 8 years old, no independent association of lumbar spine and femoral neck bone mass with DHEAS, E2 or T was found[5]. In this study, however, standard commercial radio-immunoassays were used for E2 and T measurements[5].

Finally, the influence of adrenal-derived steroids on bone maturation was studied. Serum adrenal-derived steroids (DHEAS, A, E1) were positively associated with bone maturation, independently of chronological age in the studied group of prepubertal and early pubertal boys. These associations were confirmed in a subgroup of only prepubertal boys and the associations of E1 and A with bone age remained after inclusion of respectively E2 and T in the model. We expected that the observed effects of adrenal androgens on bone maturation would be mainly due to E1, as no increase in E2 was observed in prepubertal boys and higher E1 levels in prepubertal girls in comparison with boys might contribute to their greater rate of skeletal maturation. However, after inclusion of DHEAS or A in the model the association between E1 and bone age disappeared. Possible explanations for this finding are local aromatization of androgens to estrogens at level of the growth plate[27,28] or technical issues. As to the latter, it can be mentioned that a highly performant assay[15] was used for measurement of E1 (and E2) serum levels. In any case, it should be pointed out that A, DHEAS and E1 are highly intercorrelated (r=0.77 to 0.86) so that it might not be possible to reliably estimate their relative contribution. No other studies have investigated the

association between adrenal steroids and bone age in prepubertal healthy children. Our results might enhance the understanding of bone age advancement in obese prepubertal children. It is well-known that bone age is advanced in obesity[29,30,31]. Previous research from our group showed that the advanced bone maturation in obese adolescents is probably due to the higher E2 levels as a consequence of an increased aromatization in fat tissue[32] (see chapter 3.1 and 3.2). However, bone maturation is already advanced throughout childhood[30]. Based on our present results, we hypothesize that the advanced bone maturation in prepubertal obese children is due to higher adrenal steroid levels (DHEAS, A) in these children as reported by Reinehr et al. (2013)[31]. On the other hand, patients with anorexia nervosa[33] or malnutrition[34,35] known to be associated with low DHEAS and androstenedione concentrations[36,37] often have a delayed bone maturation. Thus, alterations in adrenal steroids secretion pattern may affect progression of bone age at both extremes of the weight spectrum.

The strength of the present study is the comprehensive evaluation of (v)BMD, bone geometry, and hormonal factors. Moreover, A, T, E1 and E2 were measured by highly sensitive and accurate mass spectroscopy-based methodology as required when studying low androgen and estrogen serum levels in children and adolescents. Our study also has some limitations. Firstly, adrenal steroid levels were single point measurements executed between 8 and 10 am. Although it is possible that a single measurement is not fully representative for adrenal steroid exposure, in clinical settings, a single measurement from a serum sample drawn in early morning is commonly used and acceptable. A second limitation is the fact that not all children had a valid radius 66% measurement due to movement artefacts and one might speculate that this has influenced our results. However, we are rather confident that it is not the case since none of the associations between adrenal steroids and bone size measurements at the radius 66% showed any trend towards significance and the results of the radius 66% were in line with all other bone size measurements obtained without missing data. Finally, one should emphasize that this is a cross-sectional study that gives only information about associations and does not allow us to draw causative conclusions. In addition, we want to stress that we only studied healthy prepubertal and early pubertal boys and therefore one has to be careful when generalizing our results to other age groups, girls or pathological situations because the associations between adrenal-

derived steroids and bone mineral density, size and maturation may be age-, sex- and level-specific. In order to confirm our findings and further unravel the underlying mechanisms, prospective longitudinal studies are required and further research in healthy girls and conditions associated with altered androgen and/or estrogen levels such as obesity and anorexia nervosa, would be interesting.

In conclusion, our findings showed a significant positive association between adrenal-derived steroids (DHEAS, A and E1) and bone age, and might contribute to the understanding of bone age advancement in prepubertal obese children. In the absence of associations between adrenal-derived steroids and either (v)BMD or bone size, our data provide no evidence for a direct bone-anabolic effect of these hormones in pre-or early pubertal boys.

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2.2 ASSOCIATIONS OF SEX STEROIDS WITH BONE MATURATION, BONE MINERAL DENSITY, BONE GEOMETRY AND BODY COMPOSITION: A CROSS-SECTIONAL STUDY IN HEALTHY MALE ADOLESCENTS

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ABSTRACT

Background: Although both testosterone (T) and estradiol (E2) are considered essential in the regulation of the male skeleton, there are few data concerning the relative contribution of T and E2 on bone mineral density (BMD), bone geometry and bone maturation in healthy boys.

Objective: To analyze the relationship between T and E2 and BMD, bone geometry, skeletal maturation and body composition.

Methods: This is a cross-sectional study in 199 healthy boys (aged 6-19 y). T and E2 were determined by liquid chromatography-tandem mass spectrometry. Whole body and lumbar areal bone mineral density (aBMD) and bone area, lean mass and fat mass were determined by dual-energy X-ray absorptiometry. Trabecular (distal site) and cortical (proximal site) volumetric BMD (vBMD) and bone geometry were assessed at the non-dominant forearm and leg using peripheral QCT. Skeletal age was determined by X-ray of the left hand.

Results: T was positively associated with lean mass ($p<0.001$), lumbar and whole body bone area ($p<0.001$), trabecular and cortical area ($p<0.01$) and periosteal circumference ($p<0.01$) at the radius. E2 was positively associated with lumbar and whole body aBMD ($p<0.001$), trabecular vBMD at the radius and tibia ($p<0.01$) and cortical thickness at the radius ($p<0.05$). E2 was an independent negative predictor of the endosteal circumference ($p<0.01$). Moreover, E2 was positively associated with bone age advancement ($p<0.001$).

Conclusion: Circulating E2 is positively associated with bone maturation and areal and volumetric BMD and negatively with endosteal circumference in healthy boys, whereas T is a determinant of lean mass and bone size. These findings underscore the important role of E2 in skeletal development in boys.

INTRODUCTION

From infancy until young adulthood there is a progressive accumulation of bone mass in males. A crucial stage in bone mass acquisition is adolescence as it has been estimated that about 40% of peak bone mass is achieved during pubertal development[1]. Sex steroids, GH–IGF-1 axis and muscle mass[2] are the main determinants of pubertal bone mass. Clinical conditions as delayed puberty and primary and secondary hypogonadism stress the importance of sex steroids in bone mass accrual[2,3,4,5]. The relative contribution of androgens versus estrogens in the regulation of the build-up of the male skeleton is yet to be fully clarified. Testosterone (T) can act directly through the androgen receptor or indirectly through aromatization to estrogens and further through estrogen receptor (ER) alfa and/or beta[6]. Only very scarce data concerning the effects of T and estrogens on bone maturation, bone mineral density (BMD) and bone geometry in healthy boys at different pubertal stages have been published[1,7]. In most of these studies, BMD and bone area were evaluated by dual-energy x-ray absorptiometry (DXA)[1,7] and sex steroids were measured by immunoassays[7]. An important limitation of DXA studies is the size dependence of the areal BMD (aBMD in g/cm^2) and the lack of data on bone geometry. Consequently, there is no information at present on the possible relationship of serum sex steroid levels to trabecular versus cortical bone compartments or to bone geometric parameters in growing children and adolescents. Therefore, peripheral quantitative computed tomography (pQCT) is a useful approach in bone strength analysis, particularly during the pubertal growth spurt, because it can provide 3-dimensional information about volumetric BMD (vBMD in g/cm^3), bone size, and bone shape[8].

This study aims to describe differences in (v)BMD, bone geometry and sex steroids in healthy boys at different pubertal stages. Moreover, it investigates whether levels of estradiol (E2) and T are independently associated with geometric and densitometric properties of radius and tibia. Based on the available results from bone studies in sex steroid receptor inactivated transgenic mouse models[9,10], some human experiments of nature[11,12,13,14] and in healthy young adults[15,16], we hypothesized that during adolescence circulating E2 levels would be associated with BMD and bone maturation and circulating T with bone size.

METHODS

SUBJECTS

One hundred and ninety-nine healthy male children and adolescents aged 6-19 years (mean age: 12.5 years) were included in this cross-sectional study. Participants were recruited by letters distributed in schools within the Ghent area. Children were excluded if they were taking medication known to influence bone or mineral metabolism in the past year or if they had a metabolic bone disease, thyroid disorder or diabetes, if their height standard deviation score (SDS) was <-2.5 or >2.5 or if their BMI SDS was <-2 or >2. The study protocol was approved by the Ghent University Hospital Ethical Committee. Informed consent was obtained from the parents and all participants gave their assent.

METHODS

ANTHROPOMETRY AND WHOLE BODY COMPOSITION AND AREAL BONE PARAMETERS BY DXA

Information about medical history, lifestyle and socio-economic background was collected through a questionnaire. Calcium intake was estimated by a food questionnaire on dairy products accounting for the number of standard portions per week. Physical activity was assessed using the Flemish Physical Activity Questionnaire[17,18]. Standing and sitting height were measured to the nearest 0.1 cm using a Harpenden stadiometer (Holtain Ltd, Crymuch, UK). Body weight was measured in light indoor clothing without shoes to the nearest 0.5 kg. The length of the forearm (from the olecranon to the processus ulnaris) and tibia (from the medial knee joint line to the tip of the medial malleolus) was measured with a ruler to the nearest 0.1 cm. All anthropometric measurements were performed by the same trained physician. The SDS for body height and BMI was computed using the reference data of the 2004 Flemish growth study[19]. Pubertal status was determined by the same trained physician according to the Tanner staging method (Tanner Genital Staging: stage 1: prepuberty; stage 5: post puberty). Testicular volume was determined with a Prader orchidometer. Areal bone parameters at the lumbar spine (LS) and whole body (WB), as well as WB fat and lean mass were measured using DXA (Hologic QDR 4500, software version 11.2.1; Hologic Inc,

Bedford, MA). The coefficient of variation (CV) for both LS and WB calibration phantoms was less than 1%, as calculated from daily and weekly measurements, respectively.

BONE AGE DETERMINATION

Bone age reading of an X-ray of the left hand and wrist was done by two independent readers (a pediatric radiologist and a pediatrician), both blinded for the chronological age, using the Greulich and Pyle method[20] and the mean of both readings was taken. Skeletal age differences (SAD) were calculated by subtracting the chronological age (CA) from the skeletal age (BA) (SAD=BA–CA), positive differences reflecting an accelerated skeletal maturation.

REGIONAL BODY COMPOSITION, vBMD AND BONE GEOMETRY PARAMETERS BY pQCT

Bone variables, estimates of bone strength[21] and regional body composition of the non-dominant forearm (radius) and the lower leg (tibia) were measured using pQCT (Stratec XCT-2000, Stratec Medizintechnik, Germany, version 6.0). Two 2.0 mm slices (voxel size 0.5 mm) were performed at the 4 and 66% sites proximally from the distal end of the radius and two slices at the 4 and 38% site proximally from the distal end of the tibia. The CV for the calibration phantom was <1% as calculated from daily phantom measurements. Procedure details were as described previously[22].

HORMONAL MEASUREMENTS

Venous blood samples were collected between 0800 and 1000 h after a small breakfast. Serum samples were stored at – 80°C until batch analysis. Commercial immunoassays were used to measure serum IGF-1 (Diagnostic Systems Laboratories, Webster, TX) and SHBG (Modular, Roche Diagnostics, Mannheim, Germany). The intra- and interassay CV's for these assays were less than 10%. E2 and T were determined by liquid chromatography-tandem mass spectrometry (AB Sciex 5500 triple-quadrupole mass spectrometer; AB Sciex, Toronto Canada). Serum limit of quantification (LOQ) was <0.5 pg/mL (1.9 pmol/L) for E2 and the interassay CV was 4.0% at 21 pg/mL (77 pmol/L)[23]. Serum LOQ was 1.2 ng/dl for

T and the interassay CV was 8.3% at 36.7 ng/dl and 3.1% at 307.8 ng/dl. Free T (FT) was determined by equilibrium dialysis [24] and free E2 (FE2) was calculated from total E2, SHBG and albumin concentrations using a previously validated equation derived from the mass action law[25].

STATISTICS

Normality was checked using quantile-quantile plots and Shapiro-Wilk tests. Data are presented as mean±standard deviation or as medians (25th–75th percentile) in case of a non-normal distribution. Differences between pubertal stages were evaluated using ANOVA, when criteria for normality were met. We used LSD test as post-hoc test. In case of a non-normal distribution, Kruskal-Wallis tests were used. The independent predictors of various bone and body composition parameters were tested using linear regression analysis including age, height for the analyses of the DXA parameters and bone length for the analyses of the pQCT parameters, weight and serum E2 or T. The difference was considered statistically significant at $p<0.05$. Data were analysed using SPSS software version 19.0.

RESULTS

ANTHROPOMETRIC CHARACTERISTICS AND BODY COMPOSITION

Growth parameters and body composition results of the study population classified according to pubertal stage are shown in table 1. As expected, body height, weight and body composition differ significantly between different pubertal stages ($p<0.001$). As shown in figure 1, there is a major increase in WB lean mass and to smaller extent an increase in fat mass at the successive pubertal stages, resulting in a decrease of fat percentage from stage 3 onward. Regional body composition at the forearm and tibia follows a similar pattern (table 1; figure 1).

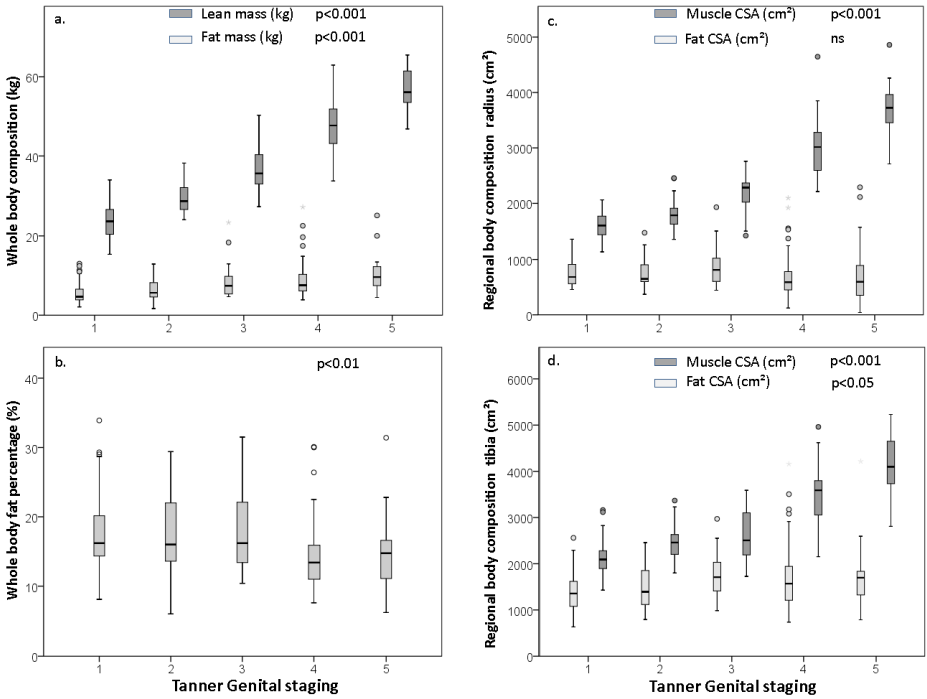

Figure 1: Lean mass and fat mass at different pubertal stages. (a) The box and whisker plots present whole body lean mass (dark-grey box) and fat mass (light-grey box) at different pubertal stages. The box and whisker plots present (b) whole body fat percentage, (c) muscle cross-sectional area (dark-grey box) and fat cross-sectional area (light-grey box) at the radius (66% from the distal end), (d) muscle cross-sectional area (dark-grey box) and fat cross-sectional area (light-grey box) at the mid-shaft tibia (38% of bone length from the distal end) at different pubertal stages.

Table 1: Anthropometric data and measures of body composition in healthy boys (n=199) at different pubertal stages

	Genital Tanner stage				
	1 (n=67)	**2 (n=35)**	**3 (n=22)**	**4 (n=49)**	**5 (n=26)**
Anthropometry	**Mean±SD**	**Mean±SD**	**Mean±SD**	**Mean±SD**	**Mean±SD**
Age (y)[a]	9.2±1.7	11.9±1.3	13.3±0.7	14.9±1.1	16.7±1.3
Bone age (y)[a]	9.0±2.0	11.5±1.0	13.1±0.4	15.0±1.4	17.6±1.3
Height (cm)[a]	136±10.3	150±6.6	160±10.2	171±7.9	179±5.8
Weight (kg)[a]	30.9±6.5	38.0±5.4	47.8±9.6	59.5±10.5	70.1±7.8
BMI (kg/m²)[a]	16.4±1.6	16.9±1.5	18.5±2.3	20.2±2.9	21.9±2.4
Sitting height (cm)[a]	71±4.8	76±3.2	81±4.5	89±4.4	94±2.6
Testicular volume (ml)[a]	3±1	6±2	11±2	18±4	24±2
Body composition	**Mean±SD**	**Mean±SD**	**Mean±SD**	**Mean±SD**	**Mean±SD**
Whole body					
Lean mass (kg)[a]	23.8±4.5	29.6±3.9	36.8±5.8	47.9±6.8	56.6±5.0
Fat mass (kg)[a]	5.5±2.4	6.4±2.6	8.7±4.6	8.9±4.7	10.4±4.4
Fat percentage (%)[b]	18±5	17±5	17±6	15±5	15±5
Radius 66%					
Muscle area (cm²)[a]	1613±215	1808±261	2196±348	3002±504	3704±483
Fat area (cm²)[d]	784±284	744±271	891±365	717±423	673±485
Tibia 38%					
Muscle area (cm²)[a]	2131±361	2451±402	2611±523	3490±568	4128±664
Fat area (cm²)[c]	1419±420	1523±488	1760±505	1717±741	1689±692

Differences between the pubertal stages were evaluated using ANOVA, since criteria for normality were met. [a]p<0.001; [b] p<0.01; [c] p<0.05; [d]non-significant.

Table 2: Hormonal data in healthy boys (n=199) at different pubertal stages

	Genital Tanner stage				
	1 (n=67)	**2 (n=35)**	**3 (n=22)**	**4 (n=49)**	**5 (n=26)**
Hormonal measurements	**Median (P25-P75)**	**Median (P25-P75)**	**Median (P25-P75)**	**Median (P25-P75)**	**Median (P25-P75)**
Estradiol (ng/l)[a]	0.5 (0.5-1.0)	0.8 (0.5-1.5)	3.9 (2.3-6.4)	12 (8.3-16.4)	16 (14.1-22.2)
Free Estradiol (ng/l)[a]	0.006 (0.004-0.01)	0.007 (0.005-0.015)	0.05 (0.03-0.09)	0.2 (0.15-0.31)	0.3 (0.27-0.44)
Testosterone (ng/dl)[a]	3.6 (2.1-6.0)	14.1 (6.0-36.8)	234 (175-315)	411 (336-502)	520 (455-624)
Free Testosterone (ng/dl)[a]	0.02 (0.01-0.04)	0.12 (0.04-0.26)	2.4 (1.4-5.0)	7.8 (6.0-9.3)	11.9 (9.4-13.6)
SHBG (nmol/l)[a]	126 (102-168)	118 (81-146)	80 (49-93)	38 (31-50)	35 (27-40)
IGF-1 (ng/ml)[a]	179 (127-210)	236 (183-277)	353 (281-432)	474 (421-558)	421 (367-507)

Differences between the pubertal stages were evaluated using Kruskal-Wallis tests. [a]$p<0.001$.

AREAL BMD AND BONE AREA AT THE LUMBAR SPINE AND WHOLE BODY, VBMD AND BONE GEOMETRY AT THE RADIUS AND TIBIA ACCORDING TO PUBERTAL STAGES.

Figure 2 shows mean values of LS and WB aBMD and bone area at different pubertal stages; aBMD and `bone area values are significantly higher at successive pubertal stages ($p<0.001$). The mean increase between prepuberty and post puberty in aBMD at LS and WB is respectively 38% ($p<0.001$) and 28% ($p<0.001$). Bone area increases respectively 40% ($p<0.001$) at LS and 39% ($p<0.001$) at WB.

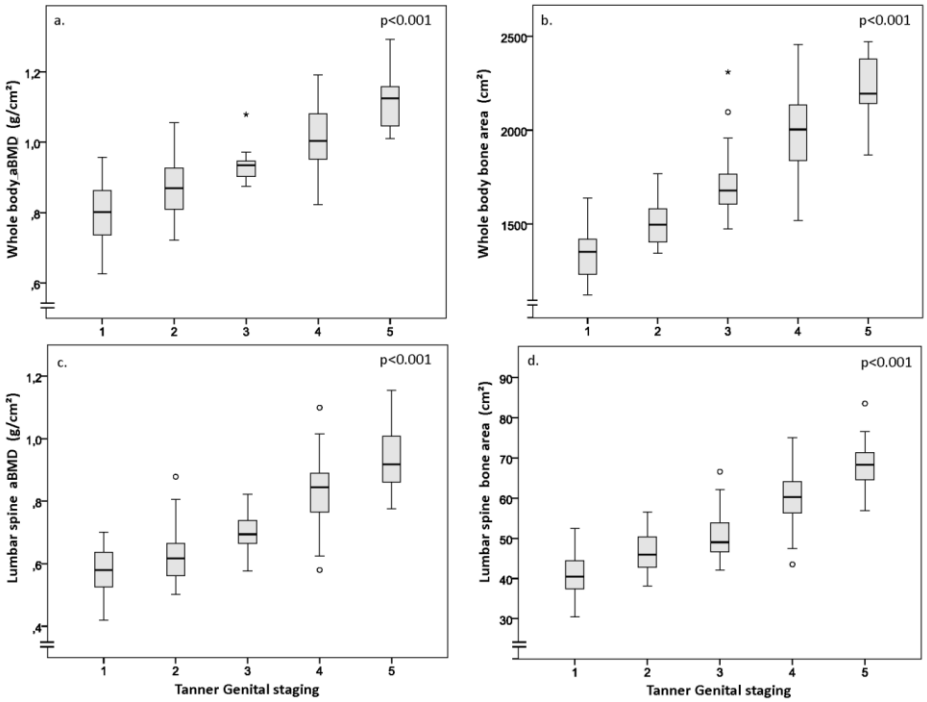

Figure 2 (a-d): Whole body and lumbar spine bone area and aBMD at different pubertal stages. The box and whisker plots present (a) whole body aBMD, (b) whole body bone area, (c) lumbar spine aBMD and (d) lumbar spine bone area at different pubertal stages.

As shown in figure 3 and figure 4, cross-sectional bone size (trabecular and cortical bone cross-sectional area (CSA), periosteal circumference and cortical thickness) and cortical vBMD are significantly different at the successive pubertal stages ($p<0.001$). At the distal radius and tibia, the increase in trabecular bone area between prepuberty and post puberty is more than 40% ($p<0.001$). Post pubertal cortical CSA, periosteal circumference, endosteal circumference and cortical thickness are respectively 46% ($p<0.001$), 18% ($p<0.001$), 11% ($p<0.01$), and 30% ($p<0.001$) larger at the proximal radius and tibia compared to the prepubertal stage. Trabecular vBMD at the radius and tibia are rather stable during puberty; however, there is a 10% ($p<0.01$) difference in trabecular vBMD between post pubertal and prepubertal boys. As a consequence of these changes, a significant increase in estimated bone strength between stage 1 to stage 5 is present (SSIp: +150% at tibia and radius).

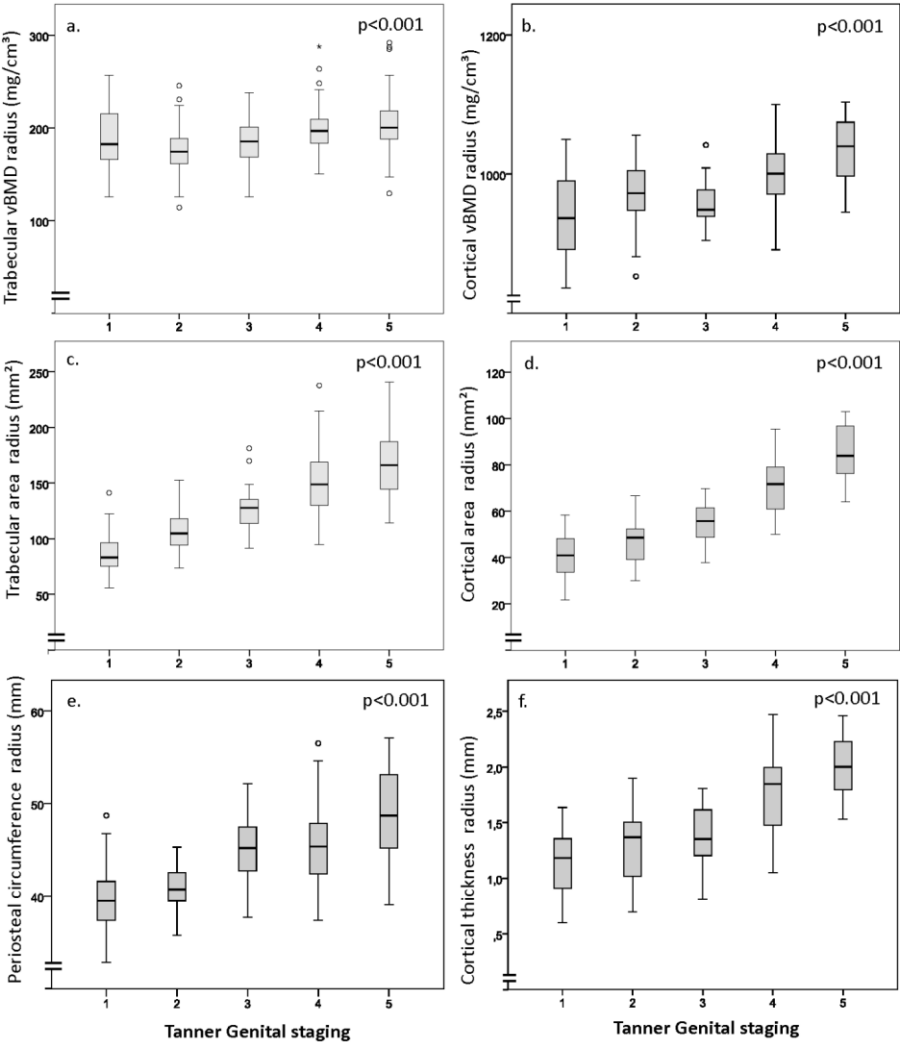

Figure 3 (a-f): Trabecular and cortical bone parameters at the radius at different pubertal stages. The box and whisker plots present (a) trabecular vBMD, (b) cortical vBMD, (c) trabecular area, (d) cortical area, (e) periosteal circumference and (f) cortical thickness at the radius at different pubertal stages.

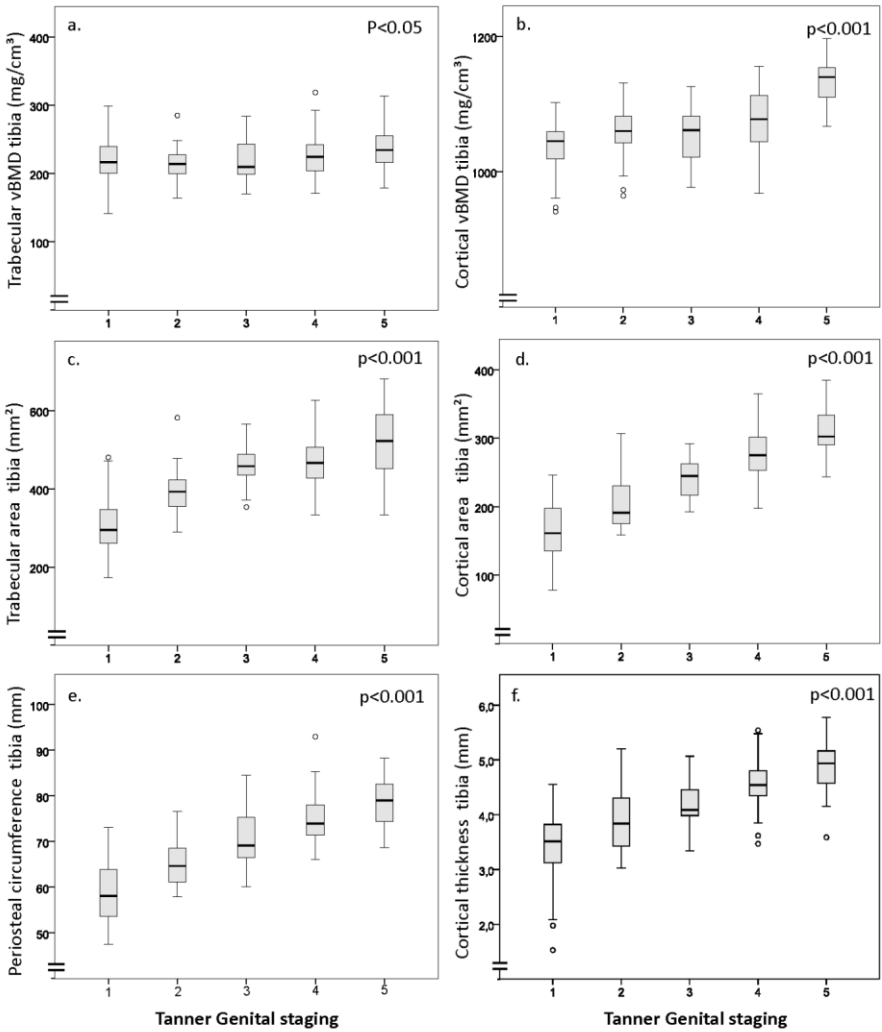

Figure 4 (a-f): Trabecular and cortical bone parameters at the tibia at different pubertal stages. The box and whisker plots present (a) trabecular vBMD, (b) cortical vBMD, (c) trabecular area, (d) cortical area, (e) periosteal circumference and (f) cortical thickness at the tibia at different pubertal stages.

SEX STEROIDS AS INDEPENDENT PREDICTORS OF BODY COMPOSITION AND BONE MATURATION.

As expected, higher sex steroids ((F)E2 and (F)T) levels are found with advancing pubertal development (table 2) ($p<0.001$). On the contrary, SHBG levels are significantly lower at the successive pubertal stages (table 1) ($p<0.001$).

In multivariable-adjusted analyses (including age, height and weight) we observe significant positive correlations of T as well as FT with WB lean mass (T: ß=0.13 $p<0.001$; FT: ß=0.14 $p<0.001$) and muscle CSA at the radius (T: ß=0.38 $p<0.001$; FT: ß=0.41 $p<0.001$) and the tibia (T: ß=0.15 $p<0.05$; FT: ß=0.19 $p<0.05$). We observe a negative association between respectively T and FT and the fat percentage (T: ß=-0.57 $p<0.001$; FT: ß=-0.66 $p<0.001$). These associations remain significant after inclusion of IGF-1 in the model ($p<0.05$).

In a regression model including height and weight, E2 and FE2 are found to be independent predictors of bone age advancement (E2: ß: 0.31 $p<0.01$; FE2: ß: 0.35 $p<0.01$). These associations remain significant after inclusion of T ($p<0.01$) or IGF-1 ($p<0.01$) in the model.

SEX STEROIDS AS INDEPENDENT PREDICTORS OF aBMD AND BONE AREA OF THE LUMBAR SPINE AND WHOLE BODY AND OF vBMD AND BONE GEOMETRY OF THE RADIUS AND TIBIA.

Regression models including age, height and weight, show that T and FT are positively associated with LS bone area (T: ß=0.26 $p<0.001$; FT: ß=0.26 $p<0.001$) and WB bone area (T: ß=0.12 $p<0.001$; FT: ß=0.15 $p<0.001$) (table 3). Moreover, in multivariable-adjusted analyses (including age, forearm length and weight) we observe significant positive associations of T as well as FT and trabecular area (T: ß=0.24 $p<0.001$; FT: ß=0.18 $p<0.05$), cortical area (T: ß=0.20 $p<0.01$; FT: ß=0.25 $p<0.01$) and periosteal circumference at the radius (T: ß=0.24 $p<0.01$; FT: ß=0.12 ns) (table 3). All these associations remain significant after controlling for physical activity ($p<0.05$), calcium intake ($p<0.05$) or inclusion of IGF-1 ($p<0.05$) in the model. After inclusion of E2 in the model, the associations remain significant ($p<0.01$) expect for the association (F)T and cortical area at the radius and T and

WB area. Significant associations between T and FT and bone area parameters were no longer present after inclusion of lean mass (in the analyses of WB and LS area) or muscle CSA (in the analyses of bone area at the radius) in the model. No significant associations could be found between T or FT and trabecular bone area, cortical bone area and periosteal circumference at the tibia.

Table 3: Sex steroids as independent predictors of aBMD and bone area of the lumbar spine and whole body and of the vBMD and bone geometry of the radius and tibia.

	Testosterone		Free Testosterone	
	ß	p	ß	p
Whole body area	0.12	<0.001	0.15	<0.001
Lumbar spine area	0.26	<0.001	0.26	<0.001
Radius				
Trabecular area 4%	0.24	<0.001	0.18	<0.05
Cortical area 66%	0.20	<0.01	0.25	<0.01
Periosteal circumference 66%	0.24	<0.01	0.12	ns
Tibia				
Trabecular area 4%	-0.02	ns	-0.19	ns
Cortical area 38%	0.03	ns	-0.03	ns
Periosteal circumference 38%	0.001	ns	-0.1	ns
	Estradiol		**Free Estradiol**	
	ß	p	ß	p
Whole body aBMD	0.17	<0.01	0.18	<0.01
Lumbar spine aBMD	0.30	<0.001	0.31	<0.001
Radius				
Trabecular vBMD 4%	0.36	<0.01	0.40	<0.001
Cortical vBMD 66%	0.09	ns	0.14	ns
Cortical thickness 66%	0.19	<0.05	0.21	<0.05
Endosteal circumference 66%	-0.12	<0.01	-0.12	<0.01
Tibia				
Trabecular vBMD 4%	0.23	0.06	0.27	<0.05
Cortical vBMD 38%	0.13	ns	0.15	ns
Cortical thickness 38%	0.03	ns	0.05	ns
Endosteal circumference 38%	-0.02	ns	-0.04	ns

All analyses included age, weight and respectively height for the DXA parameters and bone length (forearm or tibia) for the pQCT parameters as covariates. Data are presented as regression coefficients (β) and their significance level (p).

E2 and FE2 are positively associated with aBMD measured at LS (E2: ß=0.30 p<0.001; FE2: ß=0.31 p<0.001) and WB (E2: ß=0.17 p<0.01; FE2: ß=0.18 p<0.01).

Moreover, there is a positive association of E2 and FE2 with trabecular vBMD at the radius (E2: ß=0.36 p<0.01; FE2: ß=0.40 p<0.001) and the tibia (E2: ß=0.23 p=0.06; FE2: ß=0.27 p<0.05) and with cortical thickness at the radius (E2: ß=0.19 p<0.05; FE2: ß=0.21 p<0.05) (table 3). All associations remain significant after inclusion of T (p<0.05), IGF-1 (p<0.05), calcium intake (p<0.05) or physical activity (p<0.05) in the model. After inclusion of lean mass or muscle CSA in the model, all associations remain expect for the associations of (F)E2 with WB aBMD and (F)E2 with cortical thickness. No associations are found between (F)E2 and the cortical vBMD of the radius or between (F)E2 and cortical vBMD or cortical thickness of the tibia. To study the influence of E2 and FE2 on the endosteal circumference a regression model including age, weight, forearm length and periosteal circumference is used; E2 and FE2 are independent negative predictors of the endosteal circumference at the radius (E2: ß=-0.12 p<0.01; FE2: ß=-0.12 p<0.01) (table 3). However, no associations are found at the level of the tibia.

SEX STEROIDS AS INDEPENDENT PREDICTORS OF aBMD AND BONE AREA OF THE LUMBAR SPINE AND WHOLE BODY AND OF vBMD AND BONE GEOMETRY OF THE RADIUS AND TIBIA: DIFFERENCES BETWEEN EARLY-MID PUBERTAL AND LATE-POST PUBERTAL BOYS.

In order to study possible differences in the associations between sex steroids and bone parameters at different pubertal stages the study group was divided in two subgroups namely an early-mid pubertal (P2-P3; n=57) and a late-post pubertal group (P4-P5; n=75).

In the early-mid pubertal children, we only find a significant association between (F)T and periosteal circumference (T: ß=0.42 p<0.001; FT: ß=0.43 p<0.01). However, in the late-post pubertal group most of the described differences in the whole group are confirmed. Regression models including height and weight show that T and FT are positively associated with LS bone area (T: ß=0.31 p<0.001; FT: ß=0.23 p<0.01), WB bone area (T: ß=0.12 p<0.05; FT: ß=0.10 p=0.06), trabecular area (T: ß=0.32 p<0.01; FT: ß=0.22 p=0.07), cortical area (T: ß=0.20 p=0.06;FT: ß=0.24 p<0.05) and periosteal circumference at the radius (T: ß=0.37 p<0.01;FT: ß=0.24 p=0.07). As described in the whole group, there are no significant associations at the tibia. E2 and FE2 are positively associated with aBMD measured

at LS (E2: ß=0.35 p<0.001;FE2: ß=0.37 p<0.001) and WB (E2: ß=0.36 p<0.001; FE2: ß=0.39 p<0.001) and trabecular vBMD at the radius (E2: ß=0.34 p<0.01; FE2: ß=0.38 p<0.01) and cortical vBMD at the tibia (E2: ß=0.26 p<0.05; FE2: ß=0.27 p<0.05). No significant associations between (F)E2 and cortical thickness at the radius and tibia, trabecular vBMD at the tibia, cortical vBMD at the radius are found.

DISCUSSION

This is the first study presenting data about the association of sex steroids ((F)E2 and (F)T) with (v)BMD, bone geometry, bone maturation and body composition in healthy pubertal boys. The role of androgens and estrogens in bone maturation and mineralization during male pubertal development is mainly based on studies of diseases with altered steroidogenesis or steroid receptor mutations.

Firstly, the associations between sex steroids and body composition were studied. We showed that the increase in lean mass parallels the increase in T at the different pubertal stages. There is a strong positive association between (F)T and lean mass and between (F)T and muscle CSA at the radius and tibia. These findings are in line with our previous observations in transsexual men on cross-gender testosterone treatment, showing more lean mass and a larger muscle CSA in the androgen treated group compared to female controls [26].

Secondly, we analyzed areal and volumetric BMD as well as bone geometry at different pubertal stages. In line with previous studies, we showed a higher LS and WB aBMD at successive pubertal stages[1,7,27]. Post pubertal cortical and trabecular vBMD at the radius and tibia is significantly higher compared to prepubertal values. As previously described by others, trabecular vBMD remains relatively constant until the age of 15 years but increases by about 10% at the end of puberty[28,29,30,31]. On the other hand, bone CSA, periosteal circumference and cortical thickness are significantly larger at the successive pubertal stages. The increase in cortical thickness in male adolescents is explained by a greater periosteal apposition in comparison with the endosteal resorption[29,30]. In comparison to females, who add more bone to the endosteal surface, male adolescents add bone to the periosteal surface, which has the greatest effect on bone strength[30]. Most of the cortical bone expansion thus occurs during pubertal growth, where periosteal bone expansion after puberty is very limited. Pubertal mouse models also demonstrated a larger bone in male mice at the end of puberty due to a net increase in periosteal bone formation[10].

Additionally, we studied associations of sex steroids with areal and volumetric BMD and bone geometry, given the limited availability of data in healthy children and adolescents. It is well-known that bone mineralization and cross-sectional bone size increase with age, height and weight[7], all multivariate analyses were therefore adjusted for age, weight, height or bone length. (F)T levels are associated with different parameters of bone size, such as WB and LS bone area, trabecular and cortical bone area and periosteal circumference of the radius. These associations are no longer present after inclusion of lean mass or muscle CSA in the model. It is well-known that increase in bone size is driven by strain from muscle force and that muscle development precedes bone development during pubertal growth spurt[32,33]. Since there is a positive association between (F)T and muscle mass as well as a positive association between (F)T and bone size which disappears after inclusion of muscle mass in the model, we hypothesize that (F)T leads to an increase in muscle mass which causes a larger bone size due to an increase in strain exerted on the bone. Due to the cross-sectional design, however, we are not able to draw causative conclusions and a direct effect of (F)T on bone size cannot be definitely excluded.

Furthermore, the observed associations differed between early-mid pubertal boys and late-post pubertal boys. In early-mid pubertal boys, only the association (F)T and periosteal circumference was significant, however in late-post pubertal boys all described associations were present. This is not unexpected since bone diameter increases in early puberty by rapid periosteal apposition and periosteal apposition rates peak at the same time as growth in length[34]. Our results are in line with previously reported association of FT with cortical bone area and periosteal circumference at the radius in young male adults[15].

(F)E2 was found to be positively associated with LS and WB aBMD. These associations were independent of age and bone size, and remained significant after inclusion of T in the model. Conversely, there was no association of T with aBMD in the presence of E2 in the model. Our results are in line with the findings of Yilmaz et al. (2005) in healthy adolescents, showing a strong positive association of E2 with WB and LS aBMD. Pomerant et al. (2007), however found a significant association between T and WB and LS aBMD in healthy male adolescents. Since they did not study E2, they cannot exclude that the positive association with T

could be an E2 effect, related to the aromatization of T. Moreover, in our study (F)E2 is positively associated with trabecular vBMD at the radius and tibia and cortical thickness at the radius. Furthermore, there is a negative association between (F)E2 and endosteal circumference at the radius. As far as we know, there are no previous studies in male children and adolescents, studying the effects of (F)E2 on vBMD and endosteal circumference. In young adult males -at the age of peak bone mass- positive associations between (F)E2 and cortical vBMD at the radius and tibia as well as a negative association between (F)E2 and the endosteal circumference (endosteal contraction)[15,16] is described. These data suggest that for normal bone mass accrual throughout puberty an estrogen-induced suppression of endosteal expansion is needed in addition to an androgen-induced periosteal bone expansion.

Our findings on specific associations between E2 and bone parameters are supported by limited observations in rare clinical syndromes of estrogen resistance and aromatase deficiency. Men who are estrogen resistant due to a mutation in de ER α gene or have aromatase deficiency due a mutation in the CYP 19 gene present with a low BMD despite high androgen levels[11,12,14]. Aromatase deficient men respond to estrogen therapy with a marked increase in bone mass, whereas there is no effect of testosterone[11,12,13,14].

Finally, the influence of sex steroids on bone maturation was studied. The essential role of estrogens in the regulation of the human growth plate is supported by the finding that both men and women with estrogen resistance[11] or aromatase deficiency[12,13,14] have non-fused epiphyses and continue to grow after sexual maturation, whereas individuals with androgen insensitivity due to a mutation of the androgen receptor achieve epiphyseal closure[35]. Experimental studies in juvenile ovariectomized rabbits have demonstrated that E2 accelerates the programmed senescence in the proliferation rate and number and size of chondrocytes, leading finally to epiphyseal plate fusion[36]. There are no previous data on the effects of estrogen on bone maturation in healthy males. Our results show that (F)E2 is positively associated with bone maturation. This is in line with our finding in obese adolescents showing an advance in bone maturation in parallel with higher circulating estrogen levels compared to non-obese males[22]

(see chapter 3.2). The accelerated bone maturation in girls compared to boys can also be explained by the higher estradiol levels in girls, even before pubertal onset, since there is no sex difference in estrogen receptors alfa and beta expression in the human growth plate at different pubertal stages[37,38].

It should be noted however that associations found between sex hormones and bone size, are not solely or only directly caused by sex hormones, but could be caused by sex hormone-dependent alterations in IGF-1 levels. Previous research showed that circulating IGF-1 is affected by ER α signaling[39] and that it is at least partly involved in the regulation of cortical bone geometry in mice[10]. The inclusion of IGF-1 as independent predictor in the multiple linear regression analyses did, however, not change the found associations. This suggests that the observed associations between sex hormones, BMD and bone geometry are at least in part independent associations not mediated by circulating IGF-1.

To our knowledge, this is the first study reporting data on the associations of sex steroids with vBMD and bone geometry in healthy male children and adolescents. Our findings might contribute to the better understanding of sexual dimorphism of the skeletal bone geometry occurring during puberty. The strength of the present study is the comprehensive evaluation of BMD, bone geometry, body composition, pubertal development and hormonal factors involved in bone expansion. Moreover, sex steroids were measured by highly sensitive and accurate mass spectroscopy-based methodology as required when studying low androgen and estrogen serum levels in children.

Our study has some limitations. Firstly, serum T and E2 levels were single point measurements for samples obtained between 8 and 10 am. Although it is possible that a single measurement is not fully representative for E2 or T exposure, in clinical settings, a single measurement from a serum sample drawn in early morning is commonly used and acceptable. Moreover, a lot of attention was given to take the samples as early as possible and all before 10 am. A second limitation is the rather broad age range. Possibly there are different effects of sex steroids on bone mass and size at different pubertal stages. To address in part this problem, we performed subanalyses within our population. Finally, one should emphasize that this is a cross-sectional study that gives only information about associations and does not allow us to draw causative conclusions. In order to confirm our

findings and further unravel underlying mechanisms, prospective longitudinal studies are required, ideally with a yearly follow-up from start of puberty until adulthood.

In conclusion, we found that (F)E2 is significantly and positively associated with bone maturation, LS and WB aBMD, and trabecular and cortical vBMD. On the other hand, (F)E2 is negatively associated with endosteal circumference. Serum T is positively associated with muscle mass and bone size. Our findings underscore the important role of estrogens in skeletal development in boys, but need further confirmation by a longitudinal study.

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3 SEXUAL AND SKELETAL DEVELOPMENT IN OBESE BOYS

3.1 SEX STEROIDS IN RELATION TO SEXUAL AND SKELETAL MATURATION IN OBESE MALE ADOLESCENTS

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ABSTRACT

Background: Childhood obesity is associated with an accelerated skeletal maturation. However data concerning pubertal development and sex steroids levels in obese adolescents are scarce and contrasting.

Objectives: To study sex steroids in relation to sexual and skeletal maturation and to serum PSA, as a marker of androgen activity, in obese boys from early to late adolescence.

Methods: 90 obese boys (aged 10-19 y) at the start of a residential obesity treatment program and 90 age-matched controls were studied cross-sectionally. Pubertal status was assessed according to the Tanner method. Skeletal age was determined by an X-ray of the left hand. Morning concentration of total testosterone (TT) and estradiol (E2) by LC-MS-MS, free T (FT) by equilibrium dialysis, and LH, FSH, SHBG and PSA by immunoassays, were measured.

Results: Genital staging was comparable between the obese and non-obese group, whereas skeletal bone advancement (mean 1 year) was present in early and mid adolescence in the obese males. While both median SHBG and TT concentrations were significantly ($p<0.001$) lower in obese subjects during mid and late puberty, median FT, LH, FSH and PSA levels were comparable to those of controls. In contrast, serum E2 concentrations were significantly ($p <0.001$) higher in the obese group at all pubertal stages.

Conclusion: Obese boys have lower circulating SHBG and TT, but similar FT concentrations during mid and late puberty in parallel with a normal pubertal progression and serum PSA levels. Our data indicate that in obese boys, serum FT concentration is a better marker of androgen activity than TT. On the other hand, skeletal maturation and E2 were increased from the beginning of puberty, suggesting a significant contribution of hyperestrogenemia in the advancement of skeletal maturation in obese boys.

INTRODUCTION

It is well-known that obesity in childhood is associated with an accelerated growth and skeletal maturation[1,2,3,4], but it remains unclear which hormonal changes are most important for stimulating the skeletal maturation in this particular condition[5,6,7]. Moreover, only very limited and contrasting data have been published concerning pubertal development and sex steroids (testosterone (T) as well as estradiol (E2) concentrations), especially in male obese adolescents. Whereas some authors found an advanced sexual maturation in obese boys[8,9,10], others reported a normal[1,2] or even a delayed genital development[1,3,11].

Furthermore, there is controversy about T concentrations in obese children and adolescents. In prepubertal obese boys increased total T (TT) concentrations[12,13], as well as normal[14] and low TT levels have been described[2,15], whereas in pubertal boys normal[12], as well as decreased TT concentrations have been reported[2,16,17,18]. Poor assessment of pubertal staging, small study groups[14,15], lack of age-matched control group[2] and the use of direct immunoassays for TT determination[12,16,17] might explain these contrasting hormonal findings between studies. Moreover, the well-known lower SHBG production in obesity, especially during puberty, might be responsible for the finding of lower TT concentration in obese adolescents[2,12,15,16]. Little experience exits with free T (FT) in adolescent obesity[16,18]. Therefore, in the present study TT, SHBG and FT were assessed as markers of androgen secretion and both clinical (genital development) and biological (prostate-specific antigen (PSA) concentrations) markers of androgen activity were assessed in a well-described group of obese adolescents. We hypothesized a normal genital development in association with normal FT concentrations and a more rapid skeletal maturation in relation to increased E2 levels.

MATERIAL AND METHODS

SUBJECTS

Ninety male obese adolescents (BMI SDS >+2), aged 10-19 years, were investigated at the entry of a residential weight-loss program at the Zeepreventorium in De Haan, Belgium. Ninety age-matched healthy normal-weighted controls were randomly selected from an ongoing longitudinal study evaluating changes in bone geometry, bone maturation and muscle strength in relation to sex steroids in childhood and adolescence. These healthy children were recruited by letters distributed in several schools within the Ghent area. Subjects with a history of hypogonadism, panhypopituitarism, diabetes, previous or ongoing treatment with T or oral steroids were excluded. Both study protocols were approved by the Ethical Committee of the Ghent University Hospital. Informed consent was obtained from the parents and all participants gave their assent.

METHODS

ANTHROPOMETRY AND SEXUAL MATURATION

Standing height was measured to the nearest 0.1 cm using a Harpenden stadiometer (Holtain Ltd, Crymuch, UK). Body weight was measured in light indoor clothing without shoes to the nearest 0.5 kg. Waist circumference, defined as the smallest abdominal circumference if present or otherwise measured halfway between the iliac crest and the rib cage, was determined to the nearest 0.1 cm. All anthropometric measurements were performed by the same trained physician (SV). The standard deviation score (SDS) for body height, weight, waist circumference and BMI was computed using the reference data of the 2004 Flemish growth study[19]. Pubertal status of the subjects was assessed by trained pediatricians according to the method established by Tanner (Tanner Genital Staging: stage 1: prepuberty; stage 5: post puberty). Testicular volume was determined with a Prader orchidometer in a subgroup of 40 consenting obese boys and their respective controls.

BONE AGE DETERMINATION

Bone age reading of an X-ray of the left hand and wrist was done by two independent experienced pediatric radiologists, blinded for the chronological age, using the Greulich and Pyle method[20]. The mean of both readings was taken, but if the difference was more than one year a third independent reading (by a trained pediatrician) was performed and the two closest estimates were retained for final calculations. Skeletal age differences (SAD) were calculated by subtracting the chronological age (CA) from the skeletal age (BA) (SAD=BA–CA): positive differences reflecting an accelerated skeletal maturation and negative differences a delayed bone maturation.

HORMONAL MEASUREMENTS

Venous blood samples in the obese group were obtained between 0800 and 1000 h after overnight fasting. Blood samples in the age-matched control group were collected within the same time interval, but allowing a small breakfast. All samples were stored at –80°C until batch analysis. Commercial automated immunoassays were used to measure SHBG, LH, FSH, DHEAS, PSA (Roche Diagnostics, Mannheim, Germany). The intra- and interassay coefficients of variation (CV's) for all assays were less than 10%. The lower detection limit for PSA was 0.003 ng/ml and the intra-assay and interassay CV's were respectively 1.2% and 3.5%. Serum E2, TT and androstenedione (A) were determined by liquid chromatography-tandem mass spectrometry (AB Sciex 5500 triple-quadrupole mass spectrometer; AB 173 Sciex, Toronto Canada). Serum limit of quantification (LOQ) was <0.5 pg/mL (1.9 pmol/L) for E2 and the interassay CV was 4.0% at 21 pg/mL (77 pmol/L)[21]. Serum LOQ was 1.2 ng/dl for TT and the interassay CV was 8.3% at 36.7 ng/dl and 3.1% at 307.8 ng/dl. Serum LOQ was 4.25 ng/dl for A and the interassay CV was 2.9% at 59.8 ng/dl. Serum FT was determined by equilibrium dialysis (FT dialysis)[21], CV of the method calculated from duplicate measurements is 11.7%. FT was also calculated (cFT) in all subjects from the concentrations of TT, SHBG and albumin according to Vermeulen et al.[22]. The results for FT dialysis and cFT are not substantially different as can be seen from a comparison by Passing-Bablok and Bland-Altman analysis (see supplemental data).

STATISTICS

Normality was checked using QQ-plots and Shapiro-Wilk tests. The anthropometric data showed a normal distribution, hormonal data were however not normally distributed. Data are presented as mean±standard deviation or as medians (25th–75th percentile) in case of a non-normal distribution. Comparison between obese and control groups were performed using parametric independent T-tests or ANOVA, when criteria for normality were met. In other cases, Mann-Whitney U tests were used. Between-group differences of categorical variables were calculated with χ^2 tests. The difference was considered statistically significant at $p<0.05$. To study hormonal parameters and anthropometric parameters in the obese boys compared to the controls taking pubertal stage into account as presented in figure 1 and figure 2, linear regression analysis was used. Hormonal parameters TT, FT, E2, LH, SHBG, A, DHEAS and PSA underwent a Box-Cox transformation to enhance normality (transformation factors were TT: $\lambda=0.49$; FT: $\lambda=0.42$; E2: $\lambda=0.32$; LH: $\lambda=0.63$; SHBG: $\lambda=-0.29$; PSA: $\lambda=0.23$; A: $\lambda=0.40$; DHEAS: $\lambda=0.26$). Box-Cox transformations were performed using MedCalc for Windows, version 12.5 (MedCalc Software, Ostend, Belgium). Based on the available literature[13,18] on sex steroid (TT, FT) levels determined by LC-MS-MS and SHBG levels in obese prepubertal and late pubertal boys, sample size calculations were performed using Medcalc for Windows, version 12.4.00 (MedCalc Software, Ostend, Belgium) (α: 0.05; ß: 0.20). We calculated a necessary sample size of 7 to 14 children in each group at the different pubertal stages to discern the published differences in TT, FT and SHBG between both groups. Post-hoc power calculations on our E2 analyses using G*power (version 3.1.5) demonstrated a power between 70 and 99% at the different pubertal stages. Data were analyzed using SPSS software version 19.0.

RESULTS

COMPARISON OF ANTHROPOMETRIC DATA AND SEXUAL AND SKELETAL DEVELOPMENT BETWEEN OBESE BOYS AND AGE-MATCHED CONTROLS

Table 1 summarizes the anthropometric characteristics and Tanner stages of both groups. Mean body weight (SDS), BMI (SDS) and waist circumference (SDS) of the obese group were almost double of those of normal-weighted peers. Mean BMI SDS did not differ between the different pubertal stages in the control group, while BMI SDS at Tanner stage G5 was found to be highest in the obese group (BMI SDS G4: 2.6±0.25; BMI SDS G5:3.0±0.35; $p<0.001$) (figure 1). Moreover, waist circumference SDS at Tanner genital stage 5 was significantly higher than waist circumference SDS at Tanner genital stage 4 in the obese group (waist circumference SDS G4: 2.5±0.28; waist circumference SDS G5:2.7±0.24; $p<0.05$).

Table 1: Comparison of anthropometric data and skeletal and sexual development between obese and age-matched control boys.

	n	Obese boys (mean±SD)	n	Age-matched controls (mean±SD)	Significance level (p)
Anthropometry					
Age (y)	90	14.6±2.2	90	14.5±2.2	ns
Height (cm)	90	169.0±11	90	167.0±13	ns
Height (SDS)	90	0.36±1.1	90	0.11±1.0	ns
Weight (kg)	90	104.8±26.0	90	55.6±15.0	<0.001
Weight (SDS)	90	2.9±0.6	90	0.03±0.9	<0.001
BMI (kg/m²)	90	36.2±5.8	90	19.5±3.2	<0.001
BMI (SDS)	90	2.6±0.37	90	-0.04±0.97	<0.001
Waist circumference (cm)	85	109±15	85	69±8	<0.001
Waist circumference (SDS)	85	2.5±0.28	85	-0.06±0.88	<0.001
Skeletal maturation					
Bone age (y)	90	15.6±2.2	90	14.6±2.6	<0.01
Difference bone age-age (y)	90	1.1±0.9	90	0.2±1.1	<0.001
Tanner genital stage		**Obese boys (frequency)**		**Age-matched controls (frequency)**	
G1	8	8.8%	11	12.1%	ns (χ^2)
G2	17	18.7%	14	15.4%	
G3	13	14.3%	12	13.2%	
G4	30	33.0%	31	34.1%	
G5	23	25.3%	23	25.3%	

Comparison between obese boys and age-matched controls were performed using parametric independent t-tests. Between-group differences of categorical variables were calculated using chi-square tests.

Height and height SDS were not significantly different between both groups by ANOVA analysis (figure 1, table 1). Height and height SDS of prepubertal and early pubertal (G1 and G2) obese children were significantly higher compared to their healthy peers (height: G1-G2: obese: 158±6 vs. controls: 151±7 cm, p<0.001; height SDS: G1-G2: obese: 0.6±1.1 vs. controls:-0.06±1.1, p<0.05). Although skeletal maturation is advanced in obese children from Tanner stage 1 to 4 (<0.001), there is no significant difference in pubertal development between both groups (figure 1, table 1). Moreover, studying a subgroup of 40 obese adolescents and their matched

controls no difference in testicular volume was observed (figure 1). Anthropometric characteristics of this subpopulation were similar to the whole population (data not shown).

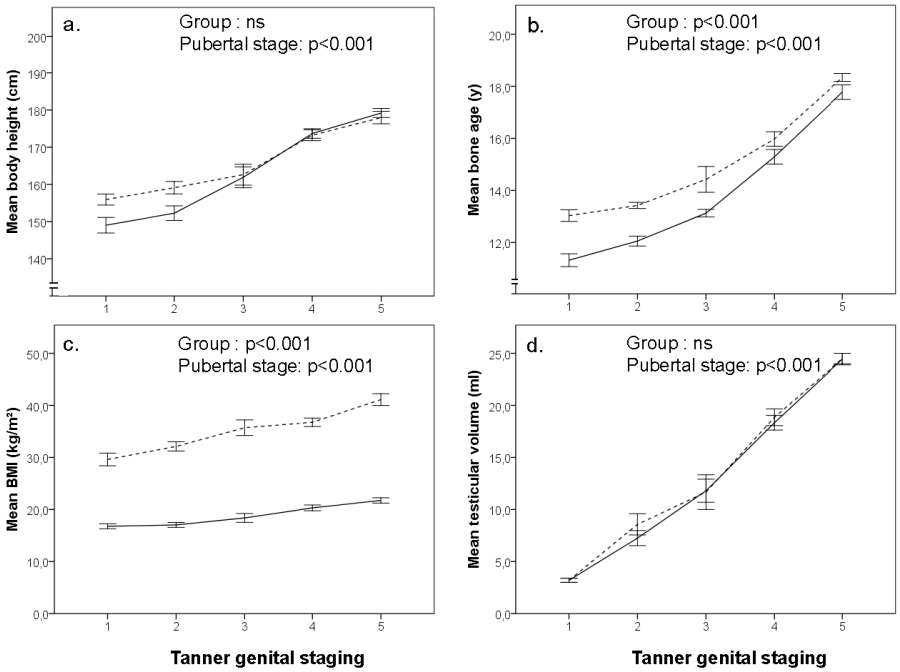

Figure 1 Height (a), bone age (b), BMI (c) and testicular volume (d) at different pubertal stages in obese adolescents compared to their age-matched controls. The line plots present mean height, BMI, bone age and testicular volume for each pubertal stage (prepuberty: 1; post puberty: 5) for the two study groups. The error bars represent 1 standard error of the mean (SEM). The obese group is presented by the dotted line and the control group (age-matched controls) by the full line. The interconnecting lines do not present longitudinal data.

COMPARISON OF HORMONAL AND BIOLOGICAL PARAMETERS BETWEEN OBESE ADOLESCENTS AND AGE-MATCHED CONTROLS.

Before pubertal onset, TT was similar, while SHBG concentrations were lower and FT, DHEAS, A and E2 higher in obese boys (table 2). There was a significant positive correlation between FT and DHEAS and between FT and A (spearman rank correlation coefficient (r) r(DHEAS-FT)=0.79 $p<0.001$; r(A-FT)=0.71 $p<0.001$). As shown in figure 2, significantly higher sex steroids (TT, FT and E2) and PSA concentrations were found with advancing pubertal stage in both groups ($p<0.001$) (figure 2, table 2). The subanalysis in the different Tanner genital stages showed that obese adolescents (at least stage G2) have lower SHBG levels at every pubertal stage, lower TT concentrations from stage G3 onward, but similar FT concentrations, except for adolescents in pubertal stage 5. There was no significant difference between values obtained from FT dialysis and cFT (cFT= 0.0109 + 0.9762 FT dialysis). No significant difference in circulating LH, FSH and PSA concentrations was found between the obese boys and their controls. Serum E2 concentrations and E2/TT ratio were significantly higher in the obese adolescents ($p<0.01$).

Table 2: Comparison of hormonal parameters and biochemical parameters between obese boys and age-matched controls.

	Tanner genital stage	n	Obese boys (median) (P25-P75)	n	Controls (median) (P25-P75)	Significance level (p)
TT (ng/dl)	G1	8	6.3 (5.8-10.3)	11	6.2 (4.8-7.3)	ns
	G2	16	19.9 (10.1-67.2)	14	20.7 (9.0-44.8)	ns
	G3	13	170 (32.6-317)	12	227 (170-293)	ns
	G4	30	300 (201-365)	30	423 (326-504)	<0.001
	G5	23	335 (265-469)	23	517 (445-616)	<0.001
FT dialysis (ng/dl)	G1	8	0.12 (0.1-0.15)	11	0.04 (0.03-0.06)	<0.001
	G2	16	0.45 (0.24-1.0)	14	0.13 (0.08-0.40)	<0.05
	G3	13	3.2 (0.5-6.2)	12	2.6 (1.5-4.7)	ns
	G4	30	6.7 (5.1-9.3)	30	8.0 (5.9-10.6)	ns
	G5	23	8.8 (6.7-10.3)	23	11.6 (9.1-13.3)	<0.01
cFT (ng/dl)	G1	8	0.13 (0.11-0.16)	11	0.04 (0.03-0.06)	<0.001
	G2	16	0.44 (0.24-1.1)	14	0.14 (0.07-0.39)	<0.05
	G3	13	3.1 (0.5-6.5)	12	2.5 (1.4-4.7)	ns
	G4	30	6.5 (5.2-8.8)	30	7.8 (5.4-9.9)	ns
	G5	23	8.9 (7.1-9.8)	23	11.3 (9.8-12.8)	<0.01
SHBG (nmol/l)	G1	8	33.6 (28.4-40.4)	11	99.0 (73.0-187)	<0.001
	G2	16	27.5 (20.2-44.9)	14	115.9(73.6-148)	<0.001
	G3	13	35.7 (23.7-43.9)	12	79.9 (52.0-93.1)	<0.001
	G4	30	21.3 (17.2-29.7)	30	36.3 (28.7-44.5)	<0.001
	G5	23	17.6 (15.4-23.8)	23	33.2 (26.4-38.5)	<0.001
E2 (ng/l)	G1	8	1.9 (1.4-3.0)	11	1.0 (0.5-1.0)	<0.001
	G2	16	3.5 (2.8-5.4)	14	1.0 (0.5-1.4)	<0.001
	G3	13	10.6 (4.0-16.9)	12	3.5 (2.7-5.3)	<0.05
	G4	30	18.6 (14.9-25.3)	30	12.4 (8.5-17.0)	<0.001
	G5	23	34.8 (25.6-41.1)	23	15.7 (13.2-21.0)	<0.001
Ratio E2/TT	G1	8	0.29 (0.20-0.44)	11	0.14 (0.08-0.19)	<0.01
	G2	16	0.16 (0.07-0.37)	14	0.04 (0.02-0.1)	<0.01
	G3	13	0.06 (0.05-0.12)	12	0.02 (0.01-0.02)	<0.001
	G4	30	0.07 (0.05-0.08)	30	0.03 (0.02-0.04)	<0.001
	G5	23	0.11 (0.06-0.13)	23	0.03 (0.02-0.05)	<0.001

Table 2 continued

	Tanner genital stage	n	Obese boys (median) (P25-P75)	n	Controls (median) (P25-P75)	Significance level (p)
LH (U/L)	G1	8	0.1 (0.1-1.1)	11	0.3 (0.1-0.5)	ns
	G2	16	1.6 (0.65-1.8)	14	1.3 (0.53-1.8)	ns
	G3	13	2.6 (1.1-3.2)	12	3.1 (2.3-3.5)	ns
	G4	30	4.4 (3.4-5.5)	30	3.7 (2.5-4.5)	<0.05
	G5	23	4.6 (3.8-6.0)	23	3.9 (3.0-5.3)	ns
FSH (U/L)	G1	8	1.8 (1.1-2.8)	11	1.6 (1.1-2.1)	ns
	G2	16	2.1 (1.6-3.1)	14	2.1 (1.3-2.3)	ns
	G3	13	3.0 (1.8-4.6)	12	2.0 (1.8-2.8)	ns
	G4	30	3.7 (2.8-5.1)	30	3.1 (2.1-4.7)	ns
	G5	23	2.6 (2.0-4.1)	23	2.7 (1.5-4.7)	ns
DHEAS (µg/dl)	G1	8	138 (114-152)	11	89.6 (41.1-106)	<0.01
	G2	16	174 (114-244)	14	133 (81.5-180)	ns
	G3	13	225 (144-288)	12	142 (87.1-181)	<0.05
	G4	30	204 (162-315)	30	221 (157-322)	ns
	G5	23	370 (268-469)	23	348 (223-438)	ns
A (ng/dl)	G1	8	26.1 (15.7-46.2)	11	17.5 (10.8-20.2)	0.06
	G2	16	46.7 (38.7-57.0)	14	23.1 (18.9-34.3)	<0.001
	G3	13	64.6 (49.6-102)	12	31.1 (25.5-47.5)	<0.001
	G4	30	79.4 (58.7-93.5)	30	47.1 (35.8-73.0)	<0.001
	G5	23	81.7 (63.0-117)	23	80.9 (66.3-85.3)	ns
PSA (µg/l)	G1	8	undetectable	11	undetectable	-
	G2	17	0.005 (0-0.01)	14	undetectable	-
	G3	13	0.05 (0.01-0.17)	12	0.03 (0.01-0.16)	ns
	G4	30	0.25 (0.16-0.35)	31	0.28 (0.19-0.44)	ns
	G5	23	0.46 (0.33-0.67)	23	0.52 (0.27-0.62)	ns

Non-Gaussian distribution: data presented as median (25th-75th percentile (P25-P75)). Comparison between obese boys and age-matched controls were performed using non-parametric Mann-Whitney-U tests. Conversion factor to SI-units for TT from ng/dl to nmol/l is 0.0347, for FT from ng/dl to nmol/l is 0.0347, for E2 from ng/l to pmol/l is 3.671, for DHEAS from µg/dl to nmol/l is 2.714, for A from ng/dl to nmol/l is 0.0349.

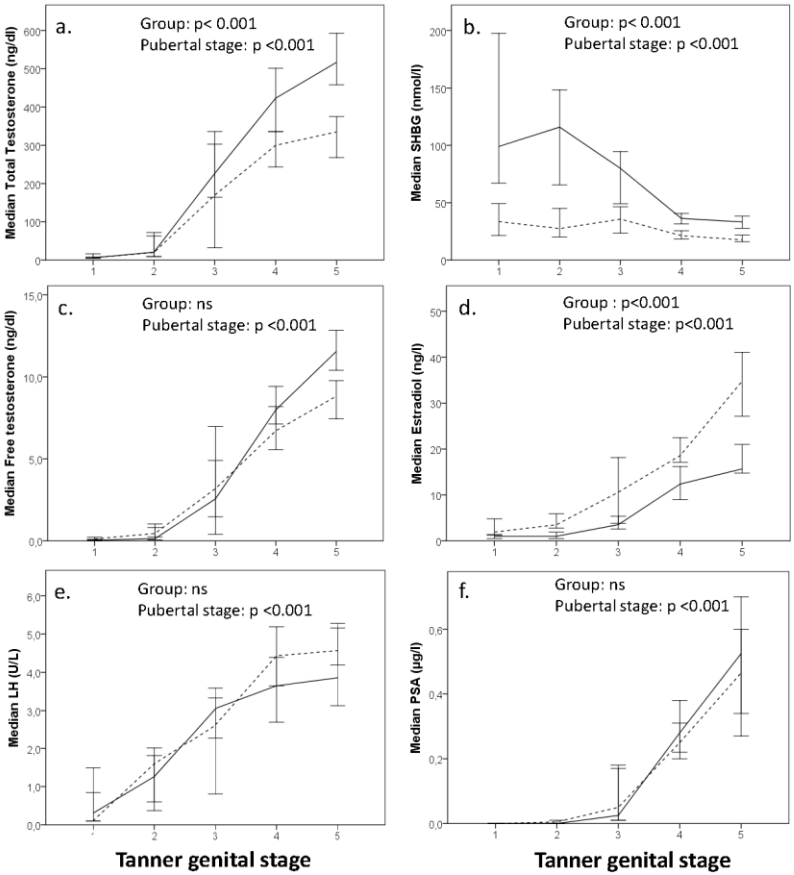

Figure 2 Total testosterone (a), SHBG (b), Free testosterone (c), Estradiol (d), LH (e) and PSA (f) in obese boys and their age-matched controls at different pubertal stages. The line plots present median TT, SHBG, FT, E2, LH and PSA levels for each pubertal stage (prepuberty: 1; post puberty: 5) for the two study groups. Since the hormonal data do not meet the criteria for a normal distribution, data are presented as medians and the error bars represent 95th confidence interval. The obese group is presented by the dotted line and the control group (age-matched controls) by the full line. The interconnecting lines do not present longitudinal data.

ASSOCIATIONS BETWEEN THE DEGREE OF OBESITY AND HORMONAL PARAMETERS IN THE OBESE BOYS

In order to study the importance of the degree of obesity on hormonal parameters, associations between the degree of obesity, expressed as BMI SDS and waist circumference SDS, and TT, FT, SHBG, E2 and PSA were studied using linear regression (including pubertal stage) in the obese group only. Waist circumference SDS was a negative predictor of TT (waist circumference SDS: ß=-0.18 $p<0.01$) and FT (waist circumference SDS: ß=-0.13 $p<0.05$) levels. However, no association between BMI SDS and TT or FT was found. There was a trend to a negative association of BMI SDS and waist circumference SDS with SHBG (BMI SDS: ß=-0.20 $p=0.08$; waist circumference SDS: ß=-0.20 $p=0.06$). BMI SDS was a positive predictor of E2 levels (BMI SDS: ß=0.20 $p<0.01$). No association was found between waist circumference SDS and E2. All results remained unchanged when FT dialysis was substituted by cFT in the analyses.

DISCUSSION

The present study investigates both androgen secretion (assessed by serum TT and FT concentrations using sensitive assays) and androgen activity using detailed clinical and biological assessment of sexual (Tanner genital staging, PSA) as well as skeletal (bone age readings of the left hand and wrist) maturation in a group of obese prepubertal and pubertal boys. Our results demonstrate that pubertal obese boys have lower TT levels, higher E2, but normal FT levels at least during mid and late puberty. These hormonal differences might be responsible for the observed dissociation between an advanced skeletal maturation (mean advancement around 1 year) and a normal sexual maturation (similar pubertal stage distribution and PSA concentrations). Our data indicate that FT is a better indicator of androgen exposure than TT, explaining the normal pubertal progression and PSA production in male obese adolescents and suggest that the increased estrogen production and aromatization might be linked to the advanced skeletal maturation during pubertal progression.

Firstly, we confirmed the presence of an accelerated growth and skeletal maturation during childhood and early stages of puberty in our group of obese children and adolescents, who had a longstanding and persisting obesity, necessitating a residential weight loss program[1,2,3,4]. This accelerated growth and bone maturation contrasts with the finding of a normal androgen secretion and activity during this period, but is in accordance with the increased estrogen production and aromatization.

Secondly, we found a normal genital development in obese adolescents. Only few other studies have examined the pubertal development in obese adolescents[1,2,3,11,23]. Our results of a normal pubertal progression is in accordance with the findings of Denzer et al. (2007), reporting a normal genital development in German boys in comparison with the historical Swiss standard of Largo and Prader[2]. Laron et al. (2004) also reported in a short communication no difference in pubertal timing among 136 obese boys and 48 non-obese boys[23]. On the other hand, an increased prevalence of delayed pubertal development in obese males has been observed by some pediatric obesity clinics[1,24]. The reasons for this

phenomenon, is not known, although some recruitment bias might be involved, given the well-known accelerated body fat accumulation in boys occurring before pubertal onset, promoting some overrepresentation of obese boys with a delayed sexual maturation in obesity clinics.

Thirdly, we found normal serum PSA concentrations in obese adolescents. During male development, PSA concentrations correlate with the rise in T levels, being high during mini-puberty, declining to undetectable values by six months, reappearing by about age 10 years and increasing in concentration thereafter until adulthood[25]. Serum PSA concentrations have been found to correlate with T concentrations during puberty, especially when T concentrations were adjusted for SHBG levels[26,27]. This study is the first report of PSA concentrations in obese boys and suggests that PSA seems to be a better marker for evaluating androgen activity at tissue level than skeletal maturation, given it is less influenced by estrogens during pubertal development

Our study design allowed us to assess the difference in circulating sex steroids between obese and non-obese adolescents at different pubertal stages. We found clearly lower TT in obese adolescents from stage 3 onwards. Most studies have reported low TT levels in obese subjects during pubertal progression[2,16,17,18], although two studies did find normal TT concentrations at Tanner stage G2[12,14]. As previously described by Denzer et al. (2007), we found markedly lower SHBG levels at every pubertal stage possibly caused by the increased insulin levels[28]. Since approximately half of TT is bound to SHBG, it is likely that the lower SHBG concentrations in obese adolescents can account at least in part for these lower TT concentrations. Moreover, FT concentrations -as assessed by the equilibrium dialysis method- were comparable with the concentrations in non-obese subjects at mid and late puberty (G3 and G4). The findings of a normal pubertal development and normal PSA values indicate that FT levels seem to be a more representative index of androgen activity during adolescence than TT levels in obese pubertal boys. Our finding of higher FT concentrations in prepubertal and early pubertal obese boys is probably related to an increased adrenal activity[2,13] in obese children and adolescents which seems to be supported by the increased DHEAS and A levels in our prepubertal and early pubertal obese boys and the strong positive correlation between DHEAS and FT and between A and FT. Taneli

et al. (2010) reported lower FT in obese boys at Tanner stage 2, but not at Tanner stage 4[16]. However, in the latter study FT concentrations were measured by direct radioimmunoassay, an inaccurate method that underestimates FT concentrations by manifold and is dependent upon SHBG concentrations[29,30]. In accordance with Mogri et al. (2013), also using an equilibrium dialysis method, we found that post pubertal obese males (G5) had significantly lower FT concentrations compared to their lean counterparts[18]. In adult men FT concentrations have been reported to be preserved in moderately obese men and decreased in severely obese subjects due to a deficient gonadotropin secretion, as evidenced by a decreased amplitude of secretory LH pulses[31,32]. The lower FT concentrations at completion of puberty in obese boys might be related to increasing body fat accumulation since a higher degree of obesity (assessed as waist circumference SDS) was negatively associated with FT levels in the obese group. Although the obese adolescents with a G5 status studied by us had indeed the most severe degree of obesity, -as shown by their higher BMI SDS and waist SDS-, and highest E2 concentrations, known to play a major role in negative feedback regulation of LH[33,34], their E2/TT was not higher in comparison with earlier pubertal stages and a single point LH measurement was not different from lean controls.

The few studies reporting on estrogens in obese boys, did not find a significant difference in E2 levels between obese boys and lean controls, but these studies were hampered by a very small sample size[14,16,18,35] as well as the use of inaccurate immunoassays[14,16,35].

The strength of the present study is the comprehensive and reliable evaluation of pubertal development (by trained pediatricians), skeletal maturation (by two experienced radiologists) and sex steroids (measured by highly sensitive and accurate mass spectrometry-based methodology as required when studying low androgen and estrogen serum levels in children and adolescents) in a large group of obese adolescents. Since there are no universally accepted reference ranges for TT and FT concentrations in pubertal boys, we used age-matched controls, recruited in parallel with the obese study subjects to avoid secular trends. As far as we know this is the first study to present sex steroid data at different pubertal stages with an acceptable sample size in obese boys using state-of-the-art

techniques and to have evaluated PSA levels as a marker for androgen responsiveness in an obese pediatric population in relation to FT, measured by equilibrium dialysis. Given the wide usage of free testosterone calculations, the cFT was also included in the analyses. All results remained unchanged when FT by dialysis was substituted by cFT in our analyses.

Our study has some limitations. Firstly, our sample size in the prepubertal and early pubertal group is rather small especially in comparison with our late and post pubertal group. However, after sample size calculations based on the available literature we are confident that this is not a major drawback. Secondly, our study is limited by the fact that we only have assessed cross-sectional data. In order to confirm the underlying mechanisms in the dissociation between skeletal and sexual maturation, prospective longitudinal studies are required, ideally with a follow-up from early childhood at onset of obesity until adulthood.

In conclusion, obese male mid and late pubertal adolescents have lower TT concentration, but similar FT levels compared to age-matched lean controls. This normal androgen activity is reflected in a normal sexual development and similar PSA levels. On the other hand, skeletal maturation and E2 were increased from the onset of puberty, suggesting a significant contribution of hyperestrogenemia in the advancement of skeletal maturation in obese boys.

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3.2 BONE SIZE AND BONE STRENGTH ARE INCREASED IN OBESE MALE ADOLESCENTS

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ABSTRACT

Context: Controversy exists on the effect of obesity on bone development during puberty.

Objective: To determine differences in volumetric bone mineral density (vBMD) and bone geometry in male obese adolescents (ObA) in overlap with changes in bone maturation, muscle mass and force development and circulating sex steroids and IGF-1. We hypothesized that changes in bone parameters are more evident at the weight-bearing site and that changes in serum estradiol are most prominent.

Design, setting, participants: 51 male ObA (10-19y) recruited at the entry of a residential weight-loss program and 51 healthy age-matched and 51 bone age-matched controls.

Main Outcome Measures: vBMD and geometric bone parameters, as well as muscle and fat area were studied at the forearm and lower leg by pQCT. Muscle force was studied by jumping mechanography.

Results: Beside an advanced bone maturation, differences in trabecular bone parameters (higher vBMD and larger trabecular area) and cortical bone geometry (larger cortical area, periosteal and endosteal circumference) were observed in ObA both at the radius and tibia at different pubertal stages. After matching for bone age, all differences at the tibia, but only the difference in trabecular vBMD at the radius remained significant. Larger muscle area and higher maximal force were found in ObA compared to controls, as well as higher circulating free estrogen, but similar free testosterone and IGF-1 levels.

Conclusions: ObA have larger and stronger bones at both the forearm and lower leg. The observed differences in bone parameters can be explained by a combination of advanced bone maturation, higher estrogen exposure and greater mechanical loading resulting from a higher muscle mass and strength.

INTRODUCTION

Childhood and adolescence are critical periods in the development of optimal bone strength since a low peak bone mass achieved in early adulthood is a risk for osteoporosis later in life. The most crucial stage in bone mass acquisition is puberty: skeletal mass approximately doubles between the start and the end of adolescence[1,2]. Conditions that alter bone development during this particular maturational period may lead to suboptimal bone strength and higher fracture risk[2].

Given the rising prevalence and severity of obesity in adolescence and the increasing evidence that overweight in adolescence may contribute to skeletal fractures[3,4,5], it is essential to understand the effects of obesity on bone development. Some studies report higher areal bone mineral density (aBMD) in overweight children[6], whereas others conclude that obesity is linked to a lower aBMD[7]. An important limitation of these dual-energy X-ray absorptiometry (DXA) studies is the size dependence of the aBMD and the lack of data on bone geometry. Prediction of bone strength requires knowledge of both the material (e.g. volumetric bone mineral density (vBMD)) and geometric properties of bone (e.g. size and shape)[8]. Therefore, peripheral quantitative computed tomography (pQCT) is a useful approach in bone strength analysis since it can provide three-dimensional information about bone mineral density (BMD), size and shape[9].

Literature on the effects of adiposity and obesity on vBMD and bone size in children is scarce[10,11,12] with conflicting results. In prepubertal children, there is some evidence that fat mass may have a positive effect on bone, whereas fat mass has a negative effect on bone during puberty and immediately post puberty[13,14,15,16].

Main determinants of pubertal bone mass accumulation and changes in bone geometry are sex steroids, the growth hormone-insulin-like growth factor axis (GH-IGF-1 axis) and muscle mass and strength. Sex steroids and the GH-IGF-1 axis have not only a role in stimulating bone growth, but also in muscle mass accrual in adolescents[1]. Muscle strength strongly stimulates the acquisition of bone mass by

exerting strain on the bone surface[9]. The interactions of loading, IGF-1 and sex steroids are held responsible for the development of skeletal gender dimorphism, leading to greater bone size, periosteal expansion and bone strength in adolescent boys[17,18]. There is some evidence from studies in prepubertal children and adolescents that obese subjects have a higher muscle mass [11] and disturbed sex steroid and IGF-1 levels[19, 20].

As mechanical and hormonal determinants (especially estradiol) are important in bone mass acquisition in male adolescents by their effects on bone expansion and bone mineralization, this study aims to determine the changes in vBMD as well as geometry of long bones by pQCT in male obese adolescents (ObA) by studying non-weight-bearing (radius) as well as a weight-bearing (tibia) sites. Moreover, it also aims to investigate potential disturbances in muscle strength and specific hormonal parameters, such as sex steroids and IGF-1 known to influence bone mineralization and bone expansion during adolescence. We hypothesized that in ObA changes in bone parameters would be more evident at the weight-bearing site and that changes in serum estradiol would be most prominent.

METHODS

SUBJECTS

Fifty-one male ObA (BMI SDS >2) aged 10-19 years were investigated at the entry of a residential weight-loss program in July 2011 at the Zeepreventorium in De Haan, Belgium. Fifty-one age (maximal difference of six months) and body height (maximal difference of 5 cm) matched as well as 51 bone age (maximal difference of six months) and body height (maximal difference of 5 cm) matched healthy normal-weighted controls were selected blindly from an ongoing longitudinal study evaluating changes in bone geometry and muscle strength in relation to sex steroids in childhood and adolescence. These healthy children were recruited by letters distributed in several schools within the Ghent area. Obese and control subjects were not related to each other. Neither was there any relatedness between the control subjects. Obese and control children were excluded if they were taking medication known to influence bone or mineral metabolism in the past year or if they had a metabolic bone disease, thyroid disorders or diabetes. Both study protocols were approved by the Ethical Committee of the Ghent University Hospital. Informed consent was obtained from the parents and all participants gave their assent.

METHODS

ANTHROPOMETRY

Information about medical history, lifestyle, physical activity and socio-economic background was collected through a questionnaire. Standing height was measured to the nearest 0.1 cm using a Harpenden stadiometer (Holtain Ltd, Crymuch, UK). Body weight was measured in light indoor clothing without shoes to the nearest 0.5 kg. Waist circumference, defined as the smallest abdominal circumference if present or otherwise measured halfway between the iliac crest and the rib cage, was determined to the nearest 0.1 cm. All anthropometric measurements were performed by the same trained physician. The standard deviation score (SDS) for body height, weight and BMI was computed using the reference data of the 2004 Flemish growth study[21]. Pubertal status of the subjects was assessed by the same

trained physician according to the method established by Tanner (Tanner Genital Staging: stage 1: prepuberty; stage 5: post puberty).

BONE AGE DETERMINATION

Bone age reading of an X-ray of the left hand and wrist was done by two independent readers (two pediatric radiologists), both blinded for the chronological age, using the Greulich and Pyle method[22] and the mean of both readings was taken. If the difference was more than one year a third independent reading (by a trained pediatrician) was performed and the two closest estimates were retained. Skeletal age differences (SAD) were calculated by subtracting the chronological age (CA) from the skeletal age (BA) (SAD=BA–CA), with positive differences reflecting an accelerated skeletal maturation and negative differences a delayed bone maturation.

PQCT

Bone variables, estimates of bone strength and regional body composition of the forearm and the lower leg were measured using pQCT (Stratec XCT-2000, Stratec Medizintechnik, Germany, version 6.0). The scanner was positioned on the non-dominant forearm (radius) and lower leg (tibia). Two 2.0 mm slices (voxel size 0.5 mm) were measured at the 4 and 66% sites proximally from the distal end of the radius and two slices at the 4 and 38% site proximally from the end of the tibia. The cross-sectional area (CSA) of the radius/tibia was determined after detecting the outer bone contour at a threshold of 280 mg/ cm^3. For determining cortical vBMD, the threshold was set at 710 mg/cm^3, whereas for trabecular bone, it was set at 180 mg/cm^3. The cortical vBMD (mg/cm^3), cortical CSA (mm^2), muscle and fat CSA, endosteal and periosteal circumferences (mm), and cortical thickness (mm) were measured at the mid-radius (66% of bone length from the distal end) and mid-shaft tibia (38% of bone length from the distal end). The combined CSA of muscle and bone (fibula and tibia or radius and ulna) was determined at a threshold of 40 mg/cm^3 and the bone CSA was determined with the threshold set at 280 mg/cm^3. Muscle CSA was calculated by subtracting the bone CSA from the combined muscle and bone CSA. Fat CSA was calculated by subtracting the combined muscle and bone CSA from the total CSA. The strength-strain index (SSI) of the radius 66% and the tibia 38% was calculated[23]. To assess the SSI, a

threshold of 480 mg/cm^3 was used. Trabecular vBMD (mg/cm^3) and area were measured using a scan through the distal metaphysis at the radius and the tibia (at 4% of bone length). The CSA of the radius/tibia was determined after detecting the outer margin; 55% of this cross-sectional bone area was peeled off to separate trabecular bone from the cortical shell. The CV for the calibration phantom was <1% as calculated form daily phantom measurements.

JUMPING MECHANOGRAPHY

All measurements were recorded with the Leonardo Mechanography Ground Reaction Force Platform (Novotec Medical GmbH, Pforzheim, Germany). Both the multiple one-legged hopping (M1LH) and the single two-legged jump (S2LJ) were analyzed using the Leonardo Mechanography GRFP Research Edition software version 4.2-b05.46d. M1LH represents one-legged hopping on the forefoot with the aim to achieve a maximal ground reaction force. It evaluates the maximal force (F max) to which the tibia is exposed, and thus can serve to evaluate the muscle-bone unit. F max and F max relative to body mass (Fmax/body mass) of the left and the right leg were analyzed for this hop. The S2LJ is a vertical counter-movement jump to achieve maximum jump height. Parameters of this particular analysis were jump height, peak velocity, F max, F max/body mass, maximal peak power (P max), and P max/body mass[24]. Each subject performed three single two legged jumps and the recording with the highest jump height was selected. For the multiple one legged hopping a minimum of 10 accurate jumps had to be performed on each leg. All tests were performed between 10 am and 3 pm by the same observer using the same device. All subjects were fed and had exerted normal daily activity before the test.

HORMONAL MEASUREMENTS

Venous blood samples in the obese group were obtained between 0800 and 1000 h after overnight fasting. Blood samples in the age and height matched control group were collected between 0800 and 1000 h after a small breakfast [25]. All samples were stored at –80°C until batch analysis. Commercial immunoassays were used to measure serum IGF-1 (Diagnostic Systems Laboratories, Webster, TX), leptin (Linco Research Inc., Missouri, USA), and SHBG (Modular, Roche Diagnostics, Mannheim, Germany). The intra- and interassay coefficients of variation (CV's) for

all assays were less than 10%. Estradiol (E2), estrone (E1), and testosterone (T) were determined by liquid chromatography-tandem mass spectrometry (AB Sciex 5500 triple-quadrupole mass spectrometer; AB Sciex, Toronto Canada). Serum limit of quantification (LOQ) was <0.5 pg/mL (1.9 pmol/L) for E2 and E1 and the interassay CV's were 4.0% at 21 pg/mL (77 pmol/L) for E2, 7.6% at 25 pg/mL (93 pmol/L) for E1[26]. Serum LOQ was 1.2 ng/dl for T and the interassay CV was 8.3% at 36.7 ng/dl and 3.1% at 307.8 ng/dl. Free testosterone (FT) was determined by equilibrium dialysis[27] and free estradiol (FE2) was calculated from total E2, SHBG and albumin concentrations using a previously validated equation derived from the mass action law[28].

STATISTICS

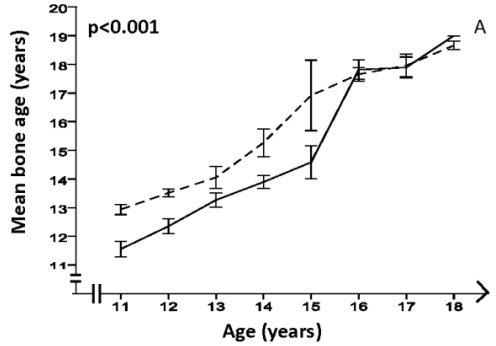
Normality was checked using QQ-plots and Shapiro-Wilk tests. Data are presented as mean±standard deviation or as medians (25th–75th percentile) in case of a non-normal distribution. Comparison between obese and control groups were performed using parametric independent T-tests or ANOVA, when criteria for normality were met. In other cases, Mann-Whitney U tests were used. Between-group differences of categorical variables were calculated with χ^2 tests. The independent predictors of the various bone parameters were tested using linear regression analysis including age, BMI and estrogen levels. The difference was considered statistically significant at $p<0.05$. In figure 1 A age categories were used children between 11 and 12 years were categorized as 11 years, children between 12 and 13 years were categorized as 12 years, etc. No rounded ages were used in the other statistical analyses. Data were analysed using SPSS software version 19.0.

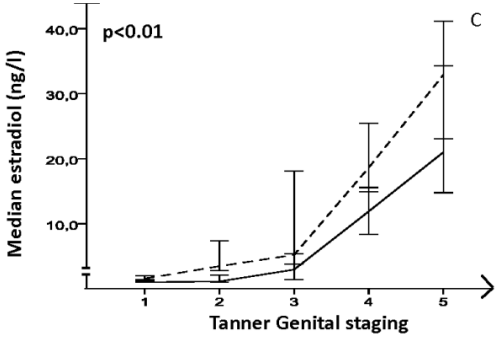
RESULTS

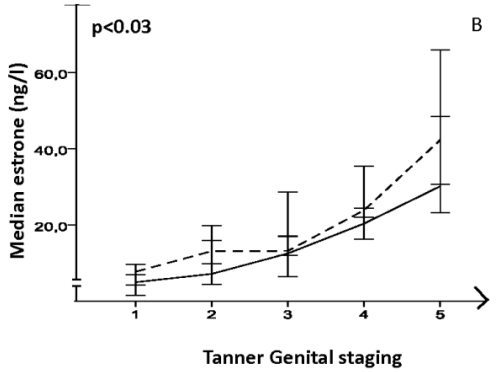
COMPARISON OF ANTHROPOMETRIC DATA AND REGIONAL BODY COMPOSITION ANALYSIS BY pQCT

Groups matched for chronological age and height

As shown in table 1, not only chronological age, and body height, but also pubertal stage were comparable between the two groups, whereas body weight (SDS) and BMI (SDS) of the obese group were almost double ($p<0.001$). Moreover, ObA had a significantly greater waist circumference ($p<0.001$). A higher absolute fat CSA, fat-muscle CSA ratio and muscle CSA at both tibia and forearm ($p<0.01$) was observed in the obese boys (table 1). There was no significant difference in mean chronological age and bone age between both groups. However, there was a significant advanced bone maturation in the obese group ($p<0.001$). Figure 1 A shows an advanced bone maturation present up to the age of 16 years.


p<0.001
A
Mean bone age (years)
20
19
18
17
16
15
14
13
12
11
11
12
13
14
15
16
17
18
Age (years)


p<0.01
C
Median estradiol (ng/l)
40,0
30,0
20,0
10,0
1
2
3
4
5
Tanner Genital staging


p<0.03
B
Median estrone (ng/l)
60,0
40,0
20,0
1
2
3
4
5
Tanner Genital staging

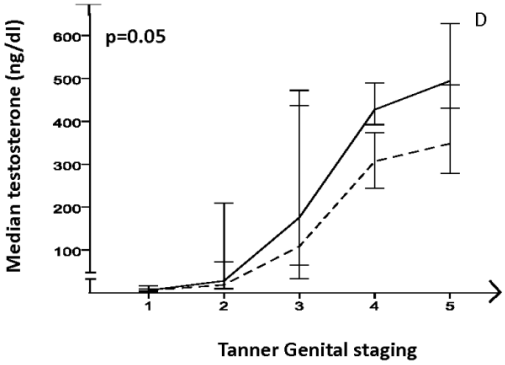

p=0.05
D
Median testosterone (ng/dl)
600
500
400
300
200
100
1
2
3
4
5
Tanner Genital staging

Figure 1:

A.Mean bone age at different ages in obese adolescents compared to their age-matched controls. The line plots present mean bone age for each age group from 11 until 19 years for the two study groups. The error bars represent 1 standard error of the mean (SEM). The obese group is presented by the dotted line and the control group (age-matched controls) by the full line. The interconnecting lines do not present longitudinal data.

B-D. Estrone (B), Estradiol (C), Testosterone (D) in obese adolescents and their age-matched controls at different pubertal stages. The line plots present median estrone levels, median estradiol levels and median testosterone levels for each pubertal stage (prepuberty: 1; post puberty: 5) for the two study groups. Since the hormonal data do not meet the criteria for a normal distribution, data are presented as medians and the error bars represent 95th confidence interval. The obese group is presented by the dotted line and the control group (age-matched controls) by the full line. The interconnecting lines do not present longitudinal data.

Groups matched for bone age and height

While body weight SDS (+2.69; p<0.001), BMI SDS (+2.47; p<0.001), waist circumference (+31 cm; p<0.001) were significantly different between obese and control group, no significant difference could be found in bone age (0.1 y; p=0.8), height SDS (0.29; p=0.13) , chronological age (-0.6 y; p=0.2), or pubertal stage distribution (P1: 12 vs 4%; P2: 18 vs 8%; P3:12 vs 18%; P4: 33 vs 37%; P5: 26 vs 33%, p=0.27).

Table 1: Comparison of anthropometric data and measures of regional body composition between obese boys and age and bone age- matched control boys.

	Obese boys (mean±SD)	Age-matched controls (mean±SD)	Bone age-matched controls (mean±SD)	Significance Age-matched (p)	Significance Bone-age matched (p)
Anthropometry					
Age (y)	14.4±2.3	14.4±2.3	15.0±2.0	ns	ns
Bone age (y)	15.5±2.3	14.6±2.7	15.4±2.5	0.07	ns
Difference bone age-age	1.12±0.90	0.18±0.90	-0.36±1.0	<0.001	<0.001
Height (cm)	167.6±11	166.3±11.3	168.7±10.5	ns	ns
Height sds	0.29±1.25	0.17±0.89	-0.04±0.9	ns	ns
Weight (kg)	99.4±24.0	53.4±12.4	57.7±11.9	<0.001	<0.001
Weight (sds)	2.74±0.59	-0.05±0.77	0.06±0.8	<0.001	<0.001
BMI (kg/m²)	35.0±5.7	19.0±2.5	19.5±2.5	<0.001	<0.001
BMI sds	2.55±0.37	-0.18±0.89	-0.36±1.0	<0.001	<0.001
Waist (cm)	102±11	68±6	71±6	<0.001	<0.001
Body composition					
Proximal forearm					
Fat CSA (cm²)	2660±817	683±380	709±388	<0.001	<0.001
Muscle CSA (cm²)	3106±840	2673±799	2969±799	<0.01	ns
Fat/Muscle ratio	91±31.9	29±20.8	27±19.2	<0.001	<0.001
Proximal tibia					
Fat CSA (cm²)	4402±1426	1574±498	1656±580	<0.001	<0.001
Muscle CSA (cm²)	3737±776	3213±850	3429±735	<0.01	<0.05
Fat/Muscle ratio	121±41	51±21	51±22	<0.001	<0.001
Tanner genital stage	**Obese boys (frequency)**	**Age-matched controls (frequency)**	**Bone-age matched controls (frequency)**		
G1	11.8%	9.8%	3.9%	ns (χ^2)	ns
G2	17.6%	15.7%	7.8%		
G3	11.7%	15.7%	17.6%		
G4	33.3%	33.3%	37.3%		
G5	25.5%	25.5%	33.3%		

Comparison between obese and control groups were performed using parametric independent t-tests. Between-group differences of categorical variables were calculated using chi-square tests.

COMPARISON OF BONE PARAMETERS AT THE UPPER AND LOWER LIMB USING PQCT

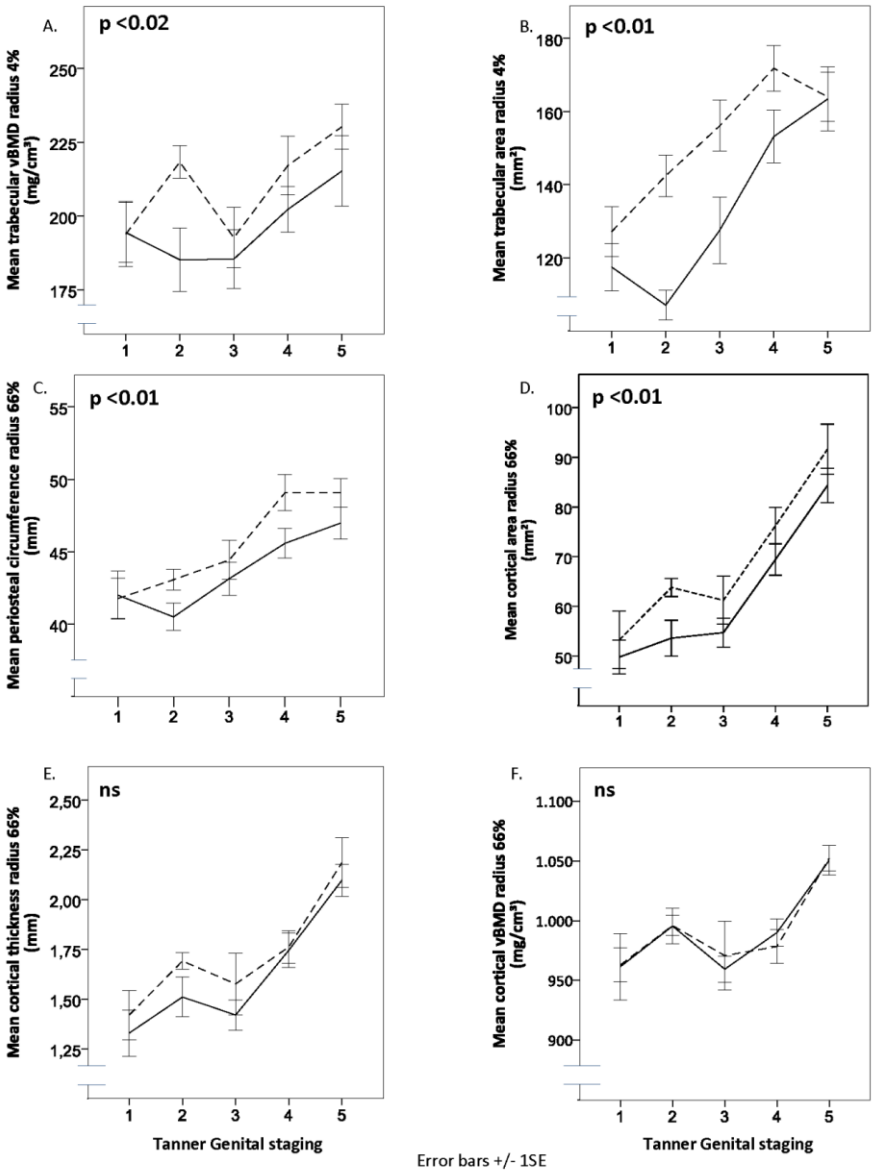
RADIUS

Groups matched for chronological age and height

ObA have a higher trabecular vBMD (+7%) and a larger trabecular area (+10%) at the distal radius. At the proximal site, cortical area (+9%), periosteal circumference (+6%), endosteal circumference (+6%) and SSI (+13%) were significantly larger in the obese group (table 2). However, there was no difference in cortical vBMD and cortical thickness between the groups. Figure 2 shows higher values of trabecular vBMD, trabecular area, periosteal circumference and cortical area at the different pubertal stages in the obese group.

Groups matched for bone age and height

ObA still had a higher trabecular vBMD (+6.5%) in comparison with bone age matched controls, but there was no difference anymore in trabecular area at the distal end. Moreover, at the proximal site, cortical parameters were comparable (table 2).



Error bars +/- 1SE

Figure 2 (A-F): Trabecular and cortical bone parameters at the radius at different pubertal stages.

The line plots present (from left to right) mean trabecular vBMD (A), mean trabecular area (B), mean periosteal circumference (C), mean cortical area (D), mean cortical thickness (E) and mean cortical vBMD (F) for each pubertal stage (prepuberty, 1; post puberty, 5) for the two study groups. The error bars represent 1SEM. The obese group is presented by the dotted line and the age-matched control group by the full line. The interconnecting lines do not present longitudinal data.

Table 2: Comparison of volumetric bone parameters as measured by pQCT at the distal (trabecular parameters) and proximal radius (cortical parameters) and distal (trabecular parameters) and proximal tibia (cortical parameters) between obese boys and controls matched for age and height and controls matched for bone age and height[a].

	Obese Boys[a]	Age-matched controls[a]	Bone age-matched controls[a]	Significance(p) Age-matched[a]	Significance(p) Bone age-matched
Radius					
4% measurement site					
Trabecular vBMD (mg/cm³)	215±33	199±35	201±33	<0.02	<0.05
Trabecular area (mm²)	157±26	141±33	149±3	<0.01	ns
66% measurement site					
Cortical vBMD (mg/cm³)	1001±54	999±55	1006±56	Ns	ns
Cortical area (mm²)	74±19	67±17	71±16	<0.05	ns
Periosteal circumference (mm)	47±4.8	44±4.3	46±4.6	<0.05	ns
Endosteal circumference (mm)	35±4.9	33±6.0	35±4.9	<0.05	ns
Cortical thickness (mm)	1.8±0.4	1.7±0.4	1.8±0.4	Ns	ns
SSI (mm³)	295±94	258±81	281±85	<0.05	ns

Table 2 continued

	Obese Boys[a]	Age-matched controls[a]	Bone age-matched controls[a]	Significance (p) Age-matched[a]	Significance (p) Bone age-matched
Tibia					
4% measurement site					
Trabecular vBMD (mg/cm³)	239±28	225±34	225±33	<0.05	<0.05
Trabecular area (mm²)	545±88	463±79	474±76	<0.001	<0.001
38% measurement site					
Cortical vBMD (mg/cm³)	1079±37	1080±54	1093±52	Ns	ns
Cortical area (mm²)	310±60	264±46	272±39	<0.001	<0.001
Periosteal circumference (mm)	82±9.0	73±6.7	74±8	<0.001	<0.001
Endosteal circumference (mm)	53±8.2	45±6.4	46±10	<0.001	<0.001
Cortical thickness (mm)	4.6±0.6	4.4±0.6	4.5±0.5	Ns	ns
SSI (mm³)	1809±467	1424±342	1497± 325	<0.001	<0.001

[a] Results are shown as mean±SD. Comparisons between obese and control groups were performed using parametric independent t-test.

TIBIA

Groups matched for chronological age and height

As shown in table 2, trabecular vBMD (+6%) and area (+15%) at the tibia were significantly higher in the obese group. At the midshaft, tibial cortical area (+15%), periosteal circumference (+11%) and endosteal circumference (+15%) were larger in the obese group ($p<0.001$). The SSI was also significantly higher (+21%) in the obese group. Figure 3 shows clearly a larger cortical area and periosteal circumference at the different pubertal stages. Additionally, both trabecular vBMD

and area were higher in the obese group, while there was no significant difference in cortical vBMD and cortical thickness between the two groups.

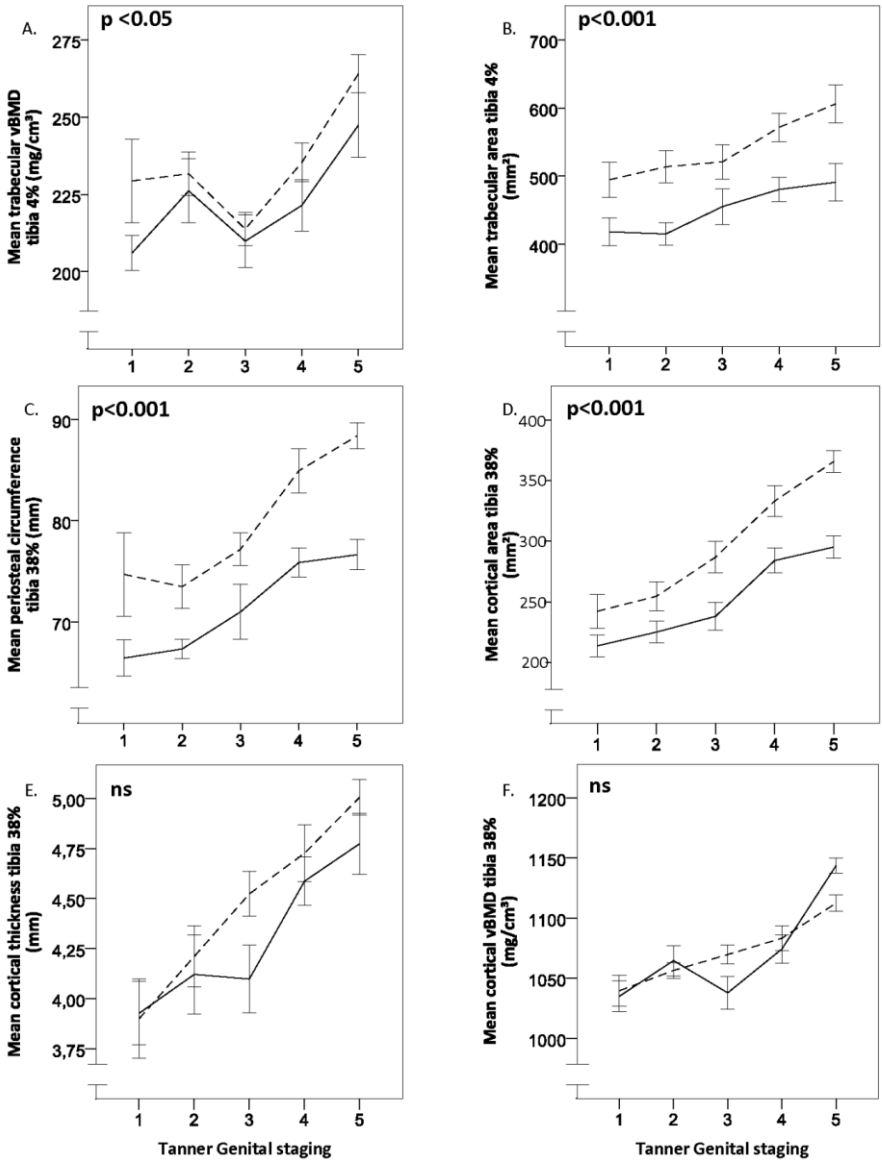


Figure 3 (A-F): Trabecular and cortical bone parameters at the tibia at different pubertal stages.

The line plots present (from left to right) mean trabecular vBMD (A), mean trabecular area (B), mean periosteal circumference (C), mean cortical area (D), mean cortical thickness (E) and mean cortical vBMD (F) for each pubertal stage (prepuberty, 1; post puberty, 5) for the two study groups. The error bars represent 1 SEM. The obese group is presented by the dotted line and the age-matched control group by the full line. The interconnecting lines do not present longitudinal data.

Groups matched for bone age and height

Matching for bone age, gave similar results: the trabecular vBMD (+6%) and area (+13%) measured at the distal end of the tibia were significantly higher in the obese group. At the midshaft, tibial cortical area (+12%), periosteal circumference (+10%) and endosteal circumference (+13%) were larger in the obese group ($p<0.001$). There was no significant difference in cortical vBMD and cortical thickness between the two groups. The SSI was significantly higher (+17%) in the obese group (table 2).

COMPARISON OF HORMONAL PARAMETERS BETWEEN OBESE ADOLESCENTS AND AGE-MATCHED CONTROLS.

ObA have significant higher serum leptin levels (28.3 (17.0-38.9) vs 2.9 (2.1-5.4) ng/ml; $p<0.001$) compared to chronological age-matched controls representative for their higher fat mass. Median serum estrogen levels (E2 (16.2 (3.7-25.7) vs. 8.4 (1.7-15.7) ng/l; $p<0.01$), FE2 (0.32 (0.07-0.54) vs. 0.14 (0.02-0.30) ng/l; $p<0.01$ and E1 (22.3 (13-35.6) vs. 17.0 (7.6-26.5) ng/l; $p<0.03$)) were markedly higher in the obese group. While both T (247 (35-355) vs. 407 (81.1-482) ng/dl; $p=0.05$) and SHBG (22.4 (17.1-38.6) vs. 48.9 (35.7-79.6) nmol/l; $p<0.001$) levels were lower in the obese group, FT levels (5.6 (0.6-9.0) vs. 5.7 (0.7-9.1) ng/dl; ns) were comparable between both groups. There was no difference in IGF-1 levels between the two groups (288 (217-412) vs. 314 (251-399) ng/ml; ns).

As expected, higher sex steroids (E2 and T) levels were found with advancing pubertal development in both groups (figure 1 C, D). Moreover, ObA had at each pubertal stage markedly higher serum estrogen levels (E2 and E1) compared to normal-weighted controls (figure 1 B, C). Median T levels tended to be lower in the obese group at different pubertal stages (figure 1 D).

COMPARISON OF MUSCLE FORCE AND MUSCLE MASS DATA BETWEEN OBESE ADOLESCENTS AND AGE-MATCHED CONTROLS.

As shown in table 3, peak force and peak power in the single two-legged jump, were respectively 43% and 21% higher in the ObA compared to the controls. However, ObA jumped on average less high than the controls, and their maximal vertical velocity during the take-off phase of the jump was lower. Weight-related peak force and power were respectively, 9% and 32% lower than in the controls. In the multiple one-legged hopping, peak force on the left and right side were 35% and 32% higher in the obese subjects. However, relative to body weight, these forces were 6% and 11% lower in the obese group than in de control group. Muscle mass as well as muscle force was higher in the obese group at any pubertal stage (data not shown).

Table 3: Comparison of single-two legged jump and multiple one-legged hopping between obese and normal–weighted boys (matched for age and height).

	Obese (median) (P25-P75)	Controls (median) (P25-P75))	Significance level (p)
Single Two-Legged Jump			
Jumping height (m)	0.2 (0.16-0.25)	0.4 (0.38-0.49)	<0.001
Peak Force (kN)	2.1 (1.5-2.4)	1.2 (1.1-1.6)	<0.001
Peak Power (kW)	2.9 (2.1-3.7)	2.3 (1.8-3.2)	<0.02
Peak Velocity (m/s)	2.0 (1.8-2.1)	2.5 (2.2-2.6)	<0.001
Peak Force per body weight	2.4 (2.1-2.6)	2.5 (2.2-2.6)	0.07
Peak Power per body weight (W/kg)	34.3 (29.5-40.6)	45.0 (40.2-52.4)	<0.001
Multiple One-Legged Hopping			
Peak Force left leg (kN)	2.1 (1.7-2.6)	1.4 (1.2-1.8)	<0.001
Peak Force right leg (kN)	2.0 (1.6-2.5)	1.4 (1.2-1.8)	<0.001
Peak Force left leg per body weight	2.5 (2.2-2.7)	2.7 (2.4-3.1)	<0.001
Peak Force right leg per body weight	2.5 (2.3-2.7)	2.8 (2.4-3.1)	<0.001

Non-Gaussian distribution: data presented as median (25th-75th percentile (P25-P75)). Comparison between obese and control group was performed using Mann-Whitney U tests.

THE CORRELATION BETWEEN ESTRADIOL AND FREE ESTRADIOL AND THE BONE PARAMETERS IN THE WHOLE POPULATION

Both E2 and FE2 correlated by linear regression with trabecular vBMD at the radius (E2: ß=0.46 p<0.001; FE2: ß=0.47 p<0.001) and tibia (E2: ß=0.51 p<0.001; FE2: ß=0.53 p<0.001) and with cortical area at both sites (radius:E2: ß=0.70 p<0.001; FE2: ß=0.50 p<0.001; tibia: E2: ß=0.73 p<0.001; FE2: ß=0.73 p<0.001). Regression models including age and BMI showed that E2 and FE2 were positively associated with trabecular vBMD at the radius (E2: ß=0.38 p<0.05; FE2: ß=0.42 p<0.01) and tibia (E2: ß=0.35 p<0.05; FE2: ß=0.37 p<0.05) and with the cortical area at the radius (E2: ß=0.31 p<:0.01; FE2: ß=0.32 p<0.01) and the tibia (E2: ß=0.24 p<0.05; FE2: ß=0.23 p<0.05). No significant associations were found between (F)E2 and cortical vBMD, endosteal circumference and trabecular area.

DISCUSSION

The present study was undertaken to investigate the vBMD and bone geometry of the peripheral skeleton in obese children during late childhood and adolescence. Our results demonstrate that obese adolescents have larger and stronger bones at the lower leg (tibia) and to a lesser degree at the lower arm (radius) than their normal-weighted peers. Moreover, obese adolescents show a more advanced bone maturation in early and mid puberty, have higher circulating estrogen levels and develop higher muscle forces at jumping.

As far as we known, only three other studies investigated the effects of obesity on vBMD and bone size in male children, but they included principally prepubertal children[10,11,12]. Since our study group consists mainly of adolescents, our data can contribute to a better understanding of the effect of obesity on bone geometry and mineralization in puberty. In contrast, we used both a chronological and bone age-matched control design to explore the impact of increased adiposity. Additionally, changes in hormones involved in bone growth, as sex steroids and IGF-1, as well as alterations in muscle mass and force were investigated to explore their potential role in bone development during adolescence.

Our bone results are in line with the results of Wetzsteon et al.[10] and Ducher et al.[11] who studied only prepubertal children. Wetzsteon et al (2008) described higher vBMD, bone area and bone strength parameters at the tibia in overweight children[10]. These results were confirmed by Ducher et al. (2009) who found a significantly larger bone size and trabecular density at the forearm and the lower leg in their overweight group[11]. No difference in cortical density could be found in either study[10,11]. Ehehalt et al. (2011) studied a group of 84 overweight children and early adolescents (mean age: 12 years) and found an altered bone structure compared to normal-weight peers at the radius; bone circumferences were larger, whereas the cortex was thinner[12].

By studying bone maturation, hormones and muscle force in parallel, our data give the opportunity to speculate about the different mechanisms that may underlie the observed differences in bone geometry and bone mineralization. Firstly, in accordance with previous studies we found that up to the age of 16 years obese

adolescents have a more advanced bone maturation compared to age- and height-matched controls[19,29,30,31]. The advanced bone development might explain at least part of the observed differences in bone expansion in our study, since after matching for bone age, no difference in cortical parameters were present, at least at the radius. However, most of the geometric differences at the tibia remained, in favor of the obese group. These results indicate that advanced bone maturation is probably not the sole explanation for the observed differences in bone geometry between obese and control boys. We speculate that higher estrogen levels as a consequence of a higher aromatization rate due to excess body fat are likely to contribute to the advanced bone maturation in adolescent obesity[29]. However, this might not be the unique explanation since Johnson et al. (2012) described also a more advanced rate of bone maturation throughout childhood[30]. Some authors suggest that the advanced bone development in obese children is due to an increased IGF-1 production[19]. However, we did not find significant differences in IGF-1 levels between young obese boys and their controls. Our results of normal serum IGF-1 levels are in accordance with the more recent studies in obese children and adolescents using similar immunoassays[32,33], although we cannot exclude that the free IGF-1 concentration might be elevated, as a consequence of decreased IGF-1 binding proteins 1 and 2 concentrations and an elevated IGF-1 binding protein proteolysis in obesity[33].

Secondly, a larger muscle size and force might play an important role in a greater bone expansion in adolescent obesity. Strain from muscle force is a known major determinant of bone size during childhood and adolescence[34, 35]. Our results confirmed a significantly higher muscle CSA at the tibia and the radius and a higher muscle force and power in obese adolescents. Since muscle mass and force increase throughout puberty together with increases in bone area, it seem plausible that the larger bones and increased bone strength in obese adolescents, after correction for bone age, are caused by the higher mechanical load applied to the skeleton, not only through a greater body weight, but also by an increased muscle mass and force. This is supported in our study by the observation of more distinct differences in bone geometry at the tibia, a weight-bearing bone, compared to the radius, a non-weight-bearing bone. These features are consistent with the results of some other studies. In obese children, Ducher et al. (2009) described a

higher muscle CSA both at the tibia and at the radius[11] and Rauch et al. (2012) documented a higher peak muscle force and peak power[36]. More support for this view comes from a recent longitudinal study in overweight children showing that increases in bone size and strength were related to the larger muscle mass but not fat mass[10]. These findings support Frost's mechanostat theory [34] and the concept that bones adapt primarily to dynamic forces produced by muscle contractions[37,38] and not to static forces imposed by extra fat mass.

Finally, we hypothesized that hormonal changes related to obesity could be involved in a different bone development and bone mass accrual during puberty. Obese adolescents in our study had at the different pubertal stages higher E2 and E1 levels, which were determined using a state of the art LC-MS-MS-based methodology. It can be noted that the difference in serum E2 is even greater when considering FE2, as a consequence of lower SHBG concentrations. Only one other study addressed the influence of sex steroids on BMD in obese children. In contrast to our study, no differences in circulating estrogen levels and a similar aBMD were found in this study[29]. Both the low number of adolescents studied and the use of an immunoassay, known to have a limited reliability for measuring low levels of E2, might be responsible for not finding a difference in circulating estrogens in this particular study. To study the influence of E2 on different bone compartments, well-described determinants of bone mass were assessed using linear regression. We observed a positive association between (F)E2 and trabecular vBMD at the radius and the tibia, as well as an association between (F)E2 and cortical area at the radius and tibia.

To our knowledge, this is the first matched-control study to report data on volumetric bone parameters and bone geometry of the tibia and the radius in adolescent obesity. The strength of the present study is the comprehensive evaluation of bone geometry, muscle strength, pubertal development and hormonal factors (especially estrogens) involved in bone expansion. Although the important role of estrogens in bone homeostasis is generally acknowledged, this study is the first to look for relationship between circulating total and free estrogens in a mixed obese and lean adolescent population. Moreover, in this study sex steroids were measured by highly sensitive and accurate mass spectrometry-based methodology as required when studying low androgen and estrogen serum

levels in children and adolescents. Our study is limited by the fact that we only have assessed cross-sectional data. In order to confirm and further unravel underlying mechanisms, prospective longitudinal studies are required, ideally with follow-up from early childhood at onset of obesity until adulthood.

CONCLUSION

We observed at both forearm and lower leg larger and stronger bones in obese adolescents compared to normal-weight peers. These differences in bone development can be explained by a combination of advanced bone maturation, higher estrogen exposure and higher mechanical loading resulting from a greater muscle mass and strength.

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4 SUMMARY OF CONTRIBUTIONS AND GENERAL DISCUSSION

This thesis has enhanced the understanding of the relative contribution of androgens versus estrogens in the regulation of pubertal development, skeletal maturation, bone mass acquisition and determination of body composition in healthy and obese up-growing boys. Our main findings are summarized in section 4.1, afterwards we will discuss our results in section 4.2, in section 4.3 we will discuss the clinical relevance of our findings and finally we will state de limitations of our research and give a perspective on future research topics in section 4.4.

4.1 MAIN FINDINGS

4.1.1 ASSOCIATIONS BETWEEN SEX STEROIDS AND (V)BMD AND BONE SIZE IN HEALTHY MALE CHILDREN AND ADOLESCENTS

In Chapter 2, we investigated the associations between sex steroid levels (adrenal and gonadal steroids) and (v)BMD and bone size in healthy male children and adolescents using multivariable-adjusted regression models including age, body height and weight.

In Chapter 2.1, we studied the contribution of adrenal-derived steroids (DHEAS, A, E1) on (v)BMD and bone size in prepubertal and early pubertal male children. Only prepubertal and early pubertal boys were selected, to investigate if adrenal steroids might impact on (v)BMD and bone size before pubertal development. Although there are some reports linking conditions with an elevated adrenal secretion such as premature adrenarche and congenital adrenal hyperplasia to an increased aBMD[28,29], we found no associations between adrenal steroid concentrations (DHEAS, A and E1) and (v)BMD or bone size in healthy prepubertal and early pubertal boys. Whether adrenal androgens within physiological range may contribute to changes in bone geometry has been previously studied by Remer et al. in 59 healthy prepubertal boys using the urinary excretion of the major urinary androgen (C19) metabolites[69,73]. While in this study slightly positive effects of DHEA and its 16-hydroxylated downstream metabolites on cortical vBMD and BMC at the proximal radial diaphyseal bone were observed in prepubarchal

children, no effect on the metaphyseal site was observed[69,73]. Their finding that of all adrenal androgen metabolites studied, only androstenediol, which acts as an estrogen and androgen receptor agonist, showed a long term prediction of bone strength suggests that adrenal DHEA increases are not bone anabolic per se[74]. Based on our data, we have no arguments for a direct bone-anabolic effect of adrenal-derived steroids determined in serum on either (v)BMD or bone size in prepubertal boys. Muscle mass was however a significant positive predictor of bone size in pre-and early pubertal children. This is not unexpected since it is well-known that strain from muscle force is a major determinant of bone size during childhood and adolescence[152,256].

In Chapter 2.2, the associations between sex steroids (T, FT, E2, FE2) and (v)BMD and bone size were investigated in boys 6 to 19 years old. We found that in males (F)E2 is associated with (v)BMD and endosteal circumference and that (F)T is associated with different parameters of bone size.

E2 and FE2 were positively associated with lumbar spine and whole body aBMD. Moreover, there was a positive association of E2 and FE2 with trabecular vBMD at the radius and the tibia. However, no associations between E2 and FE2 and cortical vBMD at the radius or tibia were present. All associations remained significant after inclusion of other possible determinants of BMD in the model such as T, IGF-1, calcium intake or physical activity. After inclusion of lean mass or muscle CSA in the model, all associations remained significant except for the associations of (F)E2 with whole body aBMD. Furthermore, E2 and FE2 were negatively associated with the endosteal circumference at the radius.

T and FT levels were associated with different parameters of bone size, such as whole body and lumbar spine bone area, trabecular and cortical bone area and periosteal circumference of the radius. Moreover, there was a significant positive association of T and FT with whole body lean mass and muscle CSA at the radius and tibia. It should be noted, however, that associations found between sex hormones and bone size, might not reflect solely direct effects of sex hormones on bone, but could reflect at least in part indirect effects resulting from sex hormone-dependent alterations in IGF-1 levels. Previous research showed that circulating IGF-1 is affected by ERα signaling[257] and that it is at least partly involved in the regulation of cortical bone geometry in mice[128]. The inclusion of IGF-1 as

independent predictor in the multiple linear regression analyses did, however, not change the found associations. This suggests that the observed associations between sex hormones, (v)BMD and bone geometry are independent associations not mediated by circulating IGF-1. After inclusion of whole body lean mass or muscle CSA in the model, the associations of T and FT with bone size were however no longer present, indicating that in boys the effects of T are mainly mediated through its effects on muscle mass and strength.

4.1.2 ASSOCIATIONS BETWEEN SEX STEROIDS AND SKELETAL MATURATION IN HEALTHY MALE CHILDREN AND ADOLESCENTS

In Chapter 2, the associations between sex steroid levels and skeletal maturation were studied in healthy boys.

In Chapter 2.1, we investigated the contribution of adrenal steroids (DHEAS, A, E1) on skeletal maturation in prepubertal and early pubertal boys. Studies in conditions with an elevated adrenal secretion such as premature adrenarche and congenital adrenal hyperplasia have reported an advanced skeletal maturation[24–27]. No data are however available on the association between adrenal steroids and bone maturation in healthy prepubertal and early pubertal boys. DHEAS, A and E1 are highly intercorrelated (r=0.77 to 0.86). In multivariable-adjusted analyses (including age, body weight and height) adrenal steroids (DHEAS, A, E1) are found to be independent positive predictors of bone age in males. Since we included also early pubertal boys (ie, pubertal stage G2) it might be argued that the observed effect on bone maturation could result from gonadal steroid production. We are however confident that this is not the case since the observed associations were confirmed in a subgroup of only prepubertal children. Moreover, the associations of E1 and A with bone age remained after inclusion of respectively E2 and T in the model. Based on human[100,101,104] and animal data[131], we would expect that the observed associations are due to E1. However, after inclusion of DHEAS or A in the model the association between E1 and bone age disappeared.

In Chapter 2.2, the associations between sex steroids (E2 and T) and skeletal maturation were investigated in boys between 6 and 19 years old. There was a positive association of E2 and FE2 with bone maturation. These associations

remained significant after inclusion of T or IGF-1 in the models. Our results therefore stress the importance of E2 in epiphyseal maturation in boys and are in line with data obtained from men with estrogen resistance[104] or aromatase deficiency[100,101,110] who have non-fused epiphyses and continue to grow after sexual maturation.

4.1.3 SEX STEROIDS IN RELATION TO SEXUAL AND SKELETAL MATURATION IN OBESE MALE ADOLESCENTS

In chapter 3.1 we studied sex steroid levels in relation to sexual and skeletal maturation in a well-described group of 90 obese male adolescents and 90 age-matched controls. It is known that childhood obesity is associated with an advanced skeletal maturation[172,174,178,183,184]. Data concerning pubertal development and sex steroids are however limited and contrasting. Our results demonstrate that obese boys compared to controls have a normal genital development and similar serum PSA levels, which can be used as a marker of androgen activity in males. However, skeletal maturation was advanced (mean advancement around 1 year) from Tanner genital stage 1 to 4. Before pubertal onset, TT concentrations were similar, while SHBG concentrations were lower and FT, E2, DHEAS and A were higher in the obese boys. During puberty, obese adolescents with at least stage G2 pubertal development have lower SHBG levels at every pubertal stage, lower TT concentrations from stage G3 onward, but similar FT concentrations, except for adolescents in pubertal stage 5. No significant differences in circulating LH and FSH concentrations were found between the obese boys and their controls. On the other hand, serum E2 concentrations were significantly higher in the obese adolescents at each pubertal genital stage. Our data therefore indicate that FT is a better indicator of androgen exposure than TT, explaining the normal pubertal progression and PSA production in male obese adolescents despite lower TT levels. Our findings further suggest that increased aromatization with higher estrogen production in obese boys might be linked to the advanced skeletal maturation during pubertal progression.

4.1.4 SEX STEROIDS AS ONE OF THE DETERMINANTS OF vBMD AND BONE SIZE IN OBESE MALE ADOLESCENTS

The results of chapter 3.1 showed that obese adolescents have higher E2 levels at every pubertal stage probably due to the increased aromatization of androgen to estrogen in fat mass. Based on our results in chapter 2.2 showing positive associations between (F)E2 and trabecular vBMD in healthy children, one could expect higher vBMD in the obese subjects. Apart from hyperestrogenemia in obese children, there is some evidence from studies in prepubertal children that obese subjects also have a larger muscle mass[220,221] which is regarded as an essential contributor of pubertal bone mass[46]. To further unravel the effects of obesity on bone development, vBMD and bone size were studied in parallel with sex steroids and muscle mass and strength in 51 obese male adolescents and 51 healthy age-matched controls. Our results demonstrate that obese adolescents have larger and stronger bones at the lower leg (tibia) and to a lesser degree at the forearm (radius) than their normal-weighted peers. Obese male adolescents have a higher trabecular vBMD at the radius and the tibia compared to age-matched controls. No difference in cortical vBMD between the two groups with different adiposity was however present. This is in line with the results of two previous studies in prepubertal obese children describing a higher trabecular vBMD, but a similar cortical vBMD compared to lean controls[220,221]. Wetzsteon et al. (2008)[220] suggested that the difference in trabecular vBMD is related to an advanced skeletal maturation in the obese children; however, our results showed that the differences in trabecular vBMD remain even after correction for the advanced skeletal maturation using a bone age-matched control group. We speculate that the observed differences are rather due to higher E2 levels since there were positive associations between (F)E2 and trabecular vBMD. However, the cross-sectional design does not allow us to draw causative conclusions. Only one other study addressed the influence of sex steroids on BMD in obese children. In contrast to our study, no differences in circulating estrogen levels and a similar aBMD were found in this study[183]. Both the low number of adolescents studied and the use of an immunoassay, known to have a limited reliability for measuring low levels of E2, might be responsible for not finding a difference in circulating estrogens in this particular study.

In our study trabecular area, cortical area, periosteal circumference and endosteal circumference at the radius and the tibia were significantly larger in the obese adolescents. This is in line with the results of two studies performed in prepubertal obese children. Wetzsteon et al. (2008) described a larger bone area at the distal and proximal tibia in overweight children[220]. These results were confirmed by Ducher et al. (2009) who found a significantly larger bone area at the forearm and the lower leg in their overweight group[221]. The advanced skeletal maturation might explain at least part of the observed differences in bone expansion in our study, since after matching for bone age, no differences in cortical bone area parameters were present, at least at the radius. However, most of the geometric differences at the tibia remained, in favor of the obese group. These results indicate that advanced bone maturation is probably not the sole explanation for the observed differences in bone geometry between obese and control boys. An additional argument is given by a study of Uusi-Rasi et al. (2012), showing that overweight at the age of 12 is associated with a larger bone CSA at the radius and tibia in adulthood[225]. A larger muscle size and force might play an important role in a greater bone expansion in adolescent obesity, as has been stated by several researchers[220,221]. Our results indeed showed a significantly higher muscle CSA at the tibia and the radius and a higher muscle force and power in obese adolescents. Moreover, we found more distinct differences in bone geometry at the tibia, a weight-bearing bone, compared to the radius, a non-weight-bearing bone.

In conclusion, obese boys have larger and stronger bones at both forearm and lower leg compared to normal-weight peers. These differences in bone development can be explained by a combination of advanced bone maturation, higher estrogen exposure and higher mechanical loading resulting from a larger muscle mass and strength.

4.2 GENERAL DISCUSSION

Sex steroids play a critical role in pubertal development, skeletal maturation, peak bone mass acquisition and determination of body composition in up-growing males. Although T is regarded as the most important sex steroid in males, several observations in humans and animal models have stressed the importance of estrogen in adult males[1,2]. Information about the effects of estrogens on skeletal development, skeletal maturation and body composition during childhood and

adolescence is however scarce. This thesis has provided further insight into the understanding of the relative contribution of androgens versus estrogens in the regulation of different developmental processes in healthy and obese boys.

Firstly, our research clarified some items of discussion concerning pubertal development and sex steroid levels in obese boys. As described in the introduction, data on the effects of obesity on pubertal development[172,174–178] and sex steroid levels[174,185–191] in obese boys are scarce and contrasting. Our results demonstrate that pubertal obese boys have lower TT levels, but normal FT levels, at least during mid-and late puberty. As previously described by others, we found markedly lower SHBG levels at every pubertal stage, probably caused by the increased insulin levels in obese boys[258]. Since approximately half of TT is bound to SHBG, it is likely that the lower SHBG concentrations can account at least partially for these lower TT concentrations. Our data indicate that FT is preserved and a better indicator of androgen exposure than TT, explaining the normal pubertal progression and PSA production in male obese adolescents.

Secondly, our research illustrated the essential role of estrogens (E2 and E1) on skeletal maturation. In chapter 2.2, we demonstrated that E2 and FE2 are significant positive predictors of bone maturation in healthy boys. Our results are in line with the data from men with estrogen resistance[104] or aromatase deficiency[100,101,110] who present with non-fused epiphyses, whereas individuals with androgen insensitivity syndrome due to a mutation of the AR achieve epiphyseal closure[122]. Moreover, administration of E2 to aromatase deficient men results in closure of the epiphysis[101,102,110]. Experimental studies in juvenile ovariectomized rabbits have demonstrated that E2 accelerates the programmed senescence in the proliferation rate and number and size of chondrocytes, leading finally to epiphyseal plate fusion[131]. The essential role of estrogens in the inhibition and final cessation of growth after puberty is further supported by the finding that FE2 was an independent negative predictor of height in young men who had just reached final height[259]. The observation that obese boys have an accelerated bone maturation linked to an increased aromatization and E2 production provides additional evidence for an important role of estrogens in bone maturation. Since Johnson et al. (2012), already described an advanced skeletal maturation in obese

children throughout childhood, the high E2 levels might not be the unique explanation[184]. Some authors suggest that the advanced bone maturation in obese children is due to an increased IGF-1 production[260]. However, we did not find significant differences in IGF-1 levels between young obese boys and their controls. Another possible explanation for the bone age advancement in obese prepubertal children is elevated adrenal steroid levels. A study by Reinehr et al. (2013) has recently reported higher adrenal steroid levels (DHEAS and A) in obese prepubertal children[186] and it is known that children with conditions characterized by an increased adrenal steroid secretion as premature adrenarche present with an advanced skeletal maturation[24–27]. In favor of this hypothesis, we found a positive association between serum levels of adrenal-derived steroids and bone maturation in healthy (prepubertal and early pubertal) children. Although one would expect that the effects are due to E1, the association between E1 and bone age disappeared after inclusion of DHEAS or A in the model. Possible explanations are local aromatization of androgens to estrogens at level of the growth plate[98,261] or technical issues. As to the latter, it can be mentioned that a highly performant assay[234] was used for measurement of E1 (and E2) serum levels in our study. In any case, serum levels of the studied adrenal–derived steroids are strongly interrelated, so that it might not be possible to establish the independent contribution of single adrenal steroids from an association study.

Thirdly, our results presented in chapter 2.2 and 3.2 stress the importance of E2 as a determinant of BMD. As shown in chapter 2.2, (F)E2 is a positive predictor of lumbar spine and whole body aBMD. Our results are in line with the findings of Yilmaz et al. (2005) in healthy adolescents, showing a strong positive association of E2 with whole body and lumbar spine aBMD[38]. Pomerant et al. (2007), however reported that T was a significant positive predictor for whole body and lumbar spine aBMD in healthy boys. Since they did not study associations between E2 and aBMD, they cannot exclude that the observed positive association with T is an E2 effect, related to the aromatization of T[132]. Furthermore, we found positive associations between (F)E2 and trabecular vBMD at the radius and the tibia, whereas there were no associations between (F)E2 and cortical vBMD at the radius or tibia. These results are supported by data from our obese population, showing a higher trabecular vBMD at the radius and tibia compared to healthy lean controls. Moreover, a multivariate regression model showed that E2 was indeed a positive

predictor of trabecular vBMD in a mixed obese-lean population. Cortical vBMD of the obese boys was however similar to healthy controls. As far as we know, there are no previous studies assessing the effects of (F)E2 on vBMD in healthy or obese boys. Our results are in line with the observations of Lapauw et al. (2009), showing a positive trend between FE2 and trabecular vBMD in young adult males at the age of peak bone mass[3]. However, they also described positive associations between FE2 and cortical vBMD at the radius and tibia[3,259], which is at variance with our data in children. More evidence comes from research in men with idiopathic osteoporosis linking low FE2 concentrations to a lower trabecular and cortical vBMD[4]. Furthermore, a recent report by Smith et al. (2008) described a lower trabecular and cortical vBMD in a patient with estrogen resistance[108]. Unexpectedly and in contrast with our results, there was no increase in trabecular or cortical vBMD during estrogen treatment in a case report of an aromatase deficient male[102]. Furthermore, results of some mouse experiments indicated that AR activation may be the sole responsible for the development and maintenance of male trabecular bone mass and that both AR and ER α activation are needed to optimize acquisition of cortical bone and muscle mass[262,263]. These results seem in contrast with our observations in healthy and obese boys.

Finally, we will discuss the determinants of bone size in healthy and obese boys with particular focus on the associations between sex steroids and bone size. In chapter 2.1, we showed that (F)T levels are associated with different parameters of bone size, such as whole body and lumbar spine bone area, trabecular and cortical bone area, and periosteal circumference at the radius. The observed associations differed between early-mid pubertal boys and late-post pubertal boys. In early-mid pubertal boys, only the association of (F)T with periosteal circumference was significant. This is not unexpected since bone diameter increases in early puberty by rapid periosteal apposition and periosteal apposition rates peak at the same time as growth in length[264]. In late-post pubertal boys, there were positive associations of (F)T with lumbar spine and whole body bone area and with trabecular bone area, cortical bone area and periosteal circumference at the radius. Our results are in line with previously reported associations of FT with cortical bone area and periosteal circumference at the radius in young male adults[259]. Moreover, a role of T in bone expansion is supported by the finding of a bone size

intermediate between males and females in a patient with androgen insensitivity syndrome. Furthermore, (F)E2 are negatively associated with the endosteal circumference at the radius in our healthy boys. This finding confirms the data in young adult males describing negative associations between (F)E2 and the endosteal circumference (endosteal contraction)[3,259]. These data suggest that for normal bone mass accrual throughout puberty an estrogen-induced suppression of endosteal expansion is needed in addition to an androgen-induced periosteal bone expansion. A possible role of estrogens in bone expansion during early puberty is suggested by some mouse experiments and a case report of a boy with aromatase deficiency. Total, cortical and trabecular bone CSA of a 16 year old boy with aromatase deficiency increased significantly during estrogen treatment, suggesting that optimal cortical bone expansion requires activation of both AR and ER α[102]. In male mice, estrogen deficiency on top of androgen withdrawal further reduced radial bone expansion, at least during early stages of puberty suggesting that both AR and ER α activation appear to stimulate radial bone expansion in early pubertal male mice. However, the described estrogen-mediated stimulatory effects on periosteal bone formation and cortical bone growth may be mediated indirectly by GH-IGF-1 axis since IGF-1 levels in the orchidectomized mice treated with an aromatase inhibitor were significantly lower compared to both sham and orchidectomized mice[128]. We were not able to confirm these data in our research; further longitudinal research in healthy boys is needed to clarify if there is indeed, as was suggested, a bimodal dose-response relationship between estrogens and periosteal bone expansion with stimulating effects at low dosage and inhibiting actions at higher concentrations.

Based on our results, we suppose that an important part of the effects of T on bone size are due to an increase in muscle mass during puberty. It is known that the increase in bone size is driven by strain from muscle force and that muscle development precedes bone development during pubertal growth spurt[152,153]. Since there are positive associations between (F)T and muscle mass as well as positive associations between (F)T and bone size which disappear after inclusion of muscle mass in the model, we hypothesize that (F)T lead to an increase in muscle mass which in turn causes a larger bone size due to an increase in strain exerted on the bone. Due to the cross-sectional design of our studies, however, we are not able to draw causative conclusions and direct effects of (F)T on bone size cannot be

definitely excluded. Our results in obese boys support the importance of muscle mass and force on bone size. We showed in chapter 3.2 that obese boys have larger long bones compared to age-matched controls, in addition to a larger muscle CSA and a higher muscle force and power output measured by mechanography. These features are consistent with the results of some other studies. Ducher et al. (2009) described a higher bone and muscle CSA both at the tibia and at the radius in prepubertal obese children[221] and Rauch et al. (2012) documented a higher peak muscle force and peak power in obese children[265]. Since muscle mass and force increase throughout puberty together with increases in bone area, it seem plausible that the larger bones and the increased bone strength in our study, even after correction for bone age, are caused by the higher mechanical load applied to the skeleton, not only through a larger body weight by an increased fat mass, but also by an increased muscle mass and force. More support for this view comes from a recent longitudinal study in overweight children showing that increases in bone size and strength were related to muscle mass, but not to fat mass[220]. These findings support Frost's mechanostat theory[152] and the concept that bones adapt primarily to dynamic forces produced by muscle contractions[266,267] and not to static forces imposed by extra fat mass. Based on the negative associations between (F)E2 and endosteal circumference in healthy boys, one should expect a smaller endosteal circumference in the obese boys. In contrast, obese adolescents had a larger endosteal circumference at the radius and the tibia compared to healthy controls probably due to larger bone size as a result of higher muscle mass and force. The effects of muscle mass and force seem thus to overrule the expected sex steroid effects on bone size in obese boys.

4.3 CLINICAL RELEVANCE

In the next paragraph, we will discuss the clinical relevance of some of our findings.

The observed associations of estrogens with skeletal maturation and bone mineral density can be of importance for the treatment of boys with short stature. Since estrogens are formed by the conversion of androgens by the aromatase enzyme, aromatase inhibition may be an effective means of enhancing growth in boys with short stature by postponing epiphyseal closure. Up to now, two controlled studies have examined the effects of aromatase inhibitors on height in boys with idiopathic

short stature[268,269], showing a mean increase in predicted adult height between 4 and 6 cm. No data to adult height are however yet available. Furthermore, the safety of aromatase inhibitors is not yet established. As we have shown that estradiol is positively associated with aBMD and vBMD in healthy and obese boys, the possible side effects of aromatase inhibitors on skeletal growth and bone mass accrual need to be studied. When aromatase inhibitors are used in clinical or research setting, a conscientious follow-up is needed. At the start of the treatment, we suggest taking an X-ray of the vertebrae, a DXA scan of the lumbar spine and a whole body and pQCT scan of the forearm, followed by a yearly follow-up using DXA scans (including an instant vertebral assessment (IVA)) and pQCT scans. We would suggest a final X-ray of the vertebrae at the end of the treatment to evaluate possible vertebral deformities. Preventive strategies as encouraging physical activity and intake of dairy products, as well as vitamin D supplementation when deficient, should be implemented during treatment.

The observed advanced skeletal maturation in obese boys is of importance when interpreting bone mineral density and bone size results of this particular group. Although the advanced skeletal maturation in obese boys does not compromise final height[184,270], it can be important in the timing of dentofacial and orthopedic treatments as a patient will respond more effectively to the treatment if skeletal development has not yet reached its conclusion[271,272].

The observation that obese boys have a normal pubertal development and similar PSA concentrations (used as a marker of androgen activity) accompanied by normal FT levels but low TT levels, stresses the importance to determine FT as well as TT to evaluate gonadal function in this population. FT seems to be a better marker for androgen activity than TT which is largely influenced by the low SHBG levels.

4.4 LIMITATIONS AND PERSPECTIVES

Although our findings have contributed to the understanding of the relative role of androgens and estrogens in the regulation of pubertal development, epiphyseal maturation, the build-up of the skeleton and changes in body composition during the growth phase, we were unable to draw causative conclusions on the observed associations due to the cross-sectional design of our work. A second limitation of

our work is related to the data collection. Although for recruitment of the healthy study population particular attention was paid to recruitment of children from a representative diversified background (e.g. children following different types of education), there was among participants some bias towards children from higher socio-economic background. This is in particular the case when comparing to the children in the obese study population. Furthermore, calcium intake in our study populations was estimated with a questionnaire validated in adults but not in children. Moreover, there are some recent and better validated questionnaires available. A final limitation is the fact that the measurements of adrenal and gonadal sex steroids were single point measurements. Although it is indeed possible that a single measurement is not fully representative for adrenal or gonadal steroid exposure, in clinical settings, a single measurement from a serum sample drawn in early morning is commonly used and acceptable.

In order to confirm and further unravel underlying mechanisms in the relation between sex steroids and skeletal maturation, bone mass acquisition and body composition, prospective longitudinal studies are required, ideally with follow-up from early childhood until adulthood. Our healthy controls are currently followed up longitudinally to evaluate changes in bone geometry, bone maturation and muscle strength in relation to sex steroids and to further clarify the causes and consequences in the observed relations. However, it was not possible to collect these longitudinal data within the time frame of this doctoral dissertation.

We are convinced that a longitudinal follow-up of obese children from early childhood to adulthood would be very interesting, preferentially organized within the setting of an obesity clinic. Our data did not show any difference in Tanner genital staging between obese boys and their healthy controls, however to establish with certainty if there is a difference in timing (start) or tempo (progression) of pubertal development, a longitudinal design is required. Furthermore, it would be interesting to study at what time and at which degree of weight gain the differences in vBMD and bone size become apparent.

An interesting additional question to be addressed is the effects of weight loss not only on sex steroids, but also on bone maturation, vBMD, bone geometry and body composition. Therefore, an evaluation 3 to 6 months (for sex steroids and body composition) and 1 year (for sex steroids, body composition and bone parameters) after the start of a weight loss intervention would be interesting. The additional 3 to 6 months evaluation (mean weight loss after 6 months is about 20%) is necessary to study the effects on sex steroid levels, since an evaluation only at the end of the year, could possibly create some problems in the interpretation of the sex steroid levels as there are in fact evolutions of two variables to be taken into account, namely pubertal progression and weight loss. Furthermore, there are some other potential confounding factors that will have to be taken into account in future analyses. Increased physical activity during a weight loss program will have an effect on muscle mass, which in turn influences bone size. Possible changes in calcium, vitamin D and protein intake due to dietary changes, will have to be recorded by a structured food questionnaire and corrected for in the further analyses. Additionally, the results at one year could be compared to the evolution in bone maturation, vBMD and bone size in our healthy control group.

Beside conditions of high estrogen production in boys, such as childhood obesity, also situations of low estrogen exposure are of interest for further study. To study the effects of low estrogen exposure on skeletal maturation and bone mass acquisition two additional studies have already been started.

Firstly, young sons (<18y) of men with idiopathic osteoporosis are followed up at the Ghent University Hospital, Endocrinology department. In this particular group

of men indirect evidence was present that an impaired estrogen action during skeletal maturation might be involved in the deficient bone mass acquisition. However, definitive proof of the implication of a low estrogen status during childhood resulting in this specific bone phenotype of idiopathic osteoporosis in males and their affected sons is lacking[4,273], by lack of longitudinal data during childhood. A cross-sectional analysis of the already available bone data showed that young sons of men with idiopathic osteoporosis have a lower whole body aBMD, lower trabecular vBMD at the radius and tibia and a delayed bone maturation compared to healthy controls. These findings are suggestive that relative estrogen deficiency during childhood might be responsible for male idiopathic osteoporosis. However, sex steroid levels are not available in all children reducing the power of the study. Therefore, further recruitment and follow-up is ongoing.

Secondly, a Belgian multi-center study has started to treat a group of 25 boys diagnosed with idiopathic short stature with an aromatase inhibitor at onset of puberty to suppress the estrogen production in order to delay bone maturation and increase final adult height. In this particular study, vBMD and bone geometry at the radius will be followed during treatment to observe potential adverse effects of relative estrogen deficiency during adolescence.

About the author

Sara Vandewalle was born on the 11th of February, 1983 in Ghent, Belgium. After her high-school education at the Sint-Franciskusinstituut in Melle, she started her Medicine studies in 2001 at the Ghent University and obtained her master degree in Medicine in 2008 (magna cum laude). After 2 years of training in Pediatrics, she started as a PhD fellow of the Research Foundation-Flanders (FWO Vlaanderen) at the Department of Endocrinology of the Ghent University Hospital. Under the supervision of Prof. Dr. Jean-Marc Kaufman (Department of Endocrinology) and Prof. Dr. Jean De Schepper (Department of Pediatric Endocrinology), she performed research on the relative contribution of androgens and estrogens on pubertal development, skeletal maturation, bone mass acquisition and body composition in healthy and obese male children and adolescents. From October 2014, she will start her training in Internal Medicine.

Awards

Belgian Bone Club Award: Clinical Project of the year 2011, January 2012

Belgian Bone Club Award: Best presentation of the year 2012, January 2013

Belgian Bone Club Award: Best presentation of the year 2013, January 2014

New investigator award: on the occasion of the 6th international conference on children's bone health 22-25 June 2013 Rotterdam, Netherlands

Tutor for Master Dissertation

Charlotte Uvin (Master in Medicine): Hebben zonen van mannen met idiopathische osteoporose een afwijkend fenotype qua bot, lichaamssamenstelling en geslachtshormonen?

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ORAL PRESENTATIONS

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Vandewalle S, Zmierckzak H, Roggen I, Debode P, van Helvoirt M, Taes Y, Goemaere S, Toye K, Kaufman JM, De Schepper J. Male obese adolescents have stronger and bigger bones than their normal-weighted peers. Oral presentation at ISMNI conference in Suffolk; May 2012.

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Van Caenegem E, Wierckx K, Taes Y, Schreiner T, **Vandewalle S**, Toye K, Kaufman JM, T'Sjoen G. Preservation of bone mass in trans women during cross-sex hormonal therapy: a prospective observational study. Oral presentation at WCO-IOF-ESCEO in Sevilla; April 2014.

ABSTRACTS AND POSTER PRESENTATIONS

2011

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