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Bone Structure Assessed with pQCT in Prepubertal Males with Delayed Puberty or Congenital Hypogonadotropic Hypogonadism

Running title: Bone structure in delayed puberty

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Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request. Data will be shared according to the EU General Data Protection Regulation and national and hospital data protection regulations.

SUMMARY

OBJECTIVE: Congenital hypogonadotropic hypogonadism (CHH) is associated with impaired bone mineral density in adulthood, whereas the estimates on bone structure in adolescents with CHH has not been previously evaluated.

DESIGN: This cross-sectional study describes bone structure in CHH patients and compares it to that in boys with constitutional delay of growth and puberty (CDGP).

METHODS: Peripheral quantitative computed tomography (pQCT) of non-dominant arm and left leg were performed in five treatment-naïve males diagnosed with CHH and in 24 males with CDGP. Volumetric bone mineral density (BMD), bone mineral content, and area in trabecular and cortical bone compartments were evaluated, and bone age-adjusted Z-scores for the bone parameters were determined.

RESULTS: The participants with CHH had more advanced bone age and were older, taller, and heavier than the CDGP boys, yet they had lower trabecular BMD in distal radius (147.7 mg/mm^3 [95%CI, 128-168 mg/mm^3] *vs.* 181.2 mg/mm^3 [172-192 mg/mm^3], p=0.002) and distal tibia (167.6 mg/mm^3 [145-190 mg/mm^3] *vs.* 207.2 mg/mm^3 [187-227 mg/mm^3], p=0.012), respectively. CHH males had lower cortical thickness at diaphyseal tibia than the participants with CDGP (p=0.001). These between-group differences remained significant in corresponding Z-scores adjusted for bone age and height (p=0.001). In CDGP group, serum testosterone correlated positively with trabecular BMD (r=0.51, p=0.013) at distal radius, and estradiol levels correlated positively with trabecular BMD at the distal site of tibia (r=0.58, p=0.004).

CONCLUSIONS: Five treatment-naïve male patients with CHH exhibited poorer trabecular BMD than untreated males with CDGP. We speculate that timely low-dose sex steroid replacement in CHH males may benefit skeletal health in adulthood.

INTRODUCTION

Puberty is a crucial period for bone growth and modulation. The fastest gain in bone mineral content (BMC) occurs 2 years before and after the pubertal peak height velocity[1,2]. At the same time, approximately one third of the predicted maximal adult BMC is acquired[1]. In males, the sex-specific skeletal changes of puberty are brought about by rise in testosterone secretion, which acts either directly or through conversion to estrogen[3]. During adolescence, periosteal bone apposition and endocortical resorption are greater in boys than in girls, resulting in the distribution of bone mineral away from the central axis and in biomechanically stronger bones in males[4]. To this end, testosterone levels correlate not only with cortical bone size, but also with the number of trabecular lamellae and trabecular thickness[5,6], whereas estradiol levels correlate positively not only with trabecular density, but also with cortical thickness suggesting that, in males, both hormones have important roles in the optimal development of trabecular and cortical bone[6,7].

Pubertal delay, defined as the absence of clinical signs of puberty at the age of 2-2.5 SD above the mean of the general population (above 14 years in boys)[8], is most commonly caused by constitutional delay of growth and puberty (CDGP). This self-limited condition is a late variant of normal timing of puberty that provides a model to investigate the role of sex steroids in bone modulation and mineral acquisition. In CDGP, bone mass accrual is delayed suggesting a slender bone phenotype, but the condition has not been associated with fractures or osteoporosis in later life [9-12]. In contrast to CDGP, congenital hypogonadotropic hypogonadism (CHH) is a rare disease characterized by absent or incomplete puberty. In subjects with CHH, GnRH deficiency results in low gonadotropin and sex steroid levels making patients prone to impaired bone mineral acquisition in adolescence and adulthood. Therefore, areal BMD is impaired in CHH men who are not on adequate sex hormone replacement therapy[13-15]. The skeletal consequences of male hypogonadism may be mainly due to estrogen deficiency[16]. It is noteworthy that the bone structure in adolescents with CHH has remained unknown[17]. Peripheral quantitative computed tomography (pQCT) is a

bone imaging method, which unambiguously differentiates bone compartments from a cross-sectional image of radius or tibia allowing the evaluation of bone structure in cortical and trabecular bone compartments separately. This imaging method has not been utilized in subjects with CDGP or CHH before.

We hypothesized that late puberty associates with decreased bone mineral content and volumetric density (BMD), especially in the trabecular segment, and that the decrease is more profound in subjects with CHH (who have profound and usually permanent sex steroid deficiency) than in those with self-limiting constitutional delay of growth and puberty (CDGP). We further hypothesized that measures of cortical bone development are lower in boys with delayed puberty than in their peers. In this pilot study, we addressed these questions by pQCT measurements of bone structure in males diagnosed with either of the two conditions.

PATIENTS AND METHODS

The CDGP group comprised of 24 prepubertal boys who participated in a prospective randomized controlled trial conducted in four Finnish pediatric endocrinology outpatient clinics between 2013 and 2017 (18). In the trial, 30 CDGP boys were randomized to receive either aromatase inhibitor letrozole (n=15) or intramuscular testosterone (n=15) for six months (ClinicalTrials.gov, NCT01797718)[18]. The current study included data only from the first study visit from 24 prepubertal (testicular volume < 4 ml) boys with pQCT measurements. At the beginning of the study, the mean age was 14.8 years, the mean bone age was 12.4 years, whereas their height, weight, testicular volumes, bone ages, levels of gonadotropins, testosterone, estradiol, IGF-1, and AMH have been reported previously (Table 1)[18,19]. Two CDGP males had a previously treated unilateral undescended testis.

The CHH group included all (n=5) boys diagnosed with CHH who were evaluated at the pediatric endocrinology outpatient clinic of Helsinki University Hospital (HUH) between 2018 and 2020, and a boy who participated in our trial on delayed puberty but was subsequently diagnosed with CHH (n=5)[18]. At the time of diagnosis, all boys were 14 years or older and had presented with delayed puberty. The diagnosis of CHH was based on the following criteria: no spontaneous progression of puberty during follow-up, low gonadotropin, testosterone, and inhibin B levels, small testicular volume, no signs of other chronic disease accounting for functional hypogonadotropic hypogonadism, and otherwise normal anterior pituitary function[17]. Additionally, all participants with CHH had not received any hormonal treatment prior to the evaluation, and three had a mutation in the genes known to underlie CHH. The clinical and biochemical characteristics of the CHH patients are shown in Table 1 and Table 2. At the time of the diagnosis, none of the males with CHH or CDGP suffered from bone or back pain. A written consent was obtained from all the participants.

The bone age of CHH males was calculated from the left wrist x-ray with an automated bone age assessment (BoneXpert®)[20]. Testis length and width were measured either with a ruler or an ultrasound, and testicular volume was calculated by using the formula: length (cm) x width² (cm) x 0.52[21]. Circulating 25-hydroxyvitamin D concentration was determined with a chemiluminescent microparticle immunoassay (Abbott Laboratories, Wiesbaden, Germany) with a detection limit of less than 10 nmol/l or with an immunoluminometric assay (Siemens Healthcare Diagnostics, Tarrytown, NY, USA), with a detection limit of 6.8 nmol/l. Procollagen type 1 N-terminal propeptide levels were determined with an immunoelectroluminometric assay with a detection limit of 15 µg/l (Cobas e801, Roche Diagnostics, Germany).

At the time of evaluation, prior to any hormonal treatment, pQCT measurement (Stratec XCT 2000 L Research, Stratec Medizintechnik GmbH, Germany; software version 6.20) of the non-dominant radius and the left tibia were performed in both groups. Daily calibration of the

machine was performed with a standard phantom. The length of the radius was measured with a ruler from the ulnar styloid process to the olecranon, and the length of tibia from distal medial malleolus to the medial proximal epicondyle of the tibia. The radius was scanned at 4% and 66% sites and the tibia at 4%, 38%, 66% sites, and scout view was used to determine the measurement site. The distal site (4%) consists mainly of trabecular bone, which is covered by a narrow cortex, whereas the diaphyseal (38%) and the proximal (66%) sites include a thicker cortical bone compartment. Peripheral CT images of radius and tibia were analyzed with macro-based automated data evaluation provided by the manufacturer[22]. At the distal site, analyzed bone parameters included total bone mineral content (Tot BMC, mg/mm), total cross-sectional area (Tot CSA, mm^2), total bone density (Tot Den, mg/mm^3), trabecular area (Trab A, mm^2), and trabecular density (Trab Den, mg/mm^3). At the diaphyseal and proximal sites, Tot BMC, Tot CSA, Tot Den, cortical CSA, cortical density (Cort Den, mg/mm^3), cortical thickness (Cort Thk, mm), endocortical and periosteal circumference (mm), muscle area (CSA, mm^2) and polar stress-strain index (SSI, mm^3) were determined. The bone age, age, and height-adjusted Z-scores for bone parameters, measured at distal and proximal radius and distal and diaphyseal tibia, were calculated by using previously published equations[23-26]. A complete pQCT measurement data set was available in 21 and a partial dataset in 24 males with CDGP. None of the participants had chronic illnesses, diet restrictions, or intake of calcium supplements >1000 mg/d or vitamin D supplements >50 µg/d. Physical activity (PA) six months prior to the visit was recorded using a questionnaire[27], and PA is expressed as minutes per day (min/d) and reported as supervised and total PA.

The study protocol was approved by the Finnish National Committee on Medical Research Ethics (TUKIJA) and by the Helsinki University Hospital, and a written consent was obtained from all participants after full explanation of the purpose and nature of all procedures.

Statistical analyses

The data are presented with mean and 95% confidence intervals (CIs) unless otherwise mentioned. Analyses were performed with SPSS statistic for Windows (version 22.2, Chicago, IL). Between group comparisons of raw pQCT parameters and of parameter Z-scores were performed with Mann-Whitney U-test. Within group comparisons of Z-scores were analyzed with one sample Wilcoxon signed rank test against zero (*i.e.* comparison to age-matched peers). Correlations between hormonal markers and bone parameters were evaluated with Spearman's rank correlation. The level of statistical significance was set to p value less than 0.05.

RESULTS

Characteristics of the study population

At the time of the pQCT measurement, the males with CHH were older, taller, and heavier, and they had more mature bone age, smaller testicular size, and lower serum testosterone levels than the participants with CDGP, with no difference in serum 25-hydroxyvitamin D level (**Table 1**). The males with CDGP had higher mean total (80 min/d [range: 9-145 min/d]) and supervised (51 min/d [range: 9-110 min/d]) physical activity (PA) than the participants with CHH (32 min/d [range: 15-47 min/d]; and 8.6 min/d [range: 0-38 min/d], p=0.003 and p=0.002, respectively). Two of the CHH males had problems with balance, which did not hinder their daily life mobility. One male with CHH had a previous fracture in upper extremity, whereas none of the CDGP males had a positive fracture history.

Comparison of absolute pQCT values between CHH and CDGP groups

The males with CHH had lower Trab Den at distal radius and tibia, and lower Tot Den, and Cort CSA/Tot CSA-ratio at diaphyseal tibia than the participants with CDGP (**Figure 1 and 2**). At

proximal radius and diaphyseal tibia, Tot CSA did not differ significantly between the groups (**Figure 2**). At proximal radius and at diaphyseal tibia, polar SSI, muscle CSA or muscle CSA-total CSA-ratio did not differ significantly between the groups (**Table 3**).

Comparison of pQCT Z-scores between CHH and CDGP groups

Next we compared bone age adjusted Z-scores of pQCT values in both groups. At both sites of radius, and at distal site of tibia, the males with CHH had significantly lower Tot BMC Z-scores than the males with CDGP (**Table 3**). Similarly, CHH males had lower Cort Thk Z-scores at diaphyseal tibia than those with CDGP (**Table 3**). At distal measurement sites of radius and tibia, which comprises mainly of trabecular bone, CHH males had lower Trab Den Z-scores than the boys with CDGP (**Table 3**). Next, to account for difference in height between the groups, we compared height-adjusted Z-scores of bone parameters (23, 25). Z-scores of Tot and Cort CSA and polar SSI at proximal radius and Cort Thk at diaphyseal tibia were significantly lower in CHH males than in those with CDGP (**Supplementary Table 1**).

Within-group evaluation of the pQCT Z-scores in CHH and CDGP males

Next we compared the pQCT bone age adjusted Z-scores of both groups to zero (*i.e*. age-matched peers). In CHH males, the Z-scores for Tot BMC were significantly reduced (mean deviation ranging from –1.1 to –0.7 SDS, and p=0.043) at all measurement sites. At the distal (4%) measurement sites, their Z-scores for Trab Den (radius: mean -2.6 SDS, p=0.043, tibia: mean -1.9 SDS, p=0.043) were lower than in bone age matched peers. In addition, their Cort CSA Z-scores were reduced at proximal radius (mean -1.5 SDS, p=0.043). The CDGP males showed significant reductions in Tot Den (mean -4.2 SDS, p=0.008) at distal tibia and endocortical circumference at proximal tibia (-1.6 SDS p<0.001).

Correlations between pQCT parameters and clinical and hormonal markers of puberty at the time of pQCT measurement in boys with CDGP

In the CDGP group, serum testosterone correlated positively with Trab Den Z-scores at the distal site of radius (r=0.51, p=0.013). At the distal site of tibia, estradiol levels correlated positively with Trab Den Z-scores (r=0.58, p=0.004).

At the proximal site of radius, inhibin B correlated negatively, and serum FSH positively with Tot CSA Z-scores (r=-0.58, p=0.006, r=0.47, p=0.03, respectively). At the same site, IGF-1 levels correlated positively with Tot Den Z-scores (r=0.48, p=0.027). At the diaphyseal site of tibia, serum AMH correlated negatively with Tot CSA, Tot BMC, and periosteal circumference Z-scores (r=-0.49, p=0.021, r=-0.49, p=0.021, and r=-0.54, p=0.011, respectively).

Sensitivity analyses

The evaluation of age-adjusted Z-scores of pQCT measures between CHH and CDGP groups showed similar differences as the bone age adjusted Z-scores (*data not shown*). Within both groups, pQCT parameters deviated more from the reference population mean when Z-scores for age were used (*data not shown*). Exclusion of the 28-year-old CHH patient from the analyses did not change the results.

DISCUSSION

The main findings of this pilot study were that i) CHH males had lower BMC at all measurement sites, lower trabecular BMD at distal measurement sites, and lower cortical dimensions at proximal measurement sites than the matched reference population; ii) despite the fact that the males with

CHH were taller, heavier, and had more advanced bone age than the participants with CDGP, they had poorer BMC and trabecular BMD at distal measurement sites; iii) between-group differences in pQCT measures persisted when the results were adjusted for bone age and height, with CHH males showing significant deficits in trabecular BMD and cortical bone size. For the first time, our results show that both CHH and CDGP males have impaired BMD and cortical bone development.

CHH and CDGP patients exhibited reduced indices of bone health and development. The skeletal health of adult men with a history of delayed puberty has been addressed in several studies with variable outcomes, possibly relating to the methods in bone health assessment. In aggregate, they suggest that men with a history of delayed puberty have decreased areal BMD in adulthood[9,11,12,28], and that areal BMD does not necessarily improve with time or with a short-term androgen treatment during puberty[16,29,30]. In support of this, the age at peak height velocity, a reliable measure of puberty timing, correlates negatively with cortical and trabecular BMD, and positively with the number of previous fractures[31]. It is thus possible that there is a window of opportunity for optimal skeletal response to sex steroids in adolescence, as also suggested by the previous studies of CHH patients (reviewed in [17]). In line with this paradigm, CHH men are known to suffer from osteopenia in adulthood and although testosterone treatment appears to increase their BMD[13,15,32], it is not clear if it completely reverses impaired bone health[33], or only improves BMD[32].

The theory of a critical developmental window for optimal bone development in adolescence is further supported by our finding that the boys with CHH, in spite of being taller, heavier, and having more mature bone age than those with CDGP, had poorer BMC and trabecular BMD at distal measurement sites than CDGP males. This is of clinical significance, as forearm fractures associate with reduced volumetric BMD by pQCT at distal forearm in children and adolescents[34]. Indeed, in young adult men reduced pQCT measured volumetric trabecular BMD is more strongly associated with fracture history than areal BMD or cortical bone size[35]. What should

then be done to improve the bone health over time in boys with CHH? In our cohort, 13 (54%) of the CDGP boys showed initial testicular growth ranging from 3 to 3.9 ml, whereas all CHH patients had a testicular volume of less than 3 ml. Interestingly, serum sex steroid correlated positively with trabecular bone density in the former but not in the latter, which raises the intriguing possibility that even a slight increase in circulating sex steroids may affect favorably bone mineral accrual at distal sites with newly-formed trabecular bone. These observations, albeit from different age groups, together with the finding that in CHH males the age at hormone replacement therapy correlates negatively with pQCT measured distal radial BMD[36], suggest that early puberty is a critical period of skeletal response to sex steroids, and emphasize that timely diagnosis and sex-hormone treatment of CHH may be beneficial for adequate bone structure. This further enforces the importance of early diagnosis of CHH[17]. We speculate that low-dose androgen treatment started at the approximate age of 12 years might provide an option to improve bone mineral accrual in CHH patients, and that trabecular bone density at distal measurement sites might provide a potential new sensitive tool to monitor androgen exposure. However, this approach would require a prospective randomized controlled study. Of particular note, low doses should be used to avoid compromised adult height as a consequence of too fast skeletal maturation with high androgen doses[37,38]. Future studies are clearly warranted to optimize bone health and induction of puberty in CHH patients.

CHH males had impaired cortical bone development in relation to males with CDGP before puberty initiation. The difference was visible particularly at the weight-bearing lower extremity, despite the fact that CHH males were older, taller, heavier, and had more mature bone age than those with CDGP. In CDGP males, given that no correlations were found between circulating sex steroid levels and cortical bone parameters, and that their nascent increase in testosterone levels was most likely a recent phenomenon, it is unlikely that such a recent increase in sex steroid exposure would explain the difference in cortical bone development between the groups. Notably, FSH levels correlated positively with total CSA in CDGP males, whereas no correlation

was found in CHH males. This was interesting since FSH has been proposed to have a resorptive effect on bone[39], although the relationship between FSH and bone modeling during early puberty has not been studied.

We found that total and supervised PA was lower in CHH males than in participants with CDGP, which is also likely to contribute to the difference in cortical bone development between the groups. The positive influence of PA on the skeletal health is indisputable[40], and longitudinal studies in adolescents have shown a positive relationship between PA and bone strength (*i.e.* bone failure load, bone volume fraction) in early and mid-puberty[41]. We speculate that males with CHH may subconsciously avoid supervised PA and competitive sports due to lower testosterone levels, lean mass, and undervirilization. Indeed, the gender divergence between athletic performance begins at the age of 12 to 13 years[42], that is earlier than the age limit set for the diagnosis of delayed puberty in boys[8]. Our finding suggests that sedentary lifestyle may be an inherent feature of CHH in males before puberty induction, and such lifestyle together with the late onset of muscle anabolic androgen replacement therapy may threaten the achievement of normal peak bone mass.

The study includes limitations that warrant further discussion. The CHH study group was relatively small, and two males had mutations in *FGFR1* and some genes associated with CHH, such as *FGFR1*[43], which may theoretically influence bone development. The study did not include information on dietary habits of the participants, or circulating levels of PTH or adrenal androgens, which may have contributed to the bone development, albeit in patients with CHH, adrenarche is reported to occur within the range of normal population[44]. Additionally, vitamin D deficiency in Finnish adolescents is a very rare condition[45]. The number of CHH patients was relatively low. However, this is a rare patient group and all the participants were well-characterized and treatment-naïve at the time of pQCT measurement.

In conclusion, both CDGP and CHH males had lower BMC, lower trabecular density, and lower cortical BMD than age-matched reference population. Although, CHH males were taller, heavier, and had more mature bone age than the participants with CDGP, they had poorer BMC, trabecular BMD, and cortical bone size. In CDGP males, testosterone and estradiol levels correlated positively with trabecular BMD at distal radius. Our results suggest that preventive measures to inhibit sex steroid-associated bone loss in patients with CHH are urgently required. Finally, we show that male CHH patients displayed reduced physical activity. This finding warrants further studies in larger patient series, yet this pilot observation already suggests that special attention should be paid to the benefit of exercise also during the induction of puberty in CHH patients to optimize future bone health.

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TABLE LEGENDS

Table 1. Comparisons of baseline characteristics between the boys with congenital hypogonadotropic hypogonadism (CHH) and constitutional delay of growth and puberty (CDGP).

Mean (range). ISO-BMI, age and sex-adjusted BMI; LH, luteinizing hormone; FSH, follicle stimulating hormone; AMH, anti-müllerian hormone; PINP, amino-terminal propeptide of type 1 procollagen.

Table 2. Clinical and hormonal characteristics of the boys with congenital hypogonadotropic hypogonadism (CHH) at the time of diagnosis.

nCHH, normosmic congenital hypogonadotropic hypogonadism, KS, Kallmann syndrome, AMH, anti-Mullerian hormone, PINP, procollagen type 1 N-terminal propeptide. *Baseline LH and FSH concentrations.

Table 3. Bone parameters in five boys with congenital hypogonadotropic hypogonadism and in 24 boys with constitutional delay of growth and puberty. The Z-scores are calculated for bone age. Mean (95%CI). * P value refers to between group comparisons of Z-scores. † Z-score data of tibia was available in 4 CHH patients. CSA, cross-sectional area, BMC, bone mineral content, SSI, stress-strain index; CHH, congenital hypogonadotropic hypogonadism; CDGP, constitutional delay of growth and puberty.

Supplementary Table 1. Bone parameters in five boys with congenital hypogonadotropic hypogonadism and in 24 boys with constitutional delay of growth and puberty. The Z-scores are calculated for height. Mean (95%CI). * P value refers to between group comparisons of Z-scores. CSA, cross-sectional area, BMC, bone mineral content, SSI, stress-strain index; CHH, congenital hypogonadotropic hypogonadism; CDGP, constitutional delay of growth and puberty.

FIGURE LEGENDS

Figure 1. Total bone mineral contents (Tot BMC, *left panel*) and trabecular densities (Trab Den) in five males with congenital hypogonadotropic hypogonadism (CHH) and in 21 boys with constitutional delay of growth and puberty (CDGP) at distal (4%) measurement sites of radius and tibia as assessed with peripheral quantitative tomography (pQCT).

Figure 2. Bone parameters in five males with congenital hypogonadotropic hypogonadism (CHH) and in 21 boys with constitutional delay of growth and puberty (CDGP) at proximal site of radius and diaphyseal site of tibia.