Compromised Volumetric Bone Density and Microarchitecture in men with Congenital Hypogonadotropic Hypogonadism

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Grants or fellowships supporting the writing of the paper:

This work was supported in part by a grant from *Programme Hospitalier de Recherche Clinique* (PHRC # P081212 HYPOPROTEO) to JY. The contribution of GP to the project was enabled thanks to a fellowship financed in part by a SICPA Foundation grant (Lausanne, Switzerland).

Disclosure statement: The authors have nothing to disclose.

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ABSTRACT

Context: Men with Congenital Hypogonadotropic Hypogonadism (CHH) and Kallmann syndrome (KS) have both low circulating testosterone and estradiol levels. Whether bone structure is affected remains unknown.

Objective: To characterize bone geometry, volumetric density and microarchitecture in CHH/KS.

Design: Cross-sectional study.

Setting: One tertiary academic French center.

Patients and Controls: 51 genotyped CHH/KS patients and 40 healthy volunteers were included. Ninety-eight percent of CHH/KS men had received testosterone and/or combined gonadotropins.

Intervention(s): High-resolution Peripheral Quantitative Computed Tomography (HR-pQCT), Dual X-ray absorptiometry (DXA) and measurement of serum bone markers.

Main Outcome: Volumetric bone mineral density (vBMD), cortical and trabecular microarchitecture.

Results: CHH and controls did not differ for age, BMI, vitamin D and PTH levels. Despite long-term hormonal treatment (10.8 ± 6.8 years), DXA showed lower areal BMD in CHH/KS at lumbar spine, total hip, femoral neck and distal radius. Consistent with persistently higher serum bone markers, HR-pQCT revealed lower cortical and trabecular vBMD as well as cortical thickness at the tibia and the radius. CHH/KS men had altered trabecular microarchitecture with a predominant decrease of trabecular thickness. Moreover, CHH/KS men exhibited lower cortical bone area, whereas total and trabecular areas were higher only at the tibia. Earlier treatment onset (before the age of 19 years) conferred a significant advantage for trabecular bone volume/tissue volume and trabecular vBMD at the tibia.

Conclusion: Both vBMD and bone microarchitecture remain impaired in CHH/KS men despite long-term hormonal treatment. Treatment initiation during adolescence is associated with enhanced trabecular outcomes, highlighting the importance of early diagnosis.

Keywords: Congenital hypogonadotropic hypogonadism, Kallmann syndrome, bone microarchitecture, bone mineral density, HR-pQCT, androgen replacement therapy

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PRECIS

High-resolution peripheral quantitative CT in a large cohort of CHH/KS men detected persistently compromised bone structure and low bone mineral density despite long-term hormonal replacement.

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INTRODUCTION

Congenital hypogonadotropic hypogonadism (CHH) is a rare genetic disease (estimated prevalence 1 in 10'000), characterized by absent or decreased hypothalamic pulsatile GnRH release or action, leading to deficient pituitary gonadotropin secretion from fetal life to adulthood, which in turn prevents physiological secretion of gonadal sex steroids [1,2]. When CHH is accompanied by anosmia, it is termed Kallmann syndrome (KS) [1,2]. Affected males may be identified at birth because of cryptorchidism and/or micropenis [3], although the majority of CHH/KS cases is diagnosed in adolescence due to absent or incomplete pubertal development [1,2,4]. Nevertheless, some CHH/KS men are brought to medical attention only in adulthood for medical complaints such as sexual dysfunction, infertility, and/or other comorbidities related to long-standing hypogonadism including skeletal morbidity.

Multiple lines of evidence concur that estradiol (E2), produced following aromatization of testosterone (T), is the critical sex steroid in men to ensure both the attainment of peak bone mass and the skeletal maintenance across lifespan [5,6]. Direct stimulation of the androgen receptor by T is a less potent promoter of cortical and trabecular bone development, and actively contributes to the periosteal expansion of male skeleton during puberty [6]. Altered E2 production leads to adverse skeletal events in men [5,7,8]. The hypogonadism observed in CHH/KS is often severe and associated with very low circulating T levels and subsequent profound E2 deficiency [2], which has been well documented using both sensitive immunoassays and mass spectrometry measurements [9,10]. It is thus not surprising that CHH/KS men display reduced bone mass. In a seminal study investigating bone defects in CHH/KS men, Finkelstein *et al* performed Dual X-ray absorptiometry (DXA) in 23 male CHH patients, most of which had already received hormonal replacement therapy. Sixteen of the patients had low areal bone mineral density (aBMD) [11]. Since then, available data on bone health in CHH/KS has been limited to aBMD measurement by DXA in small series [12-16].

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Although DXA is the standard tool for fracture risk prediction in clinical practice, bone mass is not the sole determinant of bone health [17]. Another broadly implemented element is the use of serum markers of bone remodeling [18]. More recently, the refinement of imaging methods enabled the study of other aspects of bone health, such as bone morphology and microarchitecture [19]. In particular, high-resolution peripheral quantitative CT (HR-pQCT) allows accurate visualization of compartment-specific bone microarchitecture at peripheral skeletal sites, while simultaneously assessing three-dimensional volumetric BMD (vBMD) and bone geometry [20]. Recently, the results of a large multi-center prospective study, which followed more than 7000 men and women for a mean 4.6 years, confirmed that HR-pQCT indexes (particularly cortical density, cortical area, trabecular number and trabecular thickness) predicted incident fractures independently of aBMD levels [21]. To date, no data on bone microarchitecture in CHH/KS males has been reported.

In order to explore, with these modern tools, the hypothesis that men with CHH/KS could have deteriorated bone quality and microarchitecture in addition to the known decrease of aBMD, we performed a cross-sectional study in a relatively large cohort of adult men with CHH/KS who underwent HR-pQCT, DXA and measurement of bone remodeling serum markers. Findings were compared to data obtained from healthy volunteers of similar age and BMI. HR-pQCT-derived cortical thickness and trabecular bone volume to tissue volume fraction (BV/TV) were considered the primary outcomes. Age at onset of hormonal treatment, underlying genetic causes and the degree of GnRH deficiency were investigated as potential modifiers of bone phenotype.

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PATIENTS AND METHODS

Setting

This was a non-interventional, cross-sectional study. The study was approved by the Institutional Review Board of Bicêtre teaching hospital (RCB 2019-A02543-54). All participants provided written informed consent in accordance with French Bioethics Law and the principles of the Declaration of Helsinki.

Patients and controls

Fifty-one CHH/KS patients were recruited from a tertiary academic medical center (Department of Endocrinology and Reproductive Diseases, Bicêtre University Hospital, France) between February 2014 and March 2018. Only male subjects were enrolled. CHH/KS diagnosis was based on the following criteria: (i) absent or incomplete puberty; (ii) low serum total T levels in the setting of low or inappropriately normal serum gonadotropin levels (LH, FSH); (iii) otherwise intact anterior pituitary function evidenced by normal basal/stimulated cortisol, prolactin, thyroid function and growth hormone via insulin tolerance test; (iv) normal serum dehydroepiandrosterone-sulfate and ferritin concentrations, as well as normal age-adjusted insulin-like growth factor-1 levels; (v) normal magnetic resonance imaging of the hypothalamic-pituitary region; and (vi) absence of other causes of hypogonadotropic hypogonadism (i.e. eating disorders, opioid intake). Exclusion criteria were age > 50 years and treatment with bisphosphonates or other anti-osteoporotic drugs.

Forty healthy male volunteers, who had participated in previous studies of the Rheumatology Department of Lariboisière Hospital, were also enrolled (approval number P070121, ethical committee Ile-de-France) and underwent DXA, HR-pQCT, and measurement of reproductive hormones and bone remodeling markers. To qualify as controls, they were required to have (i) unremarkable medical history and normal timing of puberty; (ii) adequate virilization at physical examination with normal circulating T levels; (iii) normal aBMD (Z-score > -2.0 SD) at all measured sites; and (iv) no history of low-trauma fracture or bone-acting medical treatment.

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Clinical data of CHH/KS cohort

Medical files of included CHH/KS men were reviewed to retrieve key medical information at diagnosis including testicular volume (TV) by orchidometer (Prader), reproductive hormones (T, LH, FSH, E2, inhibin B) and history of cryptorchidism and/or micropenis. History of fractures was registered on a declarative base, after excluding sites that are not linked to osteoporosis (skull, fingers, toes). All patients were diagnosed at adolescence or adulthood (range 12-47 years). If the diagnosis was suspected before age 16 with subsequent initiation of hormonal treatment, the latter was withdrawn at age 18 to ascertain persistent hypogonadotropic hypogonadism consistent with CHH/KS. The degree of GnRH deficiency was defined as complete if endogenous puberty was absent (TV < 4 ml) or partial if TV ≥ 4 ml at the initial presentation or during reassessment at age 18 (in the absence of prior gonadotropin therapy) [1,2]. Patients were classified as having normosmic CHH or KS based on olfactometry testing and/or cranial imaging (olfactory bulb hypoplasia/aplasia) [22]. Information on current and previous hormonal therapy (testosterone and/or combined gonadotropin therapy) was recorded, focusing on age at onset, total duration and treatment pauses. In the previously treated subjects (n=50), serum T and E2 levels were assayed in samples drawn at 3 periods: 1-4 weeks, 4-7 months and 9-12 months prior to study inclusion. Thirty-three patients were on testosterone enanthate (Androtardyl(R); Schering; 250 mg IM, prescribed every 3 weeks), whereas seventeen were on combined gonadotropins (hCG - Gonadotrophine-chorionique(R); Laboratoires Organon, Puteau, France; 1'500 IU SC, prescribed twice or three times a week; and FSH - GONAL-f; Laboratoires Merck-Serono, Lyon, France or MENOPUR; Laboratoires Ferring Pharmaceuticals, Gentilly, France; prescribed both 150 IU SC three times a week).

Hormone assays

Fasting blood samples were drawn between 8:00 am and 10:00 am from an antecubital vein and immediately centrifuged and stored at - 80°C. FSH and LH were measured using a sensitive chemiluminescent immunometric assay (Centaur, Siemens, Deerfield, USA). The intra- and interassays coefficients of variation (CV) were respectively 2.9% and 2.7% for FSH at

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6.9 IU/L and 2.3% and 1.5% for LH at 4.2 IU/L. The detection limits were 0.1 IU/L for FSH and 0.07 IU/L for LH. Plasma total estradiol was measured by a sensitive direct radioimmunoassay with an Orion Diagnostica device (Spectria, Espoo, Finland). The minimum detectable concentration was 2 pg/mL (7.3 pmol/L). The intraassay and interassay CV were 17.6% and 18.1%, respectively, for a total estradiol concentration of 3.2 pg/mL and 2.8% and 5.8%, respectively, for a concentration of 24 pg/mL. Serum total testosterone was measured by a direct radio-immuno-assay method using Orion Diagnostica (Spectria, Espoo, Finland). The intra- and interassays CV for total testosterone were 3.8% and 4.8% at, respectively 3.2 and 2.6 ng/mL (11.1 and 9 nmol/L). The detection limit was 0.02 ng/mL (0.06 nmol/L). The plasma concentrations of inhibin B were measured in serum by enzyme immunometric assays (Oxford Bio-Innovation reagents, Serotec, Oxford, UK). The lower limit of detection was 10 pg/mL. The intra- and interassays CV were respectively 6.8% and 21.5% for 52 pg/mL, and 4.2% and 10.2% for 215 pg/mL. In addition, 25-hydroxyvitamin D was measured by a direct competitive chemiluminescent immunoassay (Liaison XL, Diasorin, and Saluggia, Italy). The intra- and interassays CV were respectively 1.8% and 8.9% at 22.8 ng/mL. The detection limit was 4 ng/mL. Serum intact PTH was measured using a chemiluminescent immunometric assay (Centaur, Siemens, and Deerfield, USA). The intra- and interassays CV were 2.8% and 4.6% at 50.3 pg/mL. The detection limit was 4.6 pg/ml.

Bone turnover markers

The following immunoassays were used to measure bone turnover markers: serum β-Crosslaps, N-terminal propeptide of type 1 collagen (P1NP), Osteocalcin (Cobas e601 Analyzer; Roche Immunodiagnostics, Meylan, France), bone alkaline phosphatase (Ysis analyzer; IDS Immunodiagnostic, Paris, France), and Tartrate-resistant acid phosphatase (TRAP5B) levels (IDS Immunodiagnostic). The intra- and interassays CV were respectively 17.9% and 18.7% for β-Crosslaps, 2.3% and 3.4 % for P1NP, 3.5% and 4.2% for Osteocalcin, 8.2% and 10% for bone alkaline phosphatase and 5.6% and 8.2% for TRAP5B. The detection limits were 10 pg/ml

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for β-Crosslaps, 5 ng/ml for P1NP, 0.5 ng/ml for Osteocalcin, 1 U/L for bone alkaline phosphatase and 0.1 U/L for TRAP5B.

Dual-energy X-ray absorptiometry

Areal BMD (g/cm²) was measured at the femoral neck / total hip, lumbar spine (L1–L4) and distal radius using the same Lunar DPX-L (Lunar Corp., Madison, WI, USA) densitometer for the whole duration of the study, operated by the same technician. For the Z-score, age-adjusted values were based on a French reference population between 20 and 89 years of age provided by Lunar Corp, France. The stability of the measurements was checked every day, using a phantom for calibration. The mean CV for measurement of the patients were as follows: lumbar spine, 0.41%; total hip, 0.53%; Femoral neck 1.36%; and Radius 1.22%. Given that the majority of CHH/KS men (49/51, 96%) were aged < 50 years, we presented the results as Z-scores in accordance with the guidelines issued by the International Society for Clinical Densitometry. BMD was considered low for age if Z-score < -2.0 SD [23].

Bone microarchitecture measurements

Volumetric bone mineral density (vBMD) and microarchitectural parameters were assessed at the left distal tibia and at the non-dominant distal radius by HR-pQCT (XtremeCT, Scanco Medical AG, Brüttisellen, Switzerland), as previously described [24] and detailed in [25]. All the scans of the study were performed using the same HR-pQCT device. The volume of interest (Supplementary Fig. 1 in [25]) was automatically separated into a trabecular and cortical regions using a threshold-based algorithm (Scanco software version, evaluation program V 6.6) to provide all parameters of interest [26]. CV for HR-pQCT parameters at the radius and tibia were respectively as follows: cortical area 0.7% and 0.5%, trabecular area 0.2% and 0.4%, total vBMD 0.8% and 0.5%, cortical vBMD 0.8% and 0.4%, cortical perimeter 0.4% and 0.2 %, cortical thickness 1% and 0.6%, trabecular vBMD 0.8% and 0.6%, BV/TV 0.8% and 0.7%, trabecular number 5.8% and 4.9%, trabecular thickness 5.6% and 4.4%, trabecular separation 5.6% and 5.2%, trabecular distribution (Tb.1/N.SD) 10.9% and 5.3%.

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Genetic testing

Genomic DNA was isolated from white blood cells. Genes known to cause CHH were tested by exome sequencing as previously described [27]. Briefly, for each patient, 500 ng genomic DNA was sonicated using a Covaris M220 (Covaris Inc., MS, Woburn, MA, USA). Illumina® adapters and other oligonucleotides were provided by Eurofins Genomics (Ebersberg, Germany). Whole genome Illumina® libraries were prepared using NEBNext® DNA Library Prep Master Mix Set for Illumina® (NEB Inc., Ipswich, MA, USA) robotized on a Biomek Span 8 workstation (Beckman, Villepinte, France). Enrichment (capture) was processed using Sureselect XT kit (Agilent, Santa Clara, CA, USA) robotized on a Biomek 4000 workstation (Beckman, Villepinte, France). Paired end 2 x 150bp sequencing was performed by batch of 23 patients on a Miseq® IlluminaTM sequencer. All detected variants were classified according to American College of Medical Genetics and Genomics 2015 classification: benign, likely benign, uncertain significance, likely pathogenic and pathogenic [28]. This classification was conducted using Varsome (https://varsome.com/). Only likely pathogenic and pathogenic variants were considered as causative of the CHH/KS phenotype.

Statistical analyses

Statistical analysis was performed using XLSTAT Version 2019.3.2 (Copyright Addinsoft 1995-2019) and R version 3.5.1 (2018-07-02 - "Spring Dance" Copyright (C) 2014 The R Foundation for Statistical Computing for windows). Student's t-tests (or Mann-Whitney tests if the distribution of the variable was not log-normal and in case of heteroscedasticity) were conducted for quantitative variables. A Welch's test was applied in case of normality but heteroscedasticity. Results were expressed as mean ± SD. The effect of potential predictors on the bone microarchitecture outcomes of CHH/KS men was assessed by a univariate analysis in a binary fashion for the degree of GnRH deficiency (complete absence of puberty vs. partial puberty) and the age at onset of testosterone/gonadotropins (before 19 years vs. after 19 years). Association between the presence of each mutated gene and micro-architectural

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parameters was studied by analysis of variance (ANOVA) in case of homoscedasticity (Kruskal-Wallis instead) followed by a posteriori comparison analysis if the ANOVA test (respectively Kruskal-Wallis test) was globally significant. A similar analysis was used to compare sex steroids serum levels between different types of hormonal treatment (TE versus combined gonadotropins) and different time intervals since last injection. The Tukey's test (respectively Steel-Dwass-Critchlow-Fligner's test) was used for the *a posteriori* multiple comparisons. Shapiro's test, Grubb's test and Bartlett's test were applied to test respectively normality, outlier, and homoscedasticity. All tests were two-sided, and the significance level was fixed at 0.05.

RESULTS

CHH/KS cohort phenotype and genotype

Data on reproductive phenotype, genetic findings and current/previous hormonal treatment of the patient group are shown in **Table 1.** The majority of subjects (76.5%) presented with absent puberty. Most of them had history of cryptorchidism and/or micropenis, consistent with severe GnRH deficiency. At study entry, almost all participants (50/51, 98%) had received hormonal replacement by testosterone and/or combined gonadotropins with treatment pauses (> 1 year) in seven cases. The mean age at hormonal treatment initiation was 20.4 ± 7.8 years, with most patients (31/50, 62%) starting before the age of 19 years. A genetic cause was found in roughly half of participants (27/51, 53%) with *FGFR1* being the most frequently mutated gene. Six cases of digenicity were observed in accordance with previous reports of oligogenicity in CHH/KS [1,2,29].

Patients and controls characteristics

Anthropometric, hormonal and DXA data are shown in **Table 2**. As expected, serum T and E2 concentrations at the time of diagnosis were dramatically reduced in the CHH/KS group. The two groups did not differ in age, BMI or height. Vitamin D deficiency (serum levels < 30 µg/l) [30] was prevalent in both CHH/KS (84%) and controls (89%) but PTH levels remained normal in the vast majority (>90%) of both groups. Higher bone turnover was noted in CHH/KS with concomitant elevation in bone resorption (1.9- and 1.4-fold for β-Crosslaps and TRAP5B

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respectively) and bone formation (1.7-, 1.4- and 1.2-fold for P1NP, osteocalcin and bone phosphate alkaline respectively) serum markers as compared to healthy volunteers. CHH/KS men had significantly lower aBMD at all sites (**Table 2**). Approximately one third of CHH/KS patients had low for age aBMD (Z-score < -2.0 SD) at the lumbar spine (16/51) and the distal radius (14/51) with 20% (10/51) exhibiting low values at both sites. Seven participants reported history of eight fragility fractures, two of which were localized in the femoral neck and the vertebral spine. The remaining affected sites were the wrist (n=3), the ankle (n=2) and the ribs (n=1).

HR-pQCT findings

Tibia: CHH/KS men tended to have higher crossectional bone area (+8%, p=0.05) (**Fig. 1A, Table 3**). The difference was ascribed to higher trabecular area (+14%, p<0.01), whereas cortical area was dramatically lower in CHH/KS subjects (-19%, p<0.0001) (**Fig. 1B-C**). CHH/KS men performed significantly worse than controls in all measured outcomes of vBMD and microarchitecture except for cortical perimeter (**Fig. 1D-H and Table 3**). More specifically, cortical (-4%) and even more trabecular (-24%) vBMD were significantly lower in CHH (**Table 3**). Cortical thickness was likewise lower in CHH/KS than in controls (-21%, p<0.0001) (**Fig. 1E**). Trabecular microarchitecture was significantly altered with lower levels of BV/TV (-24%, p<0.0001) and trabecular thickness (-19%, p<0.0001), whereas a less pronounced difference was noted for trabecular number (-9%, p=0.03) (**Table 3**). The resulting inhomogeneity of the trabecular network (Tb.1/N.SD) was higher in the patient group (+19%, p=0.01) (**Fig. 1H**).

Radius: In contrast to the tibia, we did not detect any difference between groups in total and trabecular area at the radius (**Fig. 2A and 2C)**. However, the cortical area of the radius was also severely decreased in CHH/KS men (-24%, p<0.0001) (**Fig. 2B**). Changes in vBMD, cortical thickness and trabecular microarchitecture followed a similar pattern as at the tibia with a trend for differences of greater magnitude (**Table 3, Fig. 2D-H**). For instance, the inhomogeneity of the trabecular network at the radius was 32% higher in the CHH/KS group (p<0.01).

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Representative HR-pQCT tibia scans of a CHH patient versus an age- and BMI-matched control are depicted in **Figure 3**, highlighting the presence of altered microarchitecture in the former.

Modifiers of bone microarchitecture in CHH/KS

The twelve men with partial GnRH deficiency (TV ≥ 4 ml at diagnosis) presented with higher cortical vBMD at the radius (857 ± 57 mg HA/cm^3 versus 797 ± 86 mg HA/cm^3 in men with absent puberty, p<0.01). In addition, they exhibited higher cortical area and cortical thickness (p<0.01 and p=0.03 respectively).

Compared to earlier onset (n=30, mean age of 15.2 ± 1.6 years), initiation of hormonal replacement after the age of 19 years (n=21, mean age of 27.8 ± 7.2 years) was associated with more severe compromise of trabecular structure as evidenced by significantly lower trabecular vBMD and BV/TV at the tibia (**Fig. 4**). A trend for lower trabecular thickness was also observed (p=0.08). Conversely, no difference was detected regarding cortical bone. The only significant differences at the radius were an increased total and trabecular area in the tardily treated group (p<0.01).

Impact of genetic status

Based on the pathogenic mutations found in the CHH/KS cohort (**Table 1**), three subgroups were examined: (i) *FGFR1* mutations (n=8); (ii) mutations in other known CHH/KS genes (n=19); and (iii) no identified mutations in known CHH/KS genes (n=24). Men in the FGFR1 mutated group (including three digenic cases: two with *IL17RD* and one with *PROKR2* mutations) presented with higher inhomogeneity of the trabecular network: 0.292 ± 0.112 mm vs 0.192 ± 0.078 mm in the 'other CHH/KS mutated genes' group and 0.202 ± 0.073 mm in the 'no mutations' group (p=0.02 and 0.03 respectively). When compared to 'mutations in other CHH/KS genes' group, men harboring *FGFR1* mutations also exhibited significantly lower BV/TV (9.5 ± 3.8 vs 14.0 ± 4.7%, p=0.03) and trabecular vBMD (113.7 ± 45.7 vs 167.9 ± 55.9 mg HA/cm^3, p=0.03).

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Adequacy of previous hormonal treatment

Detailed history of hormonal treatment for all patients is provided in Supplemental Table 1 in [25]. Serum T and E2 levels on treatment were available at three different periods: 9-12 months, 4-7 months and 1-4 weeks prior to inclusion (**Fig. 5**). As expected, hormonal treatment produced a significant increase in the levels of both sex steroids as compared to basal levels at diagnosis (see Supplemental Fig. 2 in [25]). Testosterone enanthate (TE) and hCG/FSH therapies generated similar T levels (**Fig. 5A**), whereas patients on combined gonadotropins exhibited significantly higher serum E2 levels than those on TE (**Fig. 5C**). Given the wide range of observed serum T and E2 levels, we explored the relationship between different intervals since the last injection of each therapy and levels of both steroids. Mean serum T levels varied greatly and attained 11.2±3.4, 6.4±1.9, 4.1±1.3, 1.1±0.9 ng/mL at 1 respectively -7, 8-14, 15-21, > 22 days after the last TE injection, and 7.2±2.5 and 4.1±1.4 ng/mL at respectively 1-3 and 4-6 days after the last hCG injection (**Fig. 5B**, $p<0.01$ for all comparisons between consecutive subgroups). Similar differences of a somewhat smaller magnitude were observed for serum E2 levels (**Fig 5D**).

DISCUSSION

The aim of the current study was to evaluate bone health in CHH/KS, with particular focus to characterizing the skeletal morphology and microarchitecture of affected patients. Despite long-term hormonal treatment in the majority of our CHH/KS cohort, the patient group exhibited lower aBMD levels than controls at all relevant sites. More importantly, CHH/KS men exhibited impaired cortical and trabecular vBMD, as well as lower cortical thickness at both the radius and tibia. Using HR-pQCT, a comparative defect in multiple aspects of trabecular structure (BV/TV, thickness, spacing and number) was observed, which is beyond the selective reduction of trabecular thickness associated with age-induced bone loss in men [31].

Our finding of persistently high serum remodeling markers despite long-term testosterone/gonadotropins therapy is in agreement with previous studies in CHH/KS [14,32,33].

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One potential explanation is suboptimal therapeutic adherence. Despite the absence of long treatment pauses in the majority of the participants (**Table 1**), almost one third of the available hormonal measurements during the year preceding the study initiation were performed while exceeding the prescribed interval between hormonal injections (3 weeks for TE, 3-4 days for hCG). Consequently, infraphysiological levels of both serum T and E2 levels were frequently observed in particular for men on TE (**Fig. 5**). These observations likely reflect patients' failure to comply with the prescribed hormonal regimen in real life.

Overall, our data indicate that long-term androgen replacement does not achieve normalization of bone status in CHH/KS. Previous cross-sectional studies that solely analyzed aBMD in CHH/KS men came up with similar results [14,16,32,33]. Besides the above highlighted compliance issues, it is possible that the type of gonadal replacement is of relevance. As our data have confirmed (**Fig. 5**), injectable testosterone esters produce wide variations in serum T levels (roller-coaster pharmacokinetics), with typically subnormal levels at the end of each cycle [34]. It is possible that forms of treatment ensuring more stable T levels (e.g. transdermal gels) or higher dosing could elicit a better bone response. In the longitudinal arm of their published study, Guo *et al* demonstrated that increasing doses of hCG, which in turn pushed serum T and E2 to the upper normal range, managed to reverse previously elevated bone remodeling serum markers [32]. Men treated with gonadotropins in our cohort developed higher E2 levels, presumably due to hCG-induced direct testicular stimulation of aromatase activity with subsequent higher estradiol secretion by Leydig cells. Whether this finding could translate into a comparative bone benefit merits further investigation.

In addition, our study corroborates the existence of an early window to optimize the benefit of androgen replacement on trabecular bone in CHH/KS. Initiation before age of 19 years was associated with better trabecular outcomes. Focusing on the same question, Finkelstein *et al* evaluated bone mass using quantitative CT in 21 CHH men after a mean of 23.7 months of testosterone replacement [12]. In agreement with our data, the younger group at hormonal

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therapy onset (open epiphyses) exhibited an increase of both cortical and trabecular densities, whereas the older group (fused epiphyses) displayed an increase only in cortical bone mass.

Among other modifiers of bone status, partial endogenous puberty (TV ≥ 4 ml), which is indicative of a higher exposure to endogenous sex steroids, was associated with a milder bone phenotype at the distal radius, a site rich in cortical bone. Previous studies by Khosla *et al* revealed that cortical bone is more sensitive to variations of serum E2 even at low levels ('threshold theory') [35], which is consistent with our findings. Regarding specific genetic links, patients harboring *FGFR1* mutations displayed worse trabecular parameters at the distal radius. Interestingly, this gene is indeed known to underlie syndromes with prominent skeletal manifestations including osteoglophonic dysplasia and Pfeiffer syndrome [36]. *SEMA3A* is another causative CHH/KS gene, coding for semaphorin 3A, an axon guidance molecule [37]. A specific bone action of this gene product was suggested by the finding of marked osteopenia in Sema3a $^{-/-}$ mice and the significant increase of bone volume following Sema3A administration in 5-week wild-type mice [38]. The number of pathogenic *SEMA3A* mutations in our cohort (n=4) was insufficient to unmask a clinically relevant bone impact of mutations in this gene in humans.

Recently, a cross-sectional analysis of 31 men with hypergonadotropic hypogonadism due to Klinefelter syndrome (KFS) revealed compromised trabecular microarchitecture only at the tibia as compared to age- and height-adjusted controls [39]. The relatively modest bone impairment in these men with chronically high FSH levels, taken together with the severe bone defects in our CHH/KS cohort with very low FSH levels, question the relevance of rodent studies pointing to a significant role of elevated FSH in the pathogenesis of hypogonadal bone loss [40]. The milder bone phenotype in KFS men may be linked to preserved circulating E2 levels due to increased aromatase activity in their testes [9], as opposed to the profound hypoestrogenism in CHH/KS. The crucial role of E2 for bone microarchitecture in men is in fact supported by mechanistic studies with GnRH agonists and aromatase inhibitors in healthy men [8], as well as a large cross-sectional study performing HR-pQCT in 1169 male volunteers aged 20–87 years [41].

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Our study has several limitations. Almost all included patients had received hormonal replacement. Hence, we could not explore the pure bone traits of untreated CHH/KS. Nevertheless, the current analysis constitutes a representative image of the real-life state of this rare disease. Our cohort was heterogeneous regarding the type, onset and duration of hormonal therapy. Future research should evaluate the effect of standardized testosterone vs gonadotropin therapy on bone status. Due to the cross-sectional design, we could not distinguish whether the degraded bone microarchitecture in CHH/KS derives from defective bone accrual, accelerated bone loss or both. Fracture collection was incomplete in the absence of vertebral fracture assessment during the DXA scan. Lastly, we were unable to examine important confounding factors such as body composition and physical activity, which may have contributed to the microarchitectural differences between patients and controls [42].

On the other hand, our study shows considerable strengths. This is the first report on bone microarchitecture of CHH/KS patients, enhancing the existing knowledge on the skeletal phenotype of this disease. Thanks to concomitant input on aBMD, HR-pQCT indexes and bone remodeling serum markers, complementary aspects of bone health were studied. Recruitment from a tertiary reference center allowed for inclusion of a large patient sample, considering the rarity of CHH/KS. Establishment of diagnosis and physical examination were performed by the same CHH/KS expert team. All patients underwent bone density and microarchitecture evaluation using the same DXA and HR-pQCT scan respectively, which guaranteed reproducibility of measurements.

In summary, multiple aspects of bone microarchitecture, assessed by HR-pQCT, are impaired in CHH/KS men despite long-term hormonal replacement. This finding combined with the persistently lower aBMD and higher bone turnover suggest that endocrinologists should be particularly cautious to avoid undertreatment of CHH/KS patients and to ensure bone health monitoring throughout lifetime. Prospective studies are needed to compare the bone effects of

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different hormonal regimens and to investigate whether systematic testing of bone structure effectively identifies the patients at higher risk for fractures who should receive additional therapeutic interventions. Finally, our results indicate that the beneficial effect on trabecular bone is larger if therapy with testosterone or gonadotropins is started during adolescence, highlighting the importance of early diagnosis and treatment of affected patients.

ACKNOWLEDGMENTS

The authors are indebted to Sylvie Fernandez for HR-pQCT measurements and to Amélie Gavier for the coordination of patients' recruitment.

DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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LEGEND OF FIGURES

Figure 1:

Comparison of the HR-pQCT findings at the distal tibia of CHH/KS patients (light grey) and healthy volunteers (dark grey). The selected panels demonstrate the results on total and compartmental area (A-C), cortical bone (D&E) and trabecular bone (F-H) outcomes. All shown comparisons reveal a significant defect in CHH/KS men as compared to healthy volunteers. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$.

Figure 2:

Comparison of the HR-pQCT findings at the distal radius of CHH/KS patients (light grey) and healthy volunteers (dark grey). The selected panels demonstrate the results on total and compartmental area (A-C), cortical bone (D&E) and trabecular bone (F-H) outcomes. Results follow a similar pattern as observed at the distal tibia (Figure 1) with the exception of the total and trabecular area, which is not significantly different between CHH/KS and healthy volunteers at the radius. NS: not significant; **: $p<0.01$; ****: $p<0.0001$.

Figure 3:

Representative 3D image of HR-pQCT scan (each stacks composed with 110 slices) at the distal tibia of a CHH patient (left panel) and a healthy control (right panel), matched for age and body mass index. Microarchitectural anomalies were observed including lower bone volume BV/TV (white arrows), lower cortical thickness (orange arrows) and larger total bone area.

Figure 4:

Comparison of selected HR-pQCT parameters between early (before age of 19 years) and late (after the age 19 years) onset of hormonal replacement therapy in CHH/KS men. An advantage for early starters was observed regarding certain trabecular parameters (panels A-C; for

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trabecular thickness only a non-significant trend, p=0.08), as opposed to the cortical thickness, which did not differ between the two groups (panel D). NS: not significant; *: p<0.05.

Figure 5:

Serum testosterone (T) and estradiol (E2) concentrations in CHH/KS patients treated with either testosterone enanthate (TE) (n=33) or combined hCG and FSH (n=17) - see methods for detailed treatment modalities. Panels A and C show the serum T and E2 values respectively as measured at three distinct periods for each subject (12- 9 months; 7-4 months and 1 month to 1 week prior to inclusion in the current study assessing their bone status). Panels B and D show all previous measurements of T and E2 concentrations regrouped based on the time interval (in days) between the blood sample and the last injection of either TE or hCG. The normal range of serum T and E2 levels was 3.4-8.5 ng/ml and 9-38 pg/ml respectively. Only significant differences between distinct time periods and time intervals are shown. *: p<0.05; **: p<0.01; ***: p<0.001.

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Table 1. Clinical and genetic characteristics of CHH/KS patients (n=51)

CHH subgroup	**n (%)**	
Kallmann syndrome (KS)	33 (64.7%)	
Normosmic CHH	18 (35.3%)	
Degree of GnRH deficiency at diagnosis	**n (%)**	**Mean ± SD**
Complete (testicular volume < 4 ml)	39 (76.5%)	
Testicular volume, Prader (ml)		1.8 ± 0.9
Partial (testicular volume ≥ 4 ml)	12 (23.5%)	
Testicular volume, Prader (ml)		7.3 ± 2.6
Cryptorchidism	25 (49.0%)	
Micropenis	29 (56.9%)	
Genetics	**n (%)**	
No mutations in known CHH genes	24 (47.1%)	
FGFR1	8 (15.7%)*	
KAL1	6 (11.8%)*	
SEMA3A	5 (9.8%)*	
PROKR2	3 (5.9%)*	
CHD7	2 (3.9%)*	
SOX10	2 (3.9%)*	
TAC3	2 (3.9%)	
IL17RD	2 (3.9%)*	
KISS1R	1 (2.0%)	
GNRHR	1 (2.0%)	
NR0B1	1 (2.0%)	
Hormonal therapy status at inclusion	**n (%)**	
On Testosterone enanthate	27 (53.0%)	
On combined gonadotropins (hCG+FSH)	15 (29.4%)	
Wash-out (hormonal therapy suspended)	8 (15.7%)	
Never treated	1 (1.9%)	
Hormonal therapy characteristics	**n (%)**	**Mean ± SD (range)**
Age at treatment initiation (years)		20.4 ± 7.8 (12-47)
Treatment duration (years)		10.5 ± 6.9 (0-29)
Long treatment pauses	7 (13.7%)	
Prior gonadotropins exposure	30 (58.8%)	

*****Denotes genes that presented with an oligogenic pattern of inheritance (see references 1,2,4). Six digenic cases were detected as the following combinations: *FGFR1* & *IL17RD* (twice), *FGFR1* & *PROKR2*, *SOX10* & *CHD7*, *KAL1* & *SEMA3A*, *SEMA3A* & *SOX10*. The variant with the higher class according to ACGM2015 (reference 28) classification is shown first. A long treatment pause was defined as the absence of any hormonal replacement for more than one year following the initial diagnosis.

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Table 2. Clinical, hormonal status at diagnosis and areal bone mineral density in CHH/KS and control subjects

	Controls (n=40)	**CHH/KS** (n=51)	p-value
Clinical data	Mean ± SD	Mean ± SD	
Age (years)	35 ± 10	31 ± 9	0.12
Height (cm)	177.6 ± 6.6	179.5 ± 7.2	0.21
Weight (kg)	81.2 ± 14.6	82.0 ± 18.2	0.81
BMI (kg/m²)	25.7 ± 4.3	25.4 ± 5.0	0.74
Hormonal status at diagnosis			
Testosterone (ng/ml)	5.2 ± 1.2	0.3 ± 0.3	<0.0001 [a]
Estradiol (pg/ml)	17.6 ± 6.6	8.8 ± 5.2	<0.0001 [a]
LH (IU/l)	3.5 ± 1.7	0.4 ± 0.4	<0.0001 [a]
FSH (IU/l)	3.5 ± 1.8	0.9 ± 1.3	<0.0001 [a]
Inhibin B (pg/ml)	178.2 ± 63.4	49.2 ± 42.5	<0.0001
PTH, vitamin D and bone markers			
PTH (pg/ml)	41.1 ± 19.7	37.8 ± 17.6	0.65 [a]
25(OH)vitamin D (µg/l)	22.1 ± 4.4	21.1 ± 10.6	0.43 [a]
Bone Alkaline Phosphatase (ng/ml)	16.7 ± 5.3	23.4 ± 12.8	0.08 [a]
P1NP (ng/ml)	46.1 ± 13.0	100.8 ± 86.4	<0.01 [a]
Osteocalcin (ng/ml)	18.0 ± 3.5	31.5 ± 19.8	<0.01 [a]
TRAP (U/l)	2.78 ± 0.45	4.07 ± 1.90	<0.01 [a]
Serum CrossLaps (pg/ml)	317.2 ± 109.4	602.3 ± 473.4	0.03 [b]
DXA, Areal Bone Mineral Density (aBMD, g/cm²)			
aBMD Spine L1-L4	1.233 ± 0.134	1.119 ± 0.194	<0.01 [c]
Z-score, Spine L1-L4	0.43 ± 1.11	-1.02 ± 1.56	<0.0001
aBMD Femoral Neck	1.120 ± 0.136	1.011 ± 0.184	<0.01
Z-score, Femoral Neck	0.95 ± 1.08	-0.43 ± 1.34	<0.0001
aBMD Total Hip	1.133 ± 0.138	1.026 ± 0.195	<0.01 [c]
Z-score, Total Hip	0.90 ± 1.13	-0.43 ± 1.43	<0.0001
aBMD UD-Distal Radius	0.564 ± 0.057	0.476 ± 0.098	<0.0001 [c]
Z-score, UD-Distal Radius	0.76 ± 1.11	-1.05 ± 1.93	<0.0001 [c]

[a]: Mann-Whitney test (median comparisons); [b]: Student's T-test following a log normalization of the variable; [c]: Welch Two Sample t-test. P1NP, Procollagen I Intact N-Terminal; TRAP, Tartrate-resistant acid phosphatase; BAP, Bone alkaline phosphatase; aBMD, areal bone mineral density. DXA: Dual-energy X-ray absorptiometry

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Table 3. HR-pQCT measurements of the tibia and radius in subjects with CHH/KS and healthy volunteers.

	Controls (n=40) Mean ± SD	**CHH/KS** (n=51) Mean ± SD	p-value
Tibia			
Area (mm²)			
Total	864.5 ± 135.6	934.9 ± 183.5	0.05 [a]
Cortical	163.4 ± 27.0	132.0 ± 36.2	<0.0001
Trabecular	700.6 ± 140.6	801.8 ± 194.3	<0.01 [a]
Volumetric Bone Density (mg HA/cm^3)			
Total	347.9 ± 48.6	268.7 ± 71.5	<0.0001 [b]
Cortical	886.4 ± 38.8	850.1 ± 53.0	<0.001 [b]
Trabecular	217.7 ± 31.8	165.8 ± 47.5	<0.0001 [b]
Microarchitecture			
Cortical Thickness (mm)	1.42 ± 0.28	1.12 ± 0.36	<0.0001
BV/TV (%)	18.1 ± 2.6	13.8 ± 4.0	<0.0001 [b]
Cortical Perimeter (mm)	116.3 ± 9.6	119.9 ± 11.6	0.11
Trabecular Number (1/mm)	2.3 ± 0.3	2.1 ± 0.4	0.03 [b]
Trabecular Thickness (mm)	0.081 ± 0.013	0.066 ± 0.013	<0.0001
Trabecular Separation (mm)	0.368 ± 0.053	0.428 ± 0.106	<0.01 [b]
Tb.1/N.SD (mm)	0.156 ± 0.033	0.186 ± 0.066	0.01 [b]
Radius			
Area (mm^2)			
Total	349.4 ± 58.4	358.3 ± 87.4	0.75 [a]
Cortical	75.6 ± 12.5	57.8 ± 14.7	<0.0001
Trabecular	269.0 ± 58.6	293.3 ± 89.1	0.19 [a]
Volumetric Bone Density (mg HA/cm^3)			
Total	364.7 ± 51.9	281.0 ± 71.2	<0.0001 [b]
Cortical	881.2 ± 42.6	811.2 ± 83.9	<0.0001 [b]
Trabecular	204.6 ± 28.5	152.0 ± 51.4	<0.0001 [b]
Microarchitecture			
Cortical Thickness (mm)	0.942 ± 0.167	0.728 ± 0.218	<0.0001
BV/TV (%)	17.0 ± 2.4	12.7 ± 4.3	<0.0001 [b]
Cortical Perimeter (mm)	80.7 ± 6.5	80.9 ± 9.7	0.76 [b]
Trabecular Number (1/mm)	2.13 ± 0.24	1.91 ± 0.37	<0.01 [b]
Trabecular Thickness (mm)	0.081 ± 0.010	0.065 ± 0.015	<0.0001 [b]
Trabecular Separation (mm)	0.396 ± 0.054	0.481 ± 0.129	<0.01 [b]
Tb.1/N.SD (mm)	0.161 ± 0.036	0.213 ± 0.087	<0.01 [b]

[a]: Student's T-test following a log normalization of the variables; [b]: Mann-Whitney test. BV/TV, trabecular bone volume to tissue volume ratio; Tb.1/N.SD: trabecular distribution (inhomogeneity of network).

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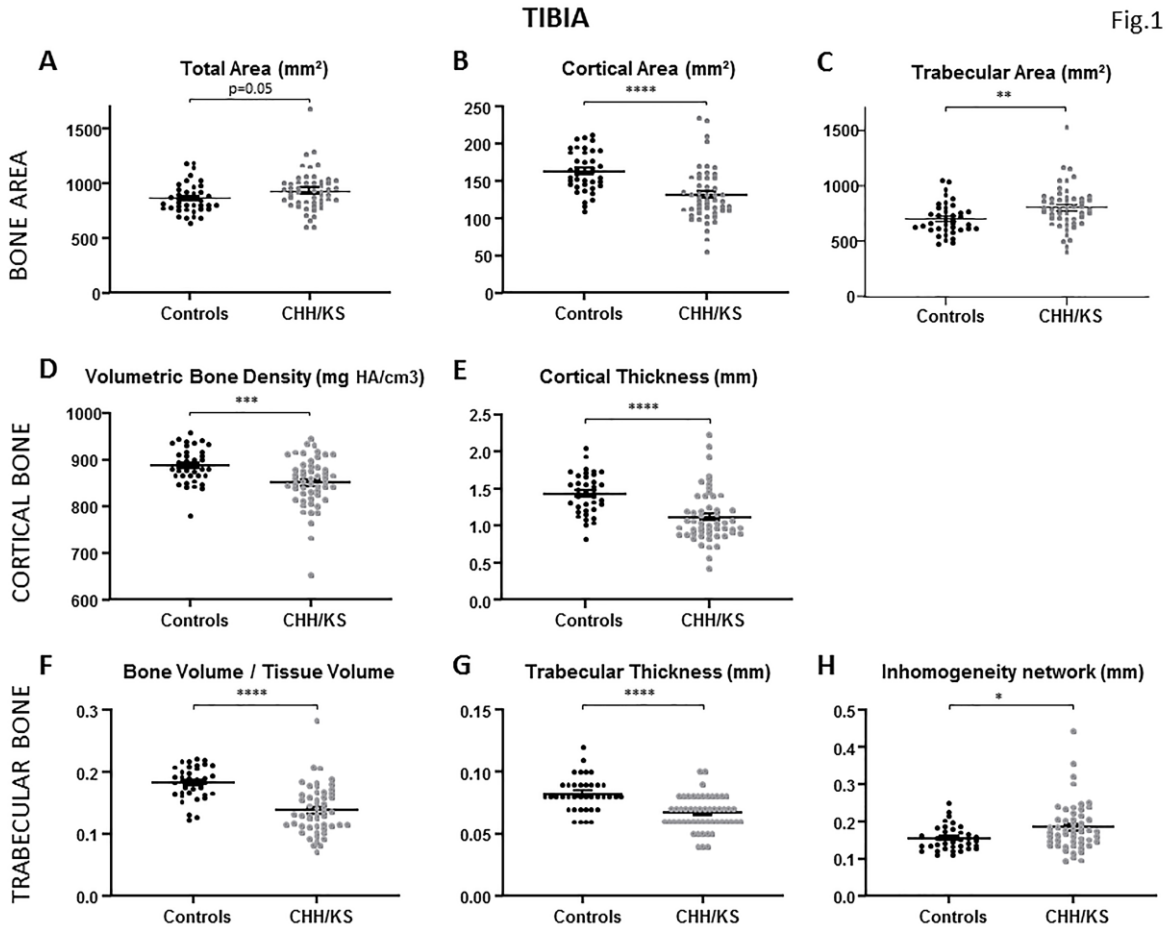

Fig.1

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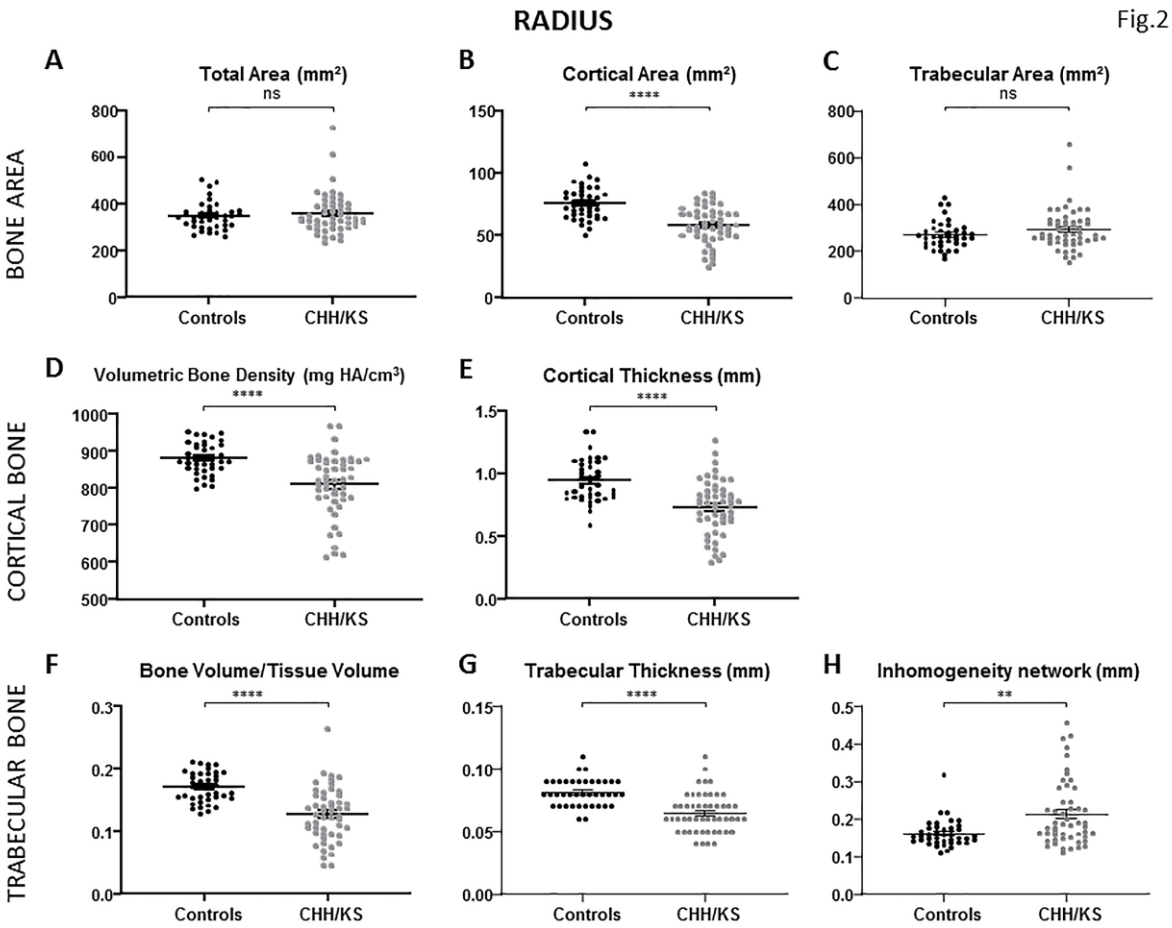

Fig.2

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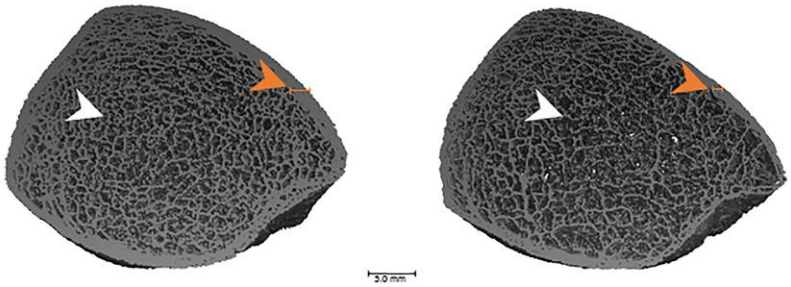
Fig.3



	Control	CHH Patient
Total area (mm^2)	749.5	919.4
BV/TV (%)	16.4	8.1
Cortical thickness (mm)	1.55	0.86

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Fig.4

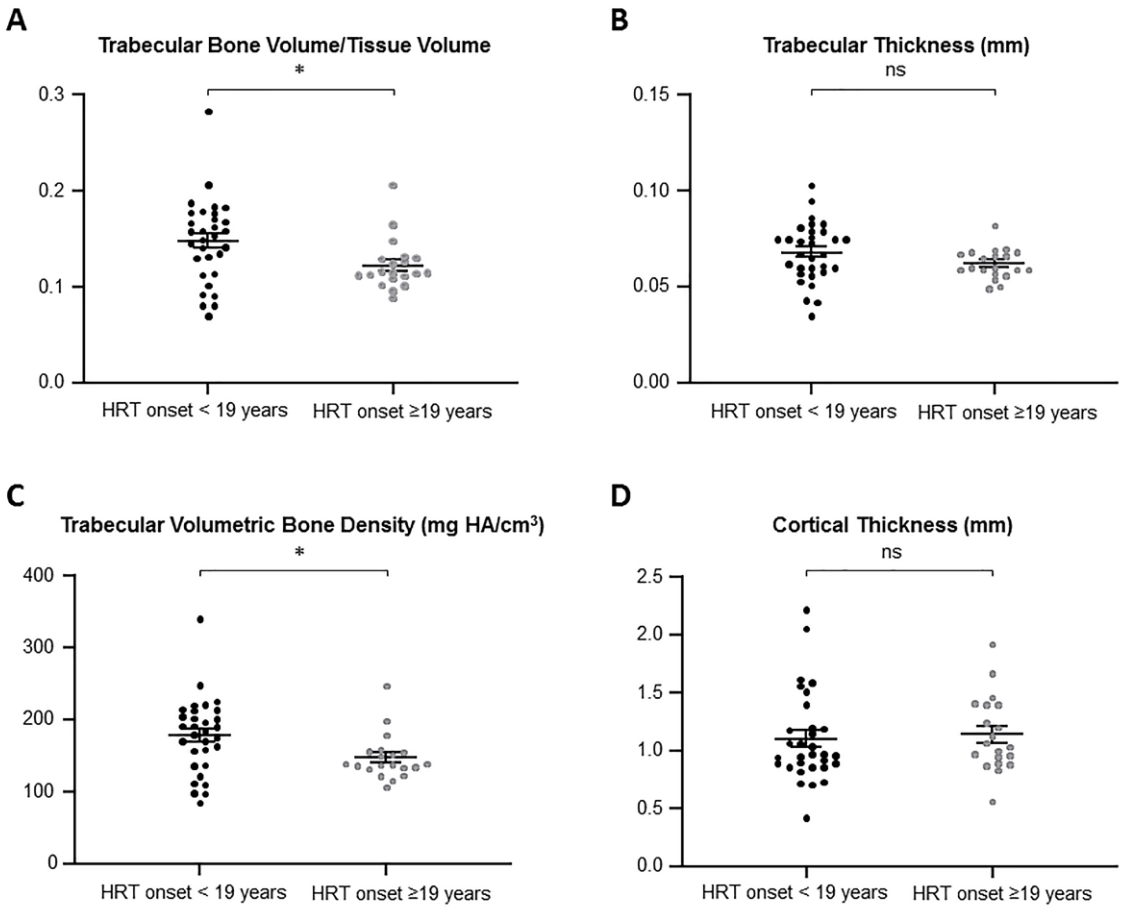

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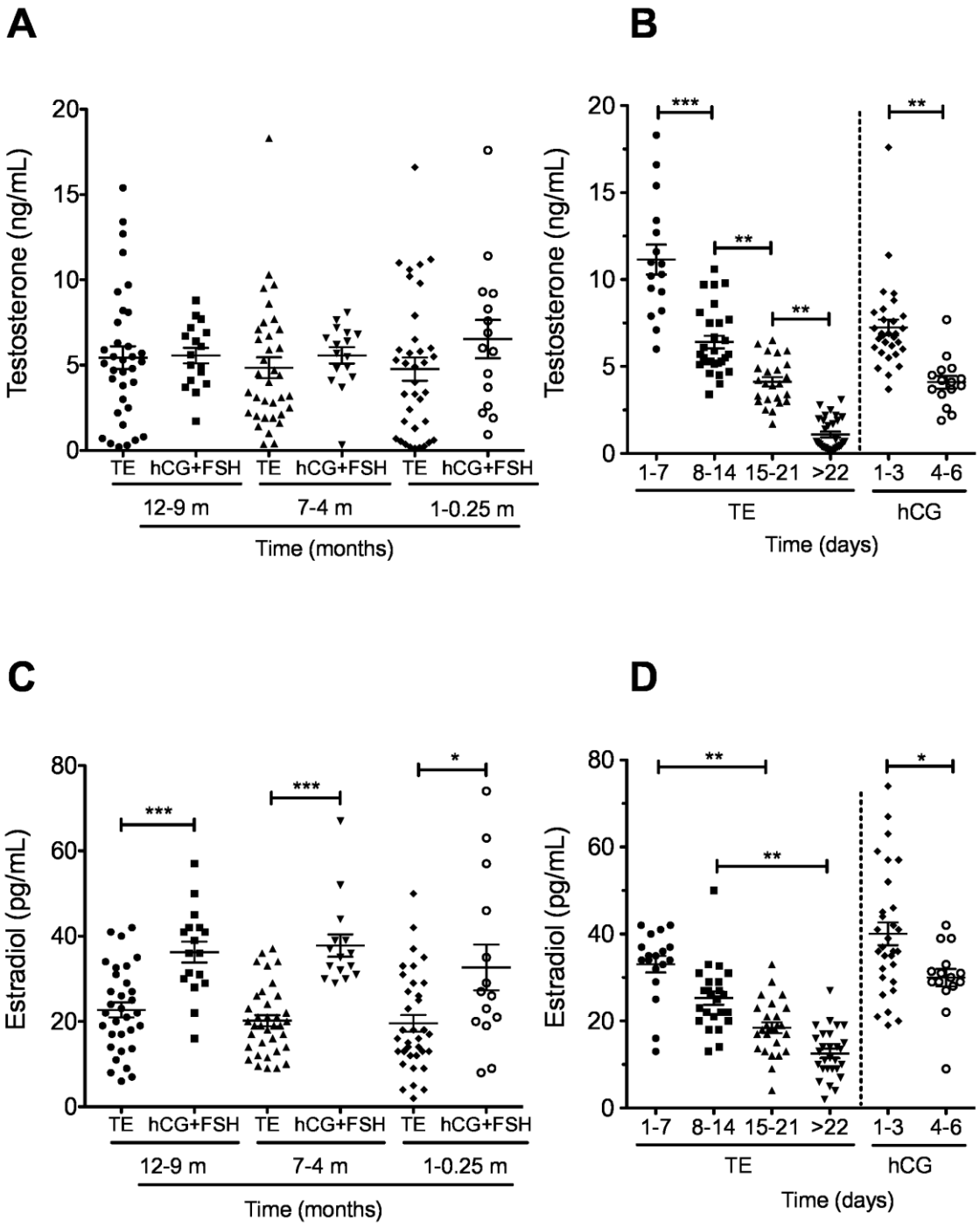
Figure 5



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