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Bone health and development in children and adolescents

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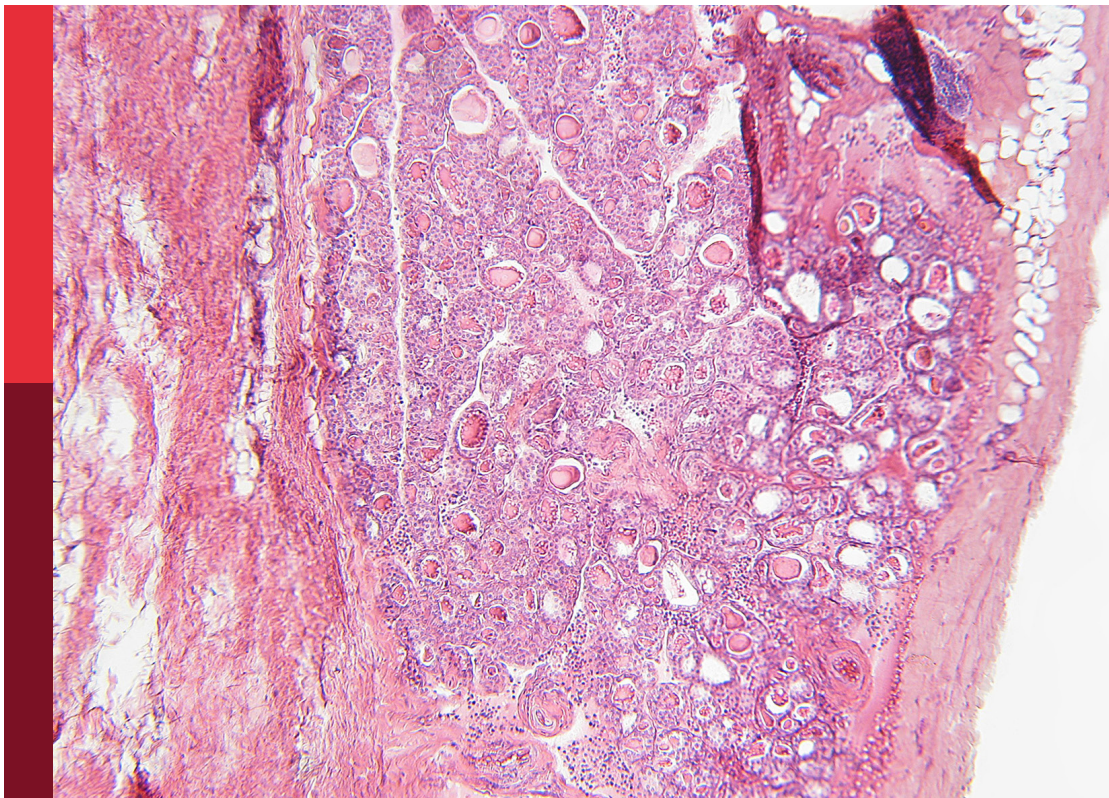

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Bone health and development in children and adolescents

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Editorial: Bone health and development in children and adolescents

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Editorial on the Research Topic
Bone health and development in children and adolescents

Bone health is understood as the bone's resistance to fracture, determined by the evaluation of bone mineral reserve, expressed as bone mineral content (BMC) or density (BMD). However, in a broader sense, bone health includes also the mechanical (support, movement, and protection) and extraskeletal (bone-derived factors) function of the skeleton (1). This Research Topic highlights several determinants of bone health (environmental, genetic, hormonal, nutritional, and mechanical) and their impact in children and adolescents.

During the growth period, the skeleton is constantly undergoing changes that go through bone modeling and remodeling, a process of bone strength optimization. Bone strength is especially "tested" during growth and aging as the incidence of fractures is higher in these periods of life (1, 2). Obesity seems to be a risk factor for bone fractures (3, 4). Despite a greater mechanical load associated with overweight and obesity, bone tissue is negatively influenced by the inflammatory state caused by cytokines released from adipose tissue (5–7). In general, overweight/obese children and adolescents have equal or higher bone mineral mass, but equal or lower cortical bone thickness than their normal body weight peers, at least in the forearm, which is the region where fractures are more frequent at these ages (6, 8–13). In this Research Topic, two studies with data from the National Health and Nutrition Examination Survey (NHANES) of the United States reveal a positive correlation between body mass index (BMI) and whole-body BMD, with a saturation effect. Both studies, conducted by Ouyang et al. and Wang et al. observed the existence of an optimal (and healthy) BMI for bone health. Another study analyzed whether serum levels of bone turnover markers (BTMs) are reduced or elevated in obese children. Cao et al. found that BTMs are reduced in overweight/obese children with significant differences according to age, sex, and pubertal stage that warrants further evaluations.

Peak bone mass - the maximum amount of bone mass reached between the second and third decade of life, is critical for bone strength. Proia et al. emphasized the need to investigate the triangulation between nutritional, endocrine, and mechanical (in this case physical activity) factors in children and adolescents' bone tissue since most studies on this interaction were performed in adults.

The assessment of 25-hydroxyvitamin D3 serum levels should be part of the screening tests that pediatricians request for their at-risk patients to evaluate their calcium/phosphate metabolism. Xu et al. explored how in children with short stature the dosage of 25-hydroxyvitamin D2 [25 (OH) D2] and 25-hydroxyvitamin D3 [25 (OH) D3] alone could overestimate vitamin D stores. They showed that C3-epi/25(OH)D3 and 25 (OH)D2/25(OH)D3 ratios determined from high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) are more precise indicators of vitamin D reserves. The results, if confirmed in larger studies, suggest the use of LC-MS/MS as a more accurate laboratory technique for the evaluation of vitamin D status in short children.

Many genetic diseases affect bone, where osteogenesis imperfecta is one of the most serious conditions. Zhang et al. evaluated the skeletal outcomes of bisphosphonates treatment in a cohort of patients affected by osteogenesis imperfecta in which the duration of treatment was dependent on the achievement of age- and sex-specific BMD (~4 years). Overall, during three years of drug holiday, the authors showed a maintenance of BMD and fracture incidence; it was also observed a greater likelihood of restarting bisphosphonates treatment in patients with severe disease and in cases who started bisphosphonates treatment later in life.

Mindler et al. reviewed the radiological and clinical data of 43 patients with X-linked hypophosphatemia (XLH). The authors performed an analysis of the patients' gait comparing the results with 76 healthy controls. They verified the negative impact of bone deformities and BMI on the quality of gait in patients with XLH.

Among 405 cases of children with finger problems treated in a hospital setting, Hao et al. described the eight cases affected by clinodactyly due to osteochondroma. In these patients, the surgical removal of the osteochondroma resulted decisive for the cure of clinodactyly although the procedure is not without complications and requires prolonged follow-up. A level of phalanx angulation greater than 10° should be investigated by hand radiography which is also useful for the preoperative characterization of osteochondroma.

Bone health is largely dependent on a proper balance of sex hormones. In particular estrogens deficiency in both adolescent girls and boys limits the maximization of peak bone mass in adulthood (14). Misakian et al. draw attention to the degree of insufficient bone mineralization in ~16-year-old adolescents with complete androgen insensitivity syndrome (CAIS), and showed that it may be even more severe in early gonadectomized cases. The authors observed that hormone replacement therapy did not lead to an optimal BMD in most of their patients, and pointed some explanations.

Classically, precocious puberty is treated with GnRH analogs, a well-established, effective, and safe therapy (15). In addition to the arrest of pubertal maturation, a slowdown in growth rate is among the treatment effects and it is caused by the interruption of bone growth plate development. Using animal models, Zhu et al. described the anabolic effect of stanazolol on the bone growth plate providing insight into the pathophysiology and rationale for its use during long-term GnRH treatment in cases with impaired growth velocity. Stanazolol has been safely utilized in Turner's syndrome to increase final stature (16).

Two studies examined the impact that environmental interferents can have on bone health using data from the US (NHANES, 2005-2010). In one of the studies, Cui et al. found negative associations between blood lead levels and BMD of the lumbar spine, proximal femur, and femoral neck in boys and girls aged 8-19 years. Negative associations were greater in the spine than in the femur and greater in girls than in boys, suggesting that further studies on this topic are needed. Utilizing 1228 participants 12-19 years old from the same cohort, Xiong et al. found a negative correlation between the serum concentrations of several perfluoroalkyl substances and BMD. Again, these associations were more pronounced in the lumbar spine, in girls but also in those who were overweight/obese and had anemia.

Overall, this Research Topic renews awareness of several factors and mechanisms that affect bone health. We believe that the information provided reinforces the commitment of general health professionals, pediatricians in particular, in optimizing the growth and development of children and young people. Greater caution is needed to optimize bone health among the most vulnerable individuals, particularly those with medical conditions and those most exposed to health-threatening environments and lifestyles.

Author contributions

FeB, FáB equally contributed to conception, design, writing and revision of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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GnRHa/Stanozolol Combined Therapy Maintains Normal Bone Growth in Central Precocious Puberty

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Background: Gonadotropin-releasing hormone agonist (GnRHa) is the gold standard in the treatment of Central Precocious Puberty (CPP) with progressive puberty and accelerative growth. However, GnRHa treatment is reported to result in growth deceleration and prevents growth plate development which leads to a reduction in height velocity. Stanozolol (ST) has been used to stimulate growth in patients with delayed growth and puberty, nevertheless, the effects and mechanisms of ST on CPP with GnRHa treatment are currently unclear.

Methods and Results: In the current study, we recorded the following vital observations that provided insights into ST induced chondrogenic differentiation and the maintenance of normal growth plate development: (1) ST efficiently prevented growth deceleration and maintained normal growth plate development in rats undergoing GnRHa treatment; (2) ST suppressed the inhibitory effect of GnRHa to promote chondrogenic differentiation; (3) ST induced chondrogenic differentiation through the activation of the JNK/c-Jun/Sox9 signaling pathway; (4) ST promoted chondrogenic differentiation and growth plate development through the JNK/Sox9 signaling pathway *in vivo*.

Conclusions: ST mitigated the inhibitory effects of GnRHa and promoted growth plate development in rats. ST induced the differentiation of chondrocytes and maintained normal growth plate development through the activation of JNK/c-Jun/Sox9 signaling. These novel findings indicated that ST could be a potential agent for maintaining normal bone growth in cases of CPP undergoing GnRHa treatment.

Keywords: CPP, GnRHa, ST, bone growth, chondrogenic differentiation

Abbreviations: GnRHa, Gonadotropin-releasing hormone agonist; CPP, Central Precocious Puberty; ST, stanozolol; GH, Growth Hormone; COL-X, Collagen-X; COL-II, Collagen II; MMP13, Matrix Metalloproteinase 13; SP, JNK specific inhibitor; PD, ERK specific inhibitor; SB, p38 specific inhibitor.

INTRODUCTION

Central Precocious Puberty (CPP) refers to premature activation of the hypothalamic-pituitary-gonadal (HPG) axis, resulting in the early development of secondary sexual characteristics (1, 2). The classical definition of precocious puberty is the development of secondary sexual characteristics before the age of 8 years or menarche before the age of 9 years in girls and any secondary sexual characteristic before the age of 9 years in boys (1). Most cases of CPP are idiopathic and seen in girls. In contrast, most boys with CPP have an identifiable cause (2). The treatment of precocious puberty aims to: interrupt sexual maturation until the normal age for pubertal development is reached; revert or stabilize sexual characteristics; delay skeletal maturation; and preserve normal height potential (3–5).

Gonadotropin-releasing hormone agonist (GnRHa) is the standard agent for the treatment of CPP with progressive puberty and accelerative growth (6–8). The efficacy and safety of GnRHa treatment for CPP have been well described (1); however, recent studies demonstrate that GnRHa treatment causes growth deceleration and prevents growth plate development which leads to a marked reduction in height velocity (9–11). Thus, an agent that can mitigate the effects of GnRHa and promote bone growth and maturation is necessary in the treatment of CPP. Previous studies suggest that when height velocity is reduced (<4 cm/year), growth hormone (GH) should be added to the GnRHa treatment (1, 12). A few studies have assessed the effect of GH administration on the height of patients with CPP, and some show a positive effect of GH/GnRHa therapy in children with decreased growth during GnRHa therapy (13, 14). However, there are some disadvantages of GH treatment: its cost is high and its application is inconvenient (15). Other treatments such as estrogen mini-dose replacement, to overcome the decreased growth plate development that is induced by GnRHa, have been reported (16). Previous studies show that mini-dose estrogen treatment can normalize the slowdown of growth rate during GnRHa therapy in patients with CPP (10). However, estrogen has the potential effect of accelerating bone maturation, and dosage is difficult to control because of individual differences (16).

Stanozolol (ST), a non-aromatizable androgen that has a high anabolic to androgenic ratio has been used to stimulate growth and final adult height of patients with other disease such as Turner syndrome (TS) (17). ST also used to treat some serious disorders like aplastic anemia and hereditary angioedema. It is also indicated as an adjunct therapy for the treatment of vascular disorders and growth failures (18). The efficacy and safety of ST treatment have been well described. Patients with TS who were treated with oxandrolone, height velocity was increased without bone age progression (19). Moreover, our previous study shows that ST promotes proliferation of growth plate chondrocytes (15). Together, these studies indicate that anabolic steroid hormones may promote long bone growth without increasing bone age to achieve the final adult height. However, the mechanism of action of ST on growth plates in patients with CPP is still unknown.

The purpose of the current study was to verify the chondro-inductive capacity of ST in rats undergoing GnRHa treatment and to explore the underlying mechanism, which may provide useful information for its potential clinical application in CPP.

MATERIALS AND METHODS

Experimental Animals

All animal experiments were approved by the Medical Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University and carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. Normal healthy Sprague-Dawley rats were provided by the experimental animal center of Sun Yat-sen University and housed in a standard animal room with food and water ad libitum under controlled conditions of humidity (50%-70%) and temperature (21°C ± 2°C) and under a 12 h light/12 h dark lighting schedule.

To evaluate the effect of GnRHa and ST, 2.5mg/kg of GnRHa (Diphereline, Ipsen Pharma Biotech, France) was injected intramuscularly once every 2 weeks and 10 mg/kg of ST (Sigma-Aldrich, MO, USA) was injected once a day. PBS as a negative control. Rats were euthanized at 7 weeks of age. Hind limb specimens were dissected and fixed in 4% paraformaldehyde for histological analyses (n = 5 animals per group) (15).

Histological Analyses

All specimens were obtained from rats post-mortem and fixed with 4% paraformaldehyde. For histologic analysis, specimens were decalcified in 0.5 M EDTA (Sigma-Aldrich, MO, USA) at 4°C. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and Safraini O Fast Green (SOFG) to evaluate general structures and growth plate development. Immunohistochemical analyses of the specimens were conducted using specific antibodies. Tissue sections were quantitated according to the number of positive cells per unit area as previously described (20).

Immunofluorescence

Tissue sections were fixed in 4% PFA for 30 min and permeabilized with 0.3% Triton X-100 for 30 min. Blocking was performed with 5% normal goat serum for 1 h. The tissue sections and the cells were incubated overnight at 4°C in primary antibodies against the following antigens: anti-Sox9 antibody (Abcam, UK; 1:200) and anti-p-JNK antibody (Abcam; 1:200). After washing three times in PBS, the primary antibodies were probed with the secondary antibodies Alexa Fluor 594 goat anti-rabbit (1:500, Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 594 goat anti-mouse (1:500, Invitrogen; Carlsbad, CA, USA) for 1 h at room temperature. Finally, the coverslips were washed in PBS three times and mounted using Prolong Gold Antifade Reagent containing 4′-6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Invitrogen). The targeted marker-positive

cells in each visual field were counted under a fluorescence microscope (Carl Zeiss Axio Observer Z1, Zeiss, Oberkochen, Germany).

Cell Preparation and Treatment

The pre-chondrocyte cell line ADTC5 cells (ATCC, Manassas, VA, USA), from which we acquired the chondrocyte phenotype in the post confluent culture, were maintained in DMEM/F12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin in a humidified incubator at 37°C and 5% CO_2. When confluent, the cells were detached using a trypsin (0.25%)-EDTA (1 mM) (Sigma-Aldrich, MO, USA) solution and subcultured in 6-well or 12-well plates (21).

Human bone mesenchymal stem cells (hBMSC) were immediately isolated and purified from bone marrow samples using density gradient centrifugation as previously described (22). Briefly, hBMSC were isolated from bone marrow blood collected during surgeries. The blood was suspended in low glucose DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco, Australia) and 1% penicillin/streptomycinin in a humidified incubator at 37°C and 5% CO_2. When confluent, the cells were detached using a trypsin (0.25%)-EDTA (1 mM) (Sigma-Aldrich, MO, USA) solution and subcultured in 6-wells or 12-wells plates.

To assess chondrogenic differentiation, 1×10^5 ATDC5 cells or hBMSC were resuspended in control medium and seeded as micromasses in 24-well plates. ATDC5 cells were allowed to attach for 1 h at 37°C, then 0.5 ml of chondrogenic medium, which contained 10 ng/ml recombinant rat TGFβ (R&D) and 50 μM l-ascorbic acid 2-sulfate (Sigma-Aldrich, MO, USA), was added to the wells. Medium was changed every day and after 9 d, micromasses were either stained with alcian blue or the cells were used for PCR and WB experiments (21).

To investigate the effect of GnRHa and Stanozolol on chondrogenic differentiation, 5nM GnRHa and 10nM Stanozolol were added into ATDC5 cells.

Quantitative Real-Time PCR

For analysis of gene expression, total RNA was extracted from cells according to the manufacturer's protocol, and 2 μg of total DNA-free RNA was used to synthesize cDNA using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). The reactions were set up in 96-well plates using 1 μl cDNA with Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan), to which gene-specific forward and reverse PCR primers were added. QRT-PCR was performed under the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 55°C for 34 s. These analyses were performed to detect COL-X, COL-II, MMP13, Aggrecan, and Sox9 expression, and β-actin was used as an internal control. Primer sequences are listed in **Tables 1** and **2**.

Western Blot

Cells were lysed in RIPA buffer, total protein was extracted, and the protein concentration was determined with a BCA assay. A 10% SDS-PAGE gel was loaded with 20 μg of total protein, and the separated proteins were transferred by electro blotting to PVDF membranes. The membranes were blocked with 5% non-fat dry milk in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) and incubated with the primary antibody overnight at 4°C in 5% non-fat dry milk in TBST. Immunolabeling was detected using ECL reagent (Invitrogen, Carlsbad, CA, USA). The antibodies used for western blot were from the following sources: anti-Sox9 antibody (Abcam, UK; 1:1000), anti-COL-X antibody (Abcam, UK; 1:1000), anti-COL-II antibody (Abcam, UK; 1:1000), anti-MMP13 antibody (Abcam, UK; 1:500), anti-GAPDH antibody (Sigma-Aldrich, MO, USA; 1:10000), and anti-β-Actin antibody (Sigma-Aldrich, MO, USA; 1:10000).

RNA Interference

The small interfering RNA (siRNA) duplexes were constructed by GenePharma (GenePharma Co., Suzhou, China). The siRNA duplexes are listed in **Table 3**. Cells were plated at a concentration of 1 x 10^5 cells/well in 6-well plates and transduced with the small interfering RNA (siRNA) using lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Different amounts of 20 μM siRNA duplexes were mixed with 5 μl/well of transfection reagent and Opti-MEM reduced-serum medium (Invitrogen, Carlsbad, CA, USA) to a total volume of 500 μl and incubated for 20 min. The mixture was applied to cells for 16 h at 37°C in 5% CO_2.

Statistical Analysis

All statistical analyses were carried out using SPSS 16 software. Data, obtained from experiments in duplicate or triplicate and repeated at least three times, are represented as mean ± SD. The unpaired Student's t test was used to compare two groups with Shapiro-Wilk test for normality test. One-way ANOVA was performed with Levene's test for homogeneity of variance, followed by the Bonferroni *post hoc* test based on the

TABLE 1 | Primers for RT-qPCR analysis of gene expression for rats.

Primer	5' Forward 3'	5' Reverse 3'
Actin	CGTGCGTGACATCAAAGAGAAG	CGTTGCCAATAGTGATGACCTG
COL-X	GTTCTTGACCCTGGTTCA	CTGAGGGACCTGGGTGT
COL-II	GGGAATGTCCTCTGCGATGAC	GAAGGGGATCTCGGGGTTG
MMP13	TCCCTGGAATTGGCAACAAAG	GCATGACTCTCACAATGCGATTAC
Aggrecan	TTCCACCAGTGCGATGCAG	TGGTGTCCCGGATTCCGTA
Sox9	GAGCCGGATCTGAAGAGGGA	GCTTGACGTGTGGCTTGTTC

TABLE 2 | Primers for RT-qPCR analysis of gene expression for humans.

Primer	5' Forward 3'	5' Reverse 3'
Actin	TATTGGCAACGAGCGGTTC	ATGCCACAGGATTCCATACCC
COL-X	ATGCTGCCACAAATACCCTTT	GGTAGTGGGCCTTTTATGCCT
COL-II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
MMP13	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
Aggrecan	CACTGTTACCGCCACTTCCC	GACATCGTTCCACTCGCCCT

comparison to be made and the statistical indication of each test. Mauchly's sphericity test was used for sphericity test. For non-parametric data, difference between groups were evaluated with non-parametric Mann-Whitney U-test, and categorical and binary variables were tested by the Fisher exact test. Statistical significance was accepted at $p < 0.05$.

RESULTS

Stanozolol Prevented Growth Deceleration and Promoted Growth Plate Development in Rats Undergoing GnRHa Treatment

To evaluate the effect of ST on growth plate development, rats were intramuscularly injected with GnRHa (2.5mg/kg) and ST (10mg/kg). H&E staining revealed that there was no significant difference in growth plate width, proliferative zone width, or hypertrophic zone width at postnatal day 6 (**Figures 1A–C**). At postnatal week 4, growth plate width, proliferative zone width, and hypertrophic zone width were significantly decreased in rats given the single GnRHa treatment (Growth plate width: from 281.14 ± 15.63 to 227.12 ± 21.46; Proliferative zone width: from 119.71 ± 8.96 to 106.51 ± 7.74; Hypertrophic zone width: from 147.29 ± 9.24 to 118.57 ± 8.28). However, these same three measurements were significantly increased in the rats receiving the GnRHa/ST combined treatment (Growth plate width: from 227.12 ± 21.46 to 327.53 ± 15.25; Proliferative zone width: from 106.51 ± 7.74 to 149.85 ± 21.78; Hypertrophic zone width: from 118.57 ± 8.28 to 163.83 ± 9.89) (**Figures 1B–D**). SOFG staining showed a similar result for growth plate width (**Figure 1E**). Immunostaining analyses revealed that expressions of MMP13, COL-X, and COL-II were significantly decreased in growth plates with the GnRHa treatment (MMP13: from 51.00 ± 13.36 to 8.20 ± 3.35; COL-X: from 63.20 ± 6.18 to 42.00 ± 6.20; COL-II: from 59.80 ± 8.32 to 18.40 ± 2.70) and increased with the GnRHa/ST combined treatment (MMP13: from 8.20 ± 3.35 to 43.80 ± 8.56; COL-X: from 42.00 ± 6.20 to 55.20 ± 4.32; COL-II: from 18.40 ± 2.70 to 44.60 ± 6.19) (**Figures 1E–G**). These results suggested that ST efficiently prevented growth deceleration and promoted growth plate development in rats undergoing GnRHa treatment.

Stanozolol Suppressed the Inhibitory Effect of GnRHa to Promote Chondrogenic Differentiation

To determine the chondro-inductive capacity of ST under GnRHa treatment, ATDC5 and hBMSC cells were treated with GnRHa (5nM) and ST (10nM). The results of alcian blue staining showed that GnRHa significantly suppressed chondrogenic differentiation of ATDC5 and hBMSC cells, and this inhibitory effect was rescued by ST (**Figures 2A, B**). Similar effects were observed in the expressions of chondrogenic relative markers including COL-X, COL-II, MMP13, and Aggrecan at mRNA and protein levels. With GnRHa/ST combined treatment, mRNA levels of COL-X, COL-II, MMP13, and Aggrecan were significantly increased to 1.98-fold, 2.49-fold, 1.97-fold and 2.37-fold in ATDC5 cells; mRNA levels of COL-X, COL-II, MMP13, and Aggrecan were significantly increased to 2.38-fold, 2.48-fold, 2.17-fold and 2.38-fold in hBMSC cells (**Figures 2C–F**). These results suggested that ST suppressed the inhibitory effects of GnRHa to promote chondrogenic differentiation.

Stanozolol Induced Chondrogenic Differentiation in a Sox9 Dependent Manner

Since Sox9 is a well-known transcription factor that regulates matrix gene expression in chondrocytes (23, 24), we further explored the potential role of Sox9 in ST promotion of chondrogenic differentiation. The results of immunofluorescence staining revealed that Sox9 positive cells were significantly decreased from 57.60 ± 8.56 to 14.60 ± 5.86 at the growth plates of rats receiving the single GnRHa treatment and increased from 14.60 ± 5.86 to 32.2 ± 9.01 with the GnRHa/ST combined treatment (**Figures 3A, B**). Furthermore, Sox9-specific small

TABLE 3 | siRNA sequences for RNA interference.

Gene	Sense	Antisense
Control	UAACGACGCGACGACGUAATT	UUACGUCGUCGCGUCGUUATT
c-Jun	GCUACAGUAACCCUAAGAUTT	AUCUUAGGGUUACUGUAGCTT
c-Fos	GCUACAGUAACCCUAAGAUTT	AUCUUAGGGUUACUGUAGCTT
JunB	CACAAGAUGAACCACGUGATT	UCACGUGGUUCAUCUUGUGTT
JunD	GCCUGGAGGAGAAAGUCAATT	UUGACUUUCUCCUCCAGGCTT
Sox9	AACGAGAGCGAGAAGAGACTT	GGGUCUCUUCUCGCUCUCGTT

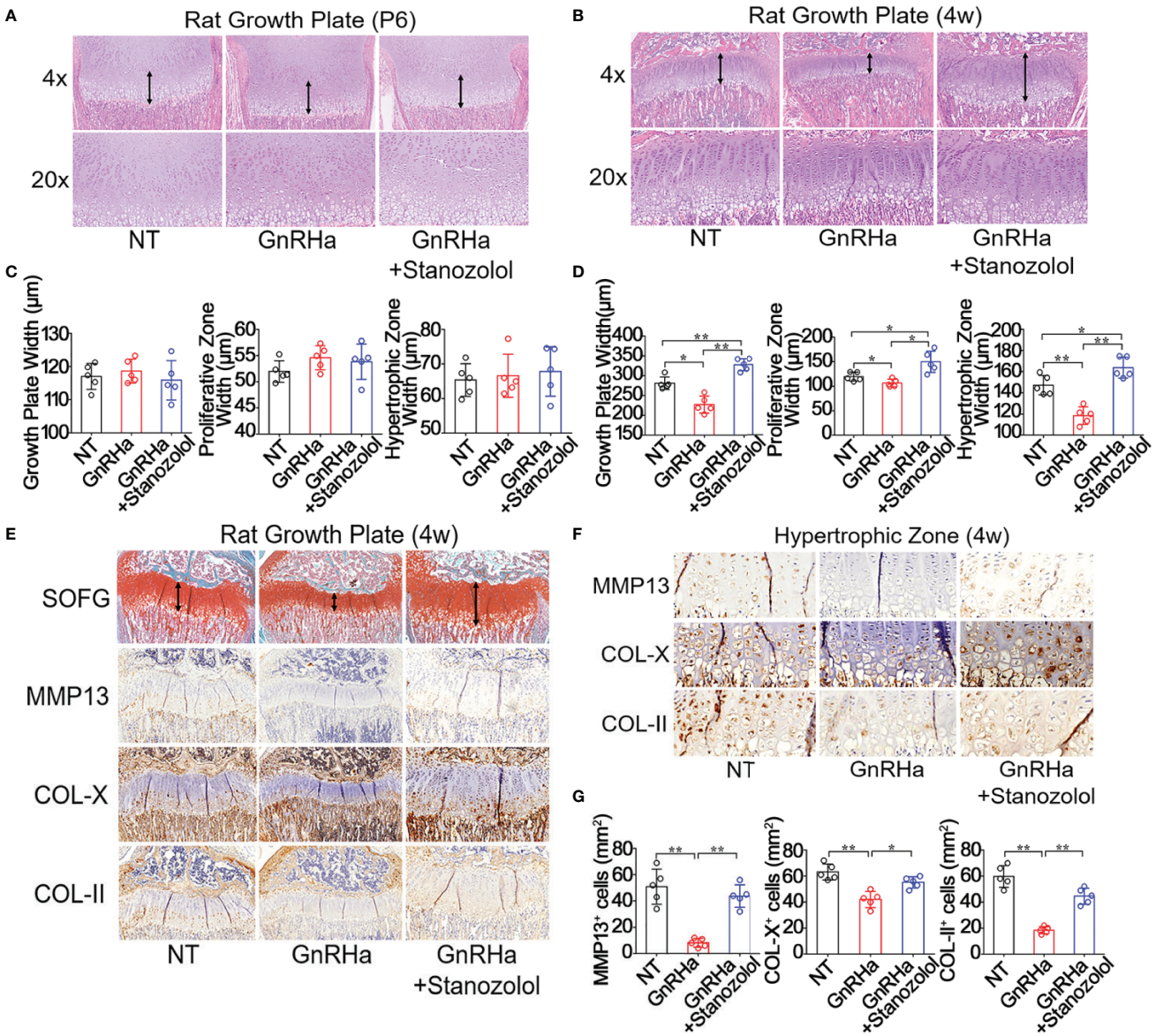

FIGURE 1 | Stanozolol prevented growth deceleration and promoted growth plate development in rats undergoing GnRHa treatment. **(A, B)** H&E staining of the growth plate in rats. **(C, D)** Quantitative analysis of growth plate width in rats. **(E)** SOFG and immunochemical staining analysis of the growth plate in rats. **(F)** Immunochemical staining analysis of the hypertrophic zone of the growth plate in rats. **(G)** Quantitative analysis of positive cell number in hypertrophic zones. Data shown as mean ± SD. n = 5. *p < 0.05 compared between groups; **p < 0.01 compared between groups.

interfering RNA (siRNA) was constructed and transfected in ATDC5 cells. The results of PCR and WB showed that Sox9 specific siRNA significantly decreased ST-induced up-regulation of chondrogenic marker expressions at both mRNA and protein levels. With silencing of Sox9, mRNA levels of COL-X, COL-II, MMP13, and Aggrecan were significantly decreased from 2.88 to 0.68-fold, 2.49 to 0.89-fold, 2.87 to 0.77-fold and 2.97 to 0.66-fold in ATDC5 cells (**Figures 3C, D**). These results suggested that ST promoted growth plate development and chondrogenic differentiation in a Sox9 dependent manner.

Stanozolol Up-Regulated Sox9 Through the JNK/c-Jun Pathway

To determine the regulatory mechanism of ST-induced Sox9 expression, ATDC5 cells were treated with pharmaceutical inhibitors of MAPKs then stimulated with ST for 24 h. Sox9 expression was then detected with RT-qPCR and western blot. PCR results showed that inhibition of JNK (SP) significantly decreased Sox9 expression from 3.71 to 1.53-fold at both mRNA. Western blot results showed similar result of the expression of Sox9 in ATDC5 cells. (**Figures 4A, B**). In addition, c-Jun was an

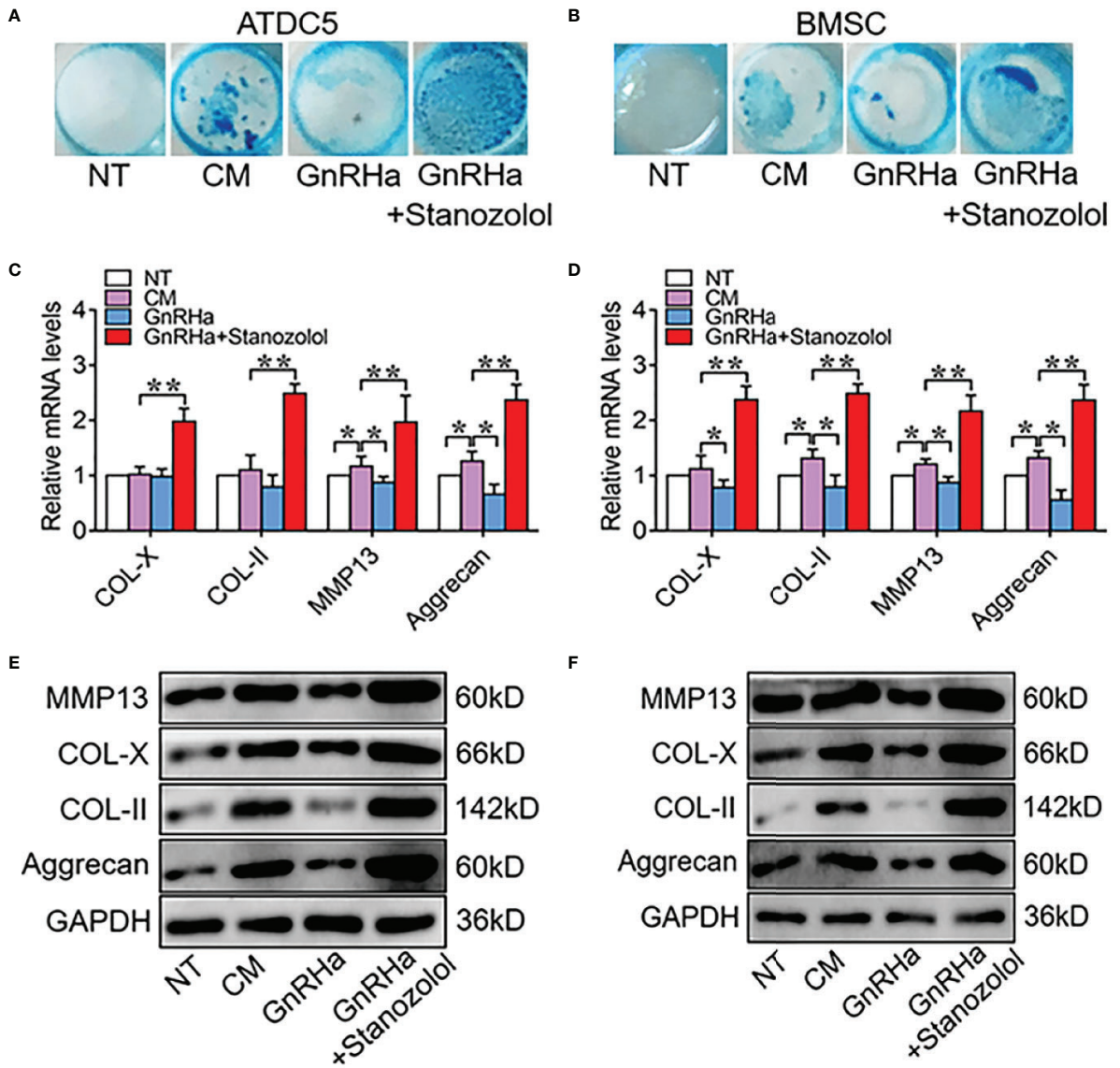

FIGURE 2 | Stanozolol suppressed the inhibitory effects of GnRHa to promote chondrogenic differentiation. **(A, B)** Alcian blue staining of ATDC5 cells and hBMSCs for 9 d. **(C, D)** RT-qPCR analysis of chondrogenic markers in ATDC5 cells and hBMSCs. **(E, F)** Western blot analysis of chondrogenic markers in ATDC5 cells and BMSC. The data are presented as means ± SD from one representative experiment of three independent experiments performed in triplicate. *p < 0.05 compared between groups; **p < 0.01 compared between groups.

important transcription factor in the MAPK/JNK pathway. The c-Jun-specific siRNA was constructed and transfected in ADTC5 cells. The PCR results showed that c-Jun specific siRNA significantly decreased ST-induced up-regulation of Sox9 expression from 3.21 to 1.85-fold at mRNA level. Western blot results showed similar result of the expression of Sox9 in ATDC5 cells. (**Figures 4C, D**). In addition, silencing of the expression of the other AP-1 subunits: c-Fos, JunB, and JunD had no effect on ST-induced Sox9 expression (**Figure 4E**). These results indicated that c-Jun, but not c-Fos, JunB, or JunD, played an important role in ST-mediated regulation of Sox9 expression. Immunofluorescence results revealed that the JNK/c-Jun signaling pathway was required for the up-regulation and translocation of Sox9 (**Figure 4F**). These results suggested that ST up-regulated Sox9 through the JNK/c-Jun pathway.

Stanozolol Promoted Chondrogenic Differentiation and Growth Plate Development Through the JNK/Sox9 Signaling Pathway *In Vivo*

To confirm the activation of the JNK/Sox9 signaling pathway in rats undergoing GnRHa/ST combined treatment, we observed

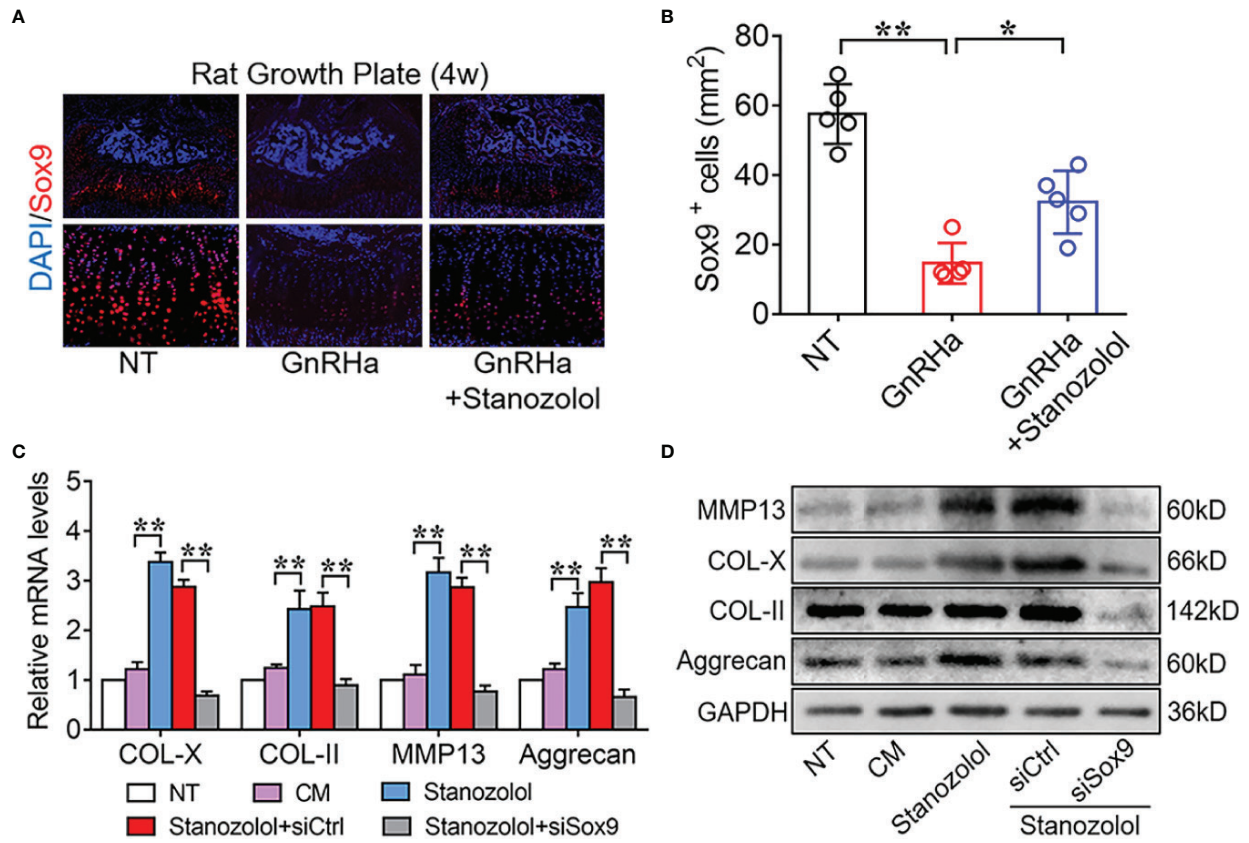

FIGURE 3 | Stanozolol-induced chondrogenic differentiation in a Sox9 dependent manner. **(A)** Immunofluorescence analysis of growth plates in rats. **(B)** Quantitative analysis of Sox9-positive cell number in the growth plates of rats. Data shown as mean ± SD. n = 5. **(C)** RT-qPCR analysis of chondrogenic markers in ATDC5 cells. **(D)** Western blot analysis of chondrogenic markers in ATDC5 cells. The data are presented as means ± SD from one representative experiment of three independent experiments performed in triplicate. *p < 0.05 compared between groups; **p < 0.01 compared between groups.

the expression of JNK and Sox9 in the growth plate of rats being administered GnRHa and ST treatment. Immunofluorescence staining demonstrated that positive staining of p-JNK and Sox9 was notably decreased at the growth plate after the single GnRHa treatment (p-JNK$^+$ cells: from 65.20 ± 9.20 to 32.40 ± 5.37; Sox9$^+$ cells: from 48.20 ± 8.58 to 11.60 ± 5.90; p-JNK$^+$ Sox9$^+$ cells: from 33.40 ± 6.80 to 8.60 ± 3.21), whereas the GnRHa/ST combined treatment led to a significant increase in positive staining of p-JNK and Sox9 at the same region (p-JNK$^+$ cells: from 32.40 ± 5.37 to 55.20 ± 7.29; Sox9$^+$ cells: from 11.60 ± 5.90 to 26.20 ± 5.26; p-JNK$^+$ Sox9$^+$ cells: from 8.60 ± 3.21 to 22.60 ± 4.39) (**Figures 5A, B**). These results indicated that ST promoted chondrogenic differentiation and growth plate development through the JNK/Sox9 signaling pathway *in vivo*.

DISCUSSION

GnRHa is the standard agent for the treatment of CPP with progressive puberty and accelerative growth (1, 2, 25). The efficacy and safety of GnRHa treatment for CPP have been well described (1). However, the side effects of GnRHa such as growth deceleration or the prevention of growth plate development, which lead to a reduction in height velocity, are also reported (3, 9, 11). Thus, an agent which can diminish the side effects of GnRHa and maintain normal growth plate development would theoretically be of great benefit for the treatment of CPP. Previous studies have reported the positive effects of GH administration on height in patients with CPP and showed that the combined use of GH and GnRHa is effective in preventing a decline in growth rate (26–28). However, GH is an expensive medicine and difficult to use in conventional treatment.

ST has a high anabolic to androgenic ratio and has been used to stimulate bone growth in patients with delayed growth and puberty (29). Previous studies reveal that it stimulates a height velocity increase without bone age progression in patients with Turner syndrome undergoing oxandrolone treatment (19). Our previous study also showed that ST promotes the proliferation of growth plate chondrocytes (15). Therefore, we speculated that ST stimulates growth plate development through the promotion of chondrogenic differentiation in patients who were experiencing an inhibitory effect of GnRHa. If that is the case, ST could be a

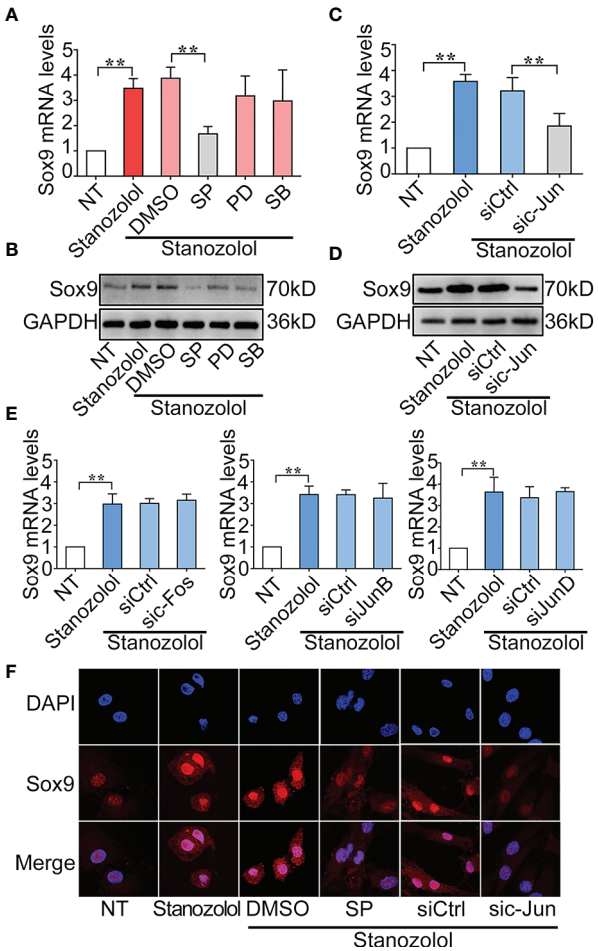

FIGURE 4 | Stanozolol up-regulated Sox9 through the JNK/c-Jun pathway. **(A, B)** RT-qPCR and western blot analysis of Sox9 expression in ATDC5 cells treated with MAPK inhibitors and ST. **(C, D)** RT-qPCR and western blot analysis of Sox9 expression in ATDC5 cells transfected with c-Jun siRNA and treated with ST. **(E)** RT-qPCR analysis of Sox9 expression in ATDC5 cells transfected with other AP-1 subunit siRNA and treated with ST. **(F)** Immunofluorescence analysis of Sox9 expression in ATDC5 cells. Data are presented as means ± SD from one representative experiment of three independent experiments performed in triplicate. **p < 0.01 compared between groups.

potential therapeutic agent for CPP patients undergoing GnRHa treatment.

In the current study, we made several critical observations that provide insights into the ST-induced chondrogenic differentiation and promotion of growth plate development. Firstly, we found that the prevention of growth plate development and growth deceleration were found in rats given a single GnRHa treatment and that ST abolished the side effects of GnRHa on growth plate development (**Figure 1**). Our previous studies report that ST activates ERα through the MAPK/ERK signaling pathway to promote the proliferation of growth plate chondrocytes (15). Several previous studies of ST have mainly focused on cartilage regeneration in osteoarthritis (OA). Castro et al. report that ST has chondroprotective effects in OA (28). In the latter study, ST treatment reduced the expression of several pro-inflammatory cytokines (MMP1, IL-6, and COX-2) in both normal and inflammatory chondrocytes to prevent the degeneration of cartilage. Spadari et al. also report that ST intra-articular treatment reduces osteophyte formation and subchondral bone reaction and promotes articular cartilage regeneration in OA (30). An important and interesting finding of our *in vitro* experiment that has not been described previously was that ST promoted chondrogenic differentiation and even suppressed the inhibitory effects of GnRHa to promote chondrogenic differentiation (**Figure 2**).

Further, we found that the chondrogenic effect of ST in chondrocytes took place through activation of MAPK/JNK and up-regulation of the expression of Sox9. ST has been reported to induce the activation of MAPK/ERK in other systems (15). In chondrogenic differentiation, the downstream signaling of ST is still unclear. In the current study, we showed that ST promoted chondrogenic differentiation in ATDC5 cells through up-regulating Sox9 gene expression (**Figure 3**). Furthermore, we confirmed that JNK regulated Sox9 expression through regulating the transcriptional factor c-Jun (AP-1 subunit). To our knowledge, this is the first reported description of a ST-JNK/c-Jun-Sox9 signaling cascade in chondrocytes (**Figure 4**). Sox9 is known to be the critical transcriptional factor in growth plate development and chondrogenic differentiation (31, 32).

In our rat model, we found that ST rescued the inhibitory effects of GnRHa to promote growth plate development (**Figure 1**). With the single GnRHa treatment, the activation of JNK was suppressed and the expression of Sox9 was decreased in growth plate chondrocytes. However, with the GnRHa/ST combined treatment, activation of the JNK/c-Jun-Sox9 signaling cascade was rescued in growth plate chondrocytes. This is the first evidence that the JNK/Sox9 signaling cascade is critical for ST-induced growth plate development (**Figure 5**).

There are several limitations to the current study. First, the mechanisms involved in preventing growth plate development with GnRHa and suppressing the inhibitory effect of GnRHa through ST are still unclear. Second, other pathways such Smad1/4 also are critical to chondrogenic differentiation, whether these pathways are involved in chondrogenic effect of ST is needed to further confirm. Third, an animal model established under the background of chondrocyte-specific ablation of ST relative downstream molecules (JNK, c-Jun, Sox9 etc.) would be the best method to specifically verify the effect of ST on growth plate development. Therefore, transgenic animal models should be established to provide more convincing evidence. In addition, clinical data from patients with CPP is also important.

CONCLUSIONS

We demonstrated that ST has a significant chondro-inductive effect and promotes growth plate development through its impact on the JNK/c-Jun/Sox9 signaling pathway. In addition, ST is a potential agent for use in CPP patients treated with

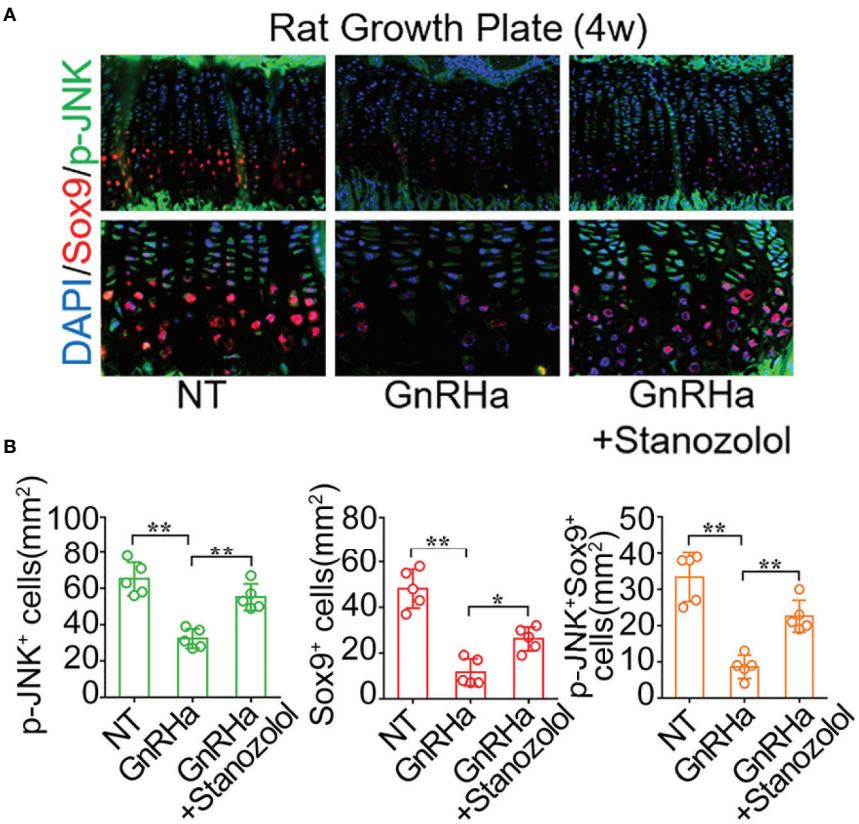

FIGURE 5 | Stanozolol promotes chondrogenic differentiation and growth plate development through the JNK/Sox9 signaling pathway *in vivo*.
(A) Immunofluorescence analysis of growth plates in rats undergoing GnRHa and ST treatments. **(B)** Quantitative analysis of p-JNK$^+$, Sox9$^+$, and p-JNK$^+$ Sox9$^+$ cell number in the growth plates of rats. Data shown as mean ± SD. n = 5. *p < 0.05 compared between groups; **p < 0.01 compared between groups.

GnRHa. Our novel findings may shed light on the mechanism of ST promotion of growth plate development and assist with its clinical application.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Medical Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University.

AUTHOR CONTRIBUTIONS

SZ and LL contributed equally to this work. Study design: SZ and ZY. Conduction of the study: LL and YT. Data collection: LL, YT, YH, and YL. Data analysis: SZ, LL, and ZY. Data interpretation: YH, YL, and ZY. Drafted the manuscript: SZ and LL. Revised the manuscript content: SZ and LL. Approved the final version of the manuscript: SZ and ZY. All authors take responsibility for the integrity of the data analysis. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Phalangeal Intra-Articular Osteochondroma Caused a Rare Clinodactyly Deformity in Children: Case Series and Literature Review

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Background: Various factors are discovered in the development of clinodactyly. The purpose of this retrospective study was to present a group of children with a rare clinodactyly deformity caused by phalangeal intra-articular osteochondroma and evaluate the efficacy of various treatment methods.

Methods: All child patients that were treated for finger problems in our center between Jan 2017 and Dec 2020 were reviewed. A detailed analysis was made of the diagnosis and treatment methods in eight rare cases. X-rays and histopathology were applied.

Results: A preliminary analysis of 405 patients in total was performed, and we included eight cases in our final analysis. This cohort consisted of 2 girls and 6 boys, with a mean age of 5.74 ± 3.22 years (range: 2y5m to 11y). Overall, four patients had their right hand affected and four patients had their left hand affected. One patient was diagnosed as having hereditary multiple osteochondroma (HMO) while the other seven patients were all grouped into solitary osteochondroma. Osteochondroma was proven in all of them by histopathology examination. Preoperative X-rays were used to allow identification and surgery planning in all cases. All osteochondromas were intra-articular and in the distal end of the phalanges, which is located opposite the epiphyseal growth area. All of the osteochondromas developed in half side of the phalanges. The angulation in the finger long axis was measured, and resulted in a mean angulation of 34.63 ± 24.93 degree (range: 10.16-88.91 degree). All of them received surgery, resulting in good appearance and fingers straightening. No recurrence was recorded.

Conclusions: This retrospective analysis indicates that 10 degrees can be selected as the angulation level for diagnosis of clinodactyly deformities. What's more important, the abnormal mass proven by X-rays should be included as the classical direct sign for diagnosis. The first choice of treatment is surgery in symptomatic osteochondromas.

Keywords: clinodactyly deformity, children, treatment, angulation, osteochondroma

INTRODUCTION

Clinodactyly is defined as a congenital curvature of a digit caused by an interphalangeal joint in the coronal plane (1). This curvature was generated by malalignment of the interphalangeal joint which was due to an abnormal trapezoidal or triangular shape of one or more phalanges (2–4). The visible curvature of the digit arises due to the abnormal shape that leads to asymmetric longitudinal growth in a direction from the original longitudinal axis of the finger. The fifth finger is the most frequently affected digit, but other fingers can be involved as well (5). Most cases are bilateral in this condition. However, the curvature angulation was reported quite differently in several pieces of research. Smith defined clinodactyly as an angulation deformity of the finger greater than 8° along the axis of phalanges (6). But some researchers argued that an angulation of less than 10° can also be normal, whereas others suggest that an angulation of greater than 15° should be abnormal (7). Samantha L. Piper reviewed thirteen digits in nine patients, and reported outcomes of opening wedge osteotomy to correct angular deformity in small finger clinodactyly, which showed all digits had greater than 20° of preoperative clinical angulation (mean 36°). But most of them agreed on curvatures with the coronal angulation of the affected digit greater than 10 degrees as a definition of clinodactyly.

Osteochondroma, one of the most common benign bone tumors, frequently occurs in the metaphysis of the long bones (8–10). According to the WHO's data, it is detectable in 35% of benign bone tumors and 8% of all surgically removed bone tumors. Most clinodactyly deformities were developed by congenital deformity and delta phalanges, however, fewer cases were reported by osteochondromas (11–13).

The aim of treatment for clinodactyly is to improve aesthetics and function. Surgery is recommended if the deformity is severe and progressive. Clinodactyly affects the little finger in most cases. Most people can tolerate the deformity and work well without functional limitation. However, some specific activities can be hard for them to finish well, such as playing musical instruments, especially when the deformity is progressive. Physical therapy or surgery will be suggested for different patients with different conditions. A wedge osteotomy can correct the coronal deformity (5). In many reports, they can achieve good recovery and prognosis after receiving surgery, such as the opening wedge osteotomy. Complications are rarely seen, but we also noted the stiffness of the distal interphalangeal joint (14, 15). Here we reported a case series of rare clinodactyly deformity in children that was caused by solitary phalangeal intra-articular osteochondroma and provide our solution methods.

MATERIALS AND METHODS

A carefully review of 405 child patients who were treated for finger problems in our center (Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology) between Jan 2017 and Dec 2020 was finished. Overall, 63.9% children cases underwent surgery for finger deformity, 24.5% of them underwent surgery for trauma, 6.4% of them underwent surgery for tumor, 5.2% of them underwent surgery for infection, while only eight child patients underwent surgery for phalangeal intra-articular osteochondroma. Therefore, only eight pediatric patients were brought into this retrospective study. Eight cases were reviewed for diagnosis of pediatric phalangeal intra-articular osteochondroma that caused a rare clinodactyly deformity.

This case series included 2 girls and 6 boys, ranked with a mean age of 5.74 ± 3.22 years (range: 2y5m to 11y). Overall, four cases had the left arm affected and four cases had the right arm affected. Patient demographics and details of the surgery treatment methods were obtained from the electronic medical records system (**Table 1**). One patient was diagnosed as having hereditary multiple osteochondroma (HMO) while the other seven patients were all grouped into solitary osteochondroma. Preoperative X-ray scans were done to make identification and surgery planning before operation for all children. We can evaluate the accurate angulation along the finger long axis between the proximal and middle phalanges using pre-operation X-rays. All fingers with angular deformities were requested for surgery.

TABLE 1 | Clinical materials and treatment methods for 8 patients.

No.	Sex	Age	Side	Finger	Joint	Phalange	Orientation	Mass location	Angle	Range of motion	K-wire fixation	Appearance	Recurrence	Joint stiffness
1	Boy	8y1m	Right	Second	DIP	Middle	Radial	Ulnar half side	42.55	Severe limitation	Yes	Straight	No	No
2	Boy	3y3m	Right	Second	PIP	Proximal	Ulnar	Radial half side	25.96	Limitation of externsion	Yes	Straight	No	No
3	Boy	9y1m	Right	Forth	PIP	Proximal	Ulnar	Radial half side	15.43	No limitation	Yes	Straight	No	No
4	Boy	2y10m	Left	Third	PIP	Proximal	Radial	Ulnar half side	41.79	No limitation	Yes	Straight	No	No
5	Girl	2y5m	Left	Forth	PIP	Proximal	Radial	Ulnar half side	88.91	Limitation of externsion	Yes	Straight	No	No
6	Girl	4y6m	Left	Third	PIP	Proximal	Radial	Ulnar half side	10.16	No limitation	No	Straight	No	No
7	Boy	4y9m	Right	Forth	PIP	Proximal	Ulnar	Radial half side	19.01	No limitation	Yes	Straight	No	No
8	Boy	11y	Left	Forth	PIP	Proximal	Ulnar	Radial half side	33.23	Limitation of externsion	Yes	Straight	No	No

Hereditary multiple exostosis is a bony dysplasia in which osteochondromas affect multiple long and flat bones. Just like osteochondromas, they are usually defined as masses occurring in the metaphyseal region and adjacent to the growth plate. However, unlike those traditional osteochondromas, we reported those eight special cases of intra-articular osteochondromas located at the distal end of the phalanges opposite the epiphyseal growth area. The fixation method for all cases but one fingers was a single 0.039-inch Kirschner wire (K-wire). Those eight patients were fixed for 4 to 6 weeks (generally 5 weeks), after which the K-wire was removed to allow rehabilitation training. The study obtained ethical approval from the Review Board of Tongji Hospital ethical committee, and all patients gave written informed consent. This was a retrospective case-control study.

RESULTS

The fourth finger was the most frequently affected finger with four patients, followed by the third and second fingers with two patients each. The proximal phalanx was the most frequently involved (seven patients), and the osteochondroma affected the proximal interphalangeal joint (PIP) in seven cases. Only in one patient was the middle phalanx affected and the distal interphalangeal joint (DIP) was involved. All osteochondromas were intra-articular and in the distal end of the phalanges opposite the epiphyseal growth area. All of the osteochondromas developed in half side of the phalanges. Four patients developed in the ulnar side and the other four patients developed in the radial side. Thus, four fingers oriented to the radial side and four of them oriented to the ulnar side. The angulation in the finger long axis was measured, resulting in a mean angulation of 34.63 ± 24.93 degree (range: 10.16-88.91 degree). All of them received operation, which resulted in good appearance and fingers straightening. No recurrence was recorded.

All of them were proven to be osteochondroma by histopathology examination. According to what was discovered in the surgery, we applied different operations for each patient. One patient received a surgery without K-wire fixation since the finger was automatically straightened after mass resection. The other seven patients were all fixated by K-wire to stretch the finger. The joint was opened in all patients while ligament reconstructions were not carried out.

Case 1 Mass Resection With K-Wire Immobilization in a Solitary Osteochondroma

A 2-year-10-month old boy presented to the pediatric orthopedic surgeon with complaints of clinodactyly deformity in his third left finger with a duration of over 2 years. There was no complaint of pain and extension motion. He had no history of symptoms of infectious diseases such as fever or significant trauma. A hard mass could be also felt near the proximal interphalangeal joint. No tenderness or pain was present, and there was also no restricted movement range. Whether for the plantar flexion or dorsiflexion, his finger's function is normal comparing with the right hand. We had no evidence of any signs of inflammation to relate to secondary deformity when analyzing the normal skin. There was no familiar history. The patient was sent to the radiologist for X-ray evaluation (**Figures 1A, B**). Anteroposterior radiographs of his hands showed of an osseous outgrowth mass from the ulnar aspect of the distal end of the proximal phalange opposite the epiphysis plate (**Figures 1A, B**). The clinical manifestation of the finger and characteristic radiographic images established the diagnosis of clinodactyly deformity. Because the patient had presented with swvere deformity, surgical treatment was considered (**Figures 1C–E**). An immediate and significant improvement was seen on X-ray, demonstrated postoperatively (**Figures 1F, G**). The physical examination showed a bending finger which twisted toward the radial side (**Figure 1H**). A dorsal approach was preferred and the proximal interphalangeal joint capsule was opened (**Figure 1I**). The abnormal outgrowth mass was excised, and an attempt to maintain finger straightening by immobilization of a K-wire was made. An immediate and significant improvement was seen on appearance, demonstrated perioperatively and postoperatively (**Figure 1J**). The pathology showed osteochondroma (**Figures 2A, B**).

Case 2 Mass Resection Without K-Wire Immobilization in a Solitary Osteochondroma

Another patient was female, aged 4 years and 6 months. No pain at the joint was present. Function was normal. The patient had no other history of diseases associated with musculoskeletal or neurological abnormal problems. An accessory growth protrusion in the distal end of the proximal third phalanx was proven by radiographys (**Figure 3A**). The lesion was located on the ulnar side of the phalanx and was resected through a surgery. K-wire was not applied in this surgery (**Figures 3B, C**). At 4 years of age, the mass was discovered on the third finger of her left hand and an axis line deflection was denoted (**Figure 3D**). After surgery, a histopathology confirmed the osteochondroma (**Figures 3E, F**). Her finger points with preservation of a correct axis with full mobility. Follow-up revealed a good prognosis.

Case 3 Mass Resection With K-Wire Immobilization in a HMO

An 11-year-old boy presented to our hospital with progressive bowing of his left fourth finger. He had no history of trauma. We detected no deficit in strength for his finger. On physical examination, there was a palpable mass located intra-articular. Plain radiography of left hand showed a mass-like tumor arising from the dotsalradio portion of the head of the fourth proximal phalanx (**Figure 4A**). We also found several osteochondromas located at the metaphysis of radius and ulnar bones (**Figure 4A**). What's more, multiple osteochondromas were discovered around his limbs. Intraarticular osteochondroma located in the intraarticular portion of proximal phalanx was identified during surgery. The tumor was completely excised, and a K-wire was implanted to make the finger straight (**Figure 4B**). On physical

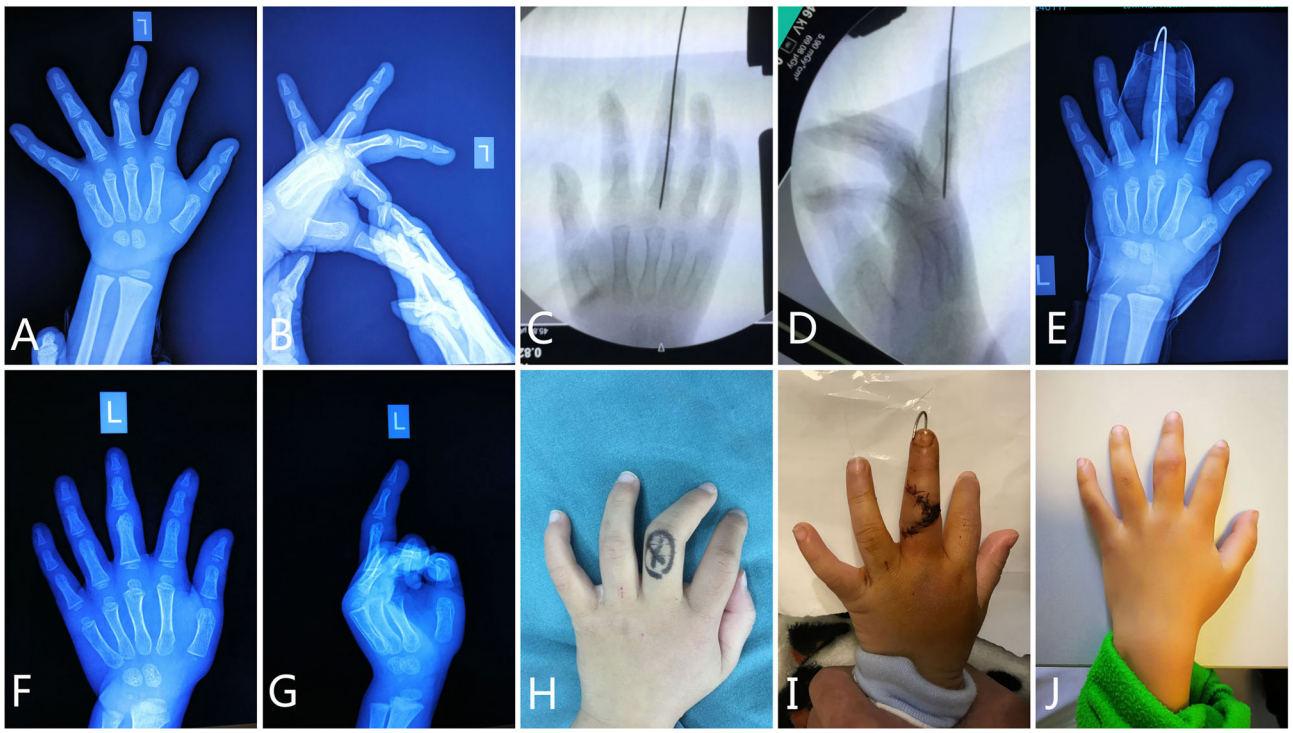

FIGURE 1 | Diagnosis and treatment of clinodactyly deformity in a 2-year-10-month-old boy. The radiograph on presentation showed an outgrowth around the distal end of the third proximal phalanx **(A, B)**. During the surgery, the abnormal mass was resected and a K-wire was implanted to keep the finger straight **(C, D)**. After operation, the radiographs showed good prognosis **(E–G)**. Clinical examination established the diagnosis of clinodactyly deformity of his third finger **(H)**, and also showed essentially normal movement of the upper extremity at the follow-up **(I, J)**.

examination, extension was limited to 30° compared to the other side (**Figure 4C**). A general histopathology confirmed the diagnosis of osteochondroma after surgery (**Figure 4D**). The lesion had a cartilage cap without signs of necrosis. No atypia or binucleate chondrocytes were found under the microscope by histologic examination. After surgery, the patient had an uneventful recovery with normal function of the joint.

DISCUSSION

Clinodactyly is a common congenital hand abnormality defined as finger deviation in the coronal plane; it affects the fifth finger most frequently. Clinodactyly occurs in approximately 1% of normal newborns, but incidence of clinodactyly has been reported to be as high as 5% in the Japanese population (16).

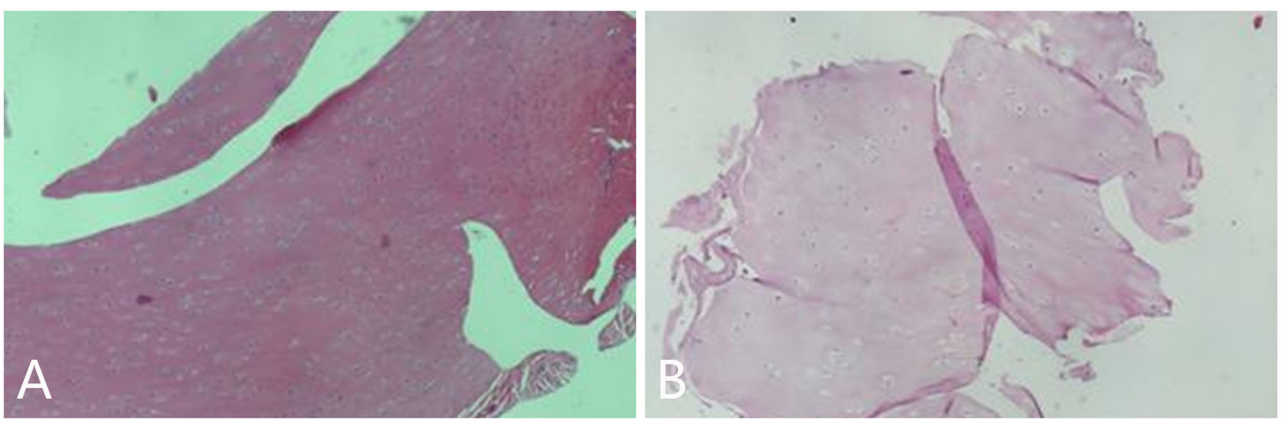

FIGURE 2 | The pathology examination of the resected mass showed cartilage map **(A, B)**.

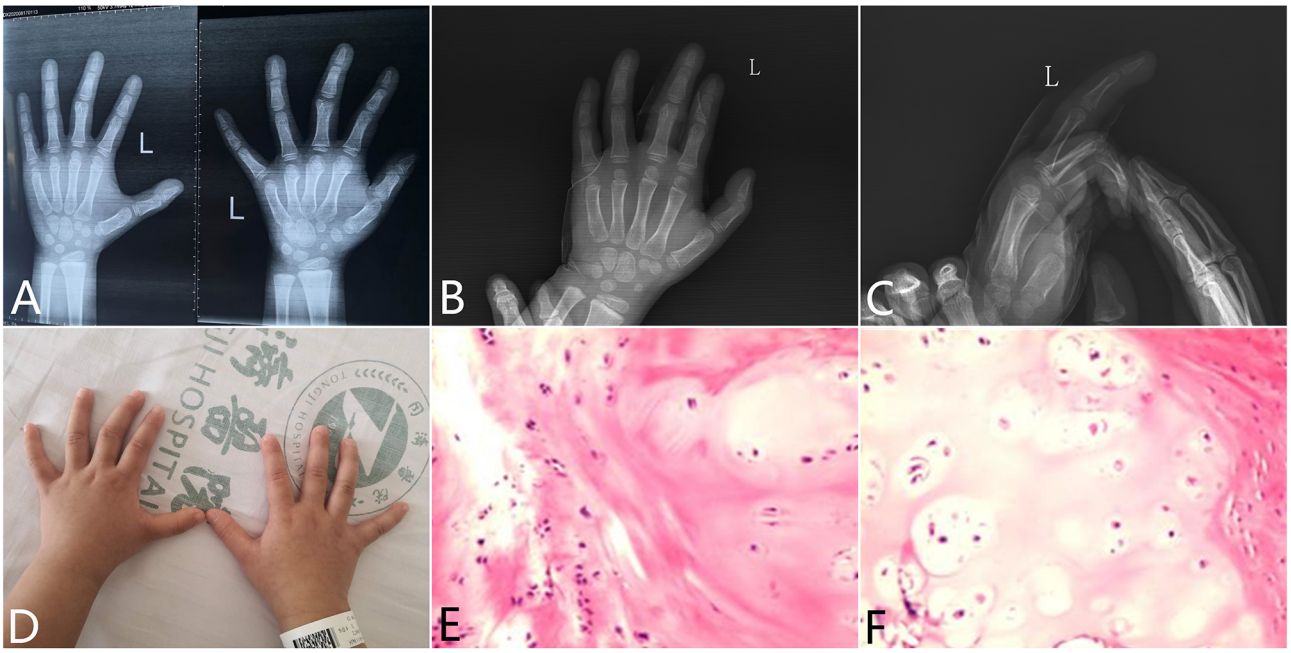

FIGURE 3 | Diagnosis and treatment of clinodactyly deformity in a 4-year-6-month-old girl. X-ray radiography showed a mass protruded into the PIP joint which made the axis declined **(A)**. During the surgery, the abnormal mass was resected without K-wire implantation **(B, C)**. Clinical examination established the diagnosis of clinodactyly deformity of her third finger **(D)**. Pathology examination proved to be osteochondroma **(E, F)**.

Some clinodactyly cases were classified as brachydactyly for its shortening change of the involved finger (17). There are many clinical features characteristic of abnormal phalanx; the most common are large, rounded phalangeal blocks as reported in Cenanie-Lenz syndrome, delta phalanges combined with a C-shaped epiphysis, and trapezoidal phalanges with a double epiphysis (1, 18–21). It is difficult to make an early differential diagnosis between these abnormal phalanges, but it is important for those patients to receive appropriate treatment. Clinodactyly in many pediatric patients is a problem of unbalanced longitudinal growth from a bracketed epiphysis. There is huge debate over which type of surgical intervention to choose and when to operate. It is a little different from our cases which were caused by phalangeal intra-articular osteochondroma prominence. Appearance but not functional impairment is the major problem that we often meet. Many studies approved an angular deviation of fewer than 10 degrees as normal variant. Therefore, clinodactyly is defined as the coronal deviation of the affected digit greater than 10 degrees. We should avoid early inappropriate surgery before correct diagnosis. However, surgery was suggested in those cases with deviation over 20 degrees and associated with shortening of the phalange (22). Clinodactyly is mistaken for many finger diseases that cause a curved finger. Clinodactyly is also found in a large group of congenital abnormalities and presented as one single sign. Therefore, it is a special group of clinical sign, and this curvature may be found at birth, may develop gradually, or may be secondary to a trauma or infection diseases. If the phalangeal epiphyses were destroyed by trauma or inflammation, the growth may be disturbed and may lead to angulation deformity. This growth disorder may also be caused by congenital reasons such as an abnormally shaped phalanx (23).

Osteochondroma, one of the most common benign bone tumors, frequently occurs in the metaphysis of the long bones (8). Osteochondroma is called a benign cartilage-forming tumor and arises from an aberrant subperiosteal cartilage. Multiple osteochondromas syndrome (MOS) is an autosomal dominant disease which has mutations in the EXT (EXT1 or EXT2) genes (9, 10). Osteochondroma is one of the most common benign bone tumors. According to the WHO data, it is detectable in 35% of benign bone tumors and 8% of all surgically removed bone tumors (24). Most clinodactyly deformities were developed by congenital deformity and delta phalanges, however, fewer cases were reported by osteochondromas (11–13, 25). In Goo Hyun Baek's report (13), only 7 of 10 patients were children aged below 12 years, and four patients presented without coronal deformity. Therefore, it is different from our cohort who all have evident coronary angulation over 10 degrees. Here we reported a special kind of clinodactyly deformity that is caused by intra-articular osteochondromas, including solitary intra-articular osteochondroma and hereditary multiple osteochondroma.

Through our case series, it was seen that boys developed this disease more frequently than girls. And this disease mainly affected the fourth finger, but can also develop on the second and third finger. The main complaint was bending of the finger. In this rare kind of clinodactyly deformity, angulation in the long finger axis between the two different phalanges ranked from 10.16 degree to 88.91 degrees, according to X-rays. Thus, we

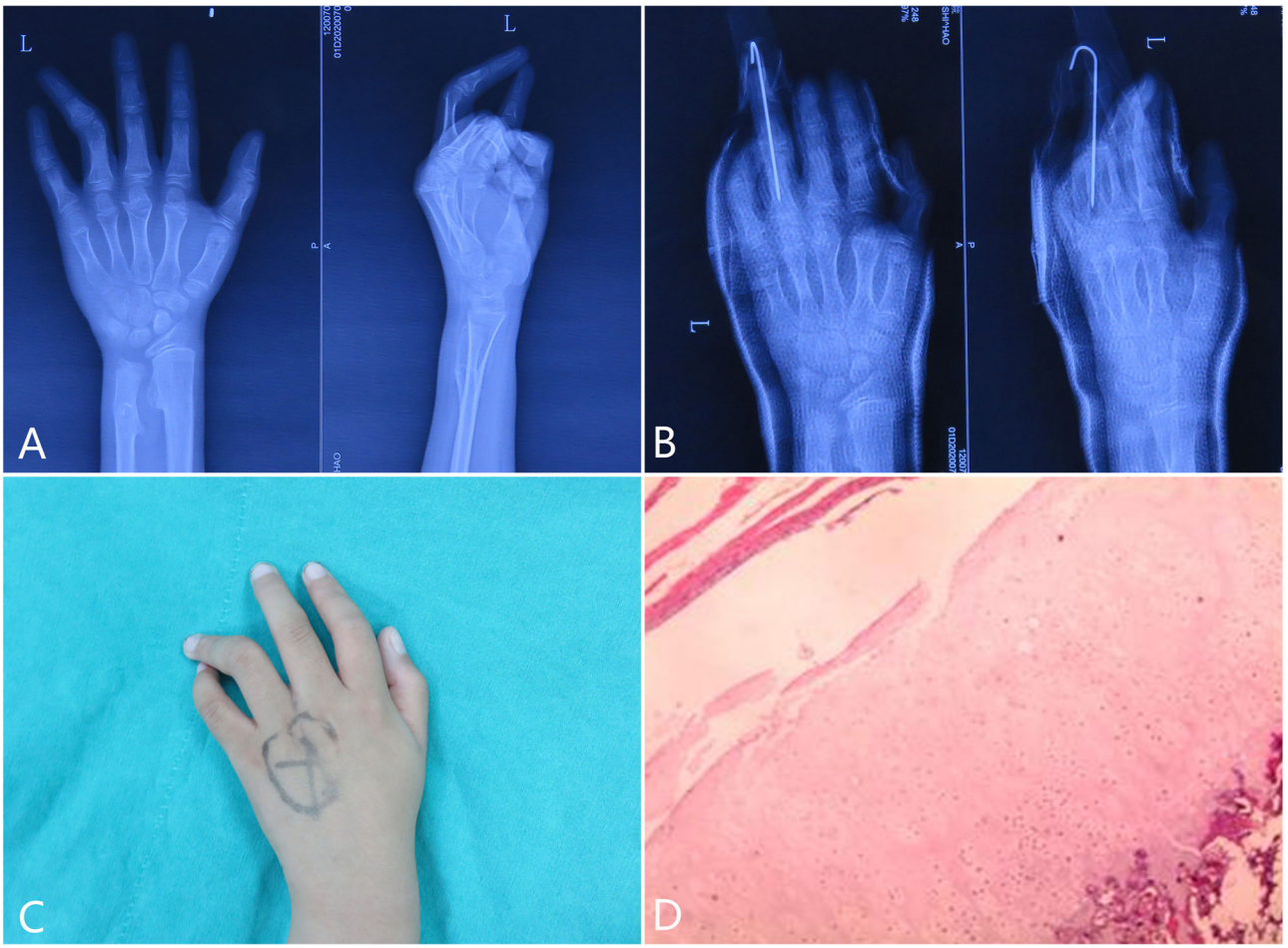

FIGURE 4 | Diagnosis and treatment of clinodactyly deformity in an 11-year-old boy. The radiograph on presentation showed an outgrowth around the distal end of the fourth proximal phalanx **(A)**. During the surgery, the abnormal mass was resected and a K-wire was implanted to keep the finger straight **(B)**. Clinical examination established the diagnosis of clinodactyly deformity of his third finger **(C)**. Pathology examination proved to be osteochondroma **(D)**.

suggested 10 degrees can be selected as the angulation level of such a group of clinodactyly deformity. What's more important, the abnormal mass proven by X-rays should be included as the classical direct sign for diagnosis. We can easily find out that the intra-articular mass protruded into the joint lead to angulation of the finger, which will worsen as the mass grows. Histopathology examination proved this mass to be diagnosed as intra-articular osteochondroma. We have found a cartilage cap with enchondral bone formation beneath the cartilaginous cap through HE staining. Dysplasia epiphysealis hemimelica (DEH), or Trevor's disease, is a relatively rare disorder for asymmetric epiphyseal cartilage overgrowth or an accessory epiphyseal ossification center (11). But it was initially reported as a kind of foot disorder (tarsomegalia). However, we are unable to diagnosis this special disease as Trevor's disease since there is no epiphysis at the distal end of phalanges.

In Goo Hyun Baek's report (13), two patients had been neglected for a long time. These two patients had relatively poor prognosis due to the development of finger osteoarthritis, residual deformity, and permanent limitation of finger motion. Thus we should be aware of neglected intra-articular phalangeal osteochondroma which will cause progressive deformity and limitation of motion. Although Vickers's physiolysis and a variety of wedge osteotomy are both common treatment options for clinodactyly, there are various forms of complications including infection, joint stiffness, nonunion of osteotomy, and disease recurrence (26). In our case series, the osseous mass was excised in seven cases, including six cases of solitary intra-articular osteochondroma and one case of hereditary multiple osteochondroma. Temporary fixation of the distal interphalangeal joint by a Kirschner wire was used in these patients. However, we also only resected this mass in one case of solitary intra-articular osteochondroma without Kirschner wire fixation since the clinodactyly reduced spontaneously. No ligamentary adjustment seemed necessary since the treatment was early and there was little destruction of those ligaments. Kirschner wire fixation was applied to correct the clinodactyly. Temporary fixation will not cause joint stiffness.

However, ligamentary adjustment may result in joint motion limitation. But the surgeons should carefully make the surgery plan and accurately define the resection margin. The follow-up was uneventful. No recurrence was found in follow-up. All fingers developed well in a normal axis combined with full ranges of motion. Our findings suggest an early surgical approach is appropriate in these definite cases.

In summary, phalangeal intra-articular osteochondroma caused a rare clinodactyly deformity in children in our report. Only several cases were discovered. The first choice of treatment is surgery. Skeletal deformity will progress throughout childhood, which will cause finger malfunction and even joint degeneration. However, a longtime follow up is still absent for this surgery. We should pay specific attention to the destruction of some articular cartilage when mass is removed. Thus we need to distinguish the osteochondroma cartilage from normal articular cartilage carefully. Furthermore, the limitations of the present study include its retrospective nature, a relatively small study group, limited follow-up in some cases, and the lack of a control group.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Review Board of Tongji Hospital ethical committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

J-PH and YH performed the research and analyzed the data. X-LW, C-JG, J-FS and J-XF analyzed the data. J-PH and J-FS designed the study. YH, J-PH and J-FS supervised the study. YH and J-PH wrote the paper. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Case Report: Low Bone and Normal Lean Mass in Adolescents With Complete Androgen Insensitivity Syndrome

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Introduction: Osteopenia and osteoporosis have been reported in adults with Complete Androgen Insensitivity Syndrome (CAIS). Little is known about changes in bone mineral density (BMD) in adolescents with CAIS and whether it is affected by early gonadectomy. Body composition data have not been reported.

Methods: Single-center, retrospective study of CAIS adolescents who underwent dual-energy x-ray absorptiometry (DXA) (Hologic, Horizon A). Body composition is presented as lean and fat mass indices (LMI, FMI). Z-scores for lumbar spine areal BMD (LBMD), total body less head (TBLH), bone mineral content (BMC), LMI, and FMI were calculated using female normative data. Results are expressed as median and min, max.

Results: Six females with genetically confirmed CAIS were identified—one with intact gonads and five with history of gonadectomy at 2–11 months. In the subject with intact gonads, LBMD-Z and TBLH BMC-Z were –1.56 and –1.26, respectively, at age 16 years. Among those with gonadectomy, LBMD-Z was –1.8 (–3.59 to 0.49) at age 15.6 years (12–16.8) and decreased in all three subjects who had longitudinal follow-up despite hormone replacement therapy (HRT). Adherence to HRT was intermittent. LMI-Z and FMI-Z were 0.1 (–1.39 to 0.7) and 1.0 (0.22 to 1.49), respectively.

Conclusions: These limited data indicate that adolescents with CAIS have bone mass deficit. Further studies are needed to understand the extent of BMD abnormalities and the effect of gonadectomy, especially early in childhood, and to establish the optimal HRT regimen for bone accrual. Data on lean mass are reassuring.

Keywords: bone mineral density, complete androgen insensitivity syndrome, gonadectomy, lean mass, osteoporosis

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INTRODUCTION

Complete androgen insensitivity syndrome (CAIS) (OMIM# 300068) is an X-linked recessive disorder characterized by mutations of the androgen receptor (AR) that render the receptor completely non-functional (1, 2) and occurs in 1:20,000 to 90,000 neonates (2). In the fetus affected by CAIS, the fully functional SRY protein on the Y chromosome permits male gonad development,

normal secretion of testosterone and anti-Mullerian hormone, and ultimately regression of the Mullerian structures destined to be the uterus and fallopian tubes (2). However, the fetus cannot properly respond to androgens (3) and virilization of external genitalia is absent (4). Male gonads remain intact with retention of testis in the abdomen or inguinal area (5). Thus, while CAIS individuals are genetically XY, they appear phenotypically female and are usually raised and legally recognized as female (1).

Treatment for CAIS is multifactorial and includes vaginal enlargement, genetic counseling, and psychosocial support. Gonadectomy for prevention of germ cell tumor development and hormone replacement therapy (HRT), typically estrogen, are usually recommended although the timing of gonadectomy is controversial (6). Because testosterone is metabolized into 5a-dihydrotestosterone (5-DHT) *via* 5a-reductase-2 or into 17B-estradiol *via* P450 aromatase, adolescents with CAIS and intact gonads are able to achieve the expected effects of puberty including typical breast development, widening of the hips, redistribution of fat, growth acceleration, and bone accrual *via* 17B-estradiol, while they lack axillary and pubic hair development (6).

Testosterone and estrogen play critical roles in bone mineral accrual during adolescence and young adulthood, a life stage during which peak bone mass is achieved and risk of osteoporosis and fracture at least partly established (7). To date, limited data explore changes in bone mineral density (BMD) in CAIS adolescents (8–10). Instead, most publications focus on bone health in adult CAIS women (11–20). Understanding the effects of early *vs.* late gonadectomy, the impact of hypogonadism, and its treatment during adolescence is important as peak bone mass and accrual occur during this time (7). Furthermore, body composition can influence both skeletal health and cardiometabolic risk factors (21–23), and particularly relevant in CAIS is the impact of hypogonadism and gonadal hormones on lean mass and fat distribution (24–27). To better understand such potential alterations in adolescents with CAIS, we describe BMD, body composition, and changes in these outcomes in a series of patients treated with HRT.

METHODS

We performed a retrospective cohort study of all CAIS females (age <20 years) who had follow-up by the Division of Endocrinology at our institution between July 1, 2015, and December 30, 2020, and underwent bone mass (lumbar area and whole body) and body composition measurements by dual-energy x-ray absorptiometry (DXA) (Hologic, Horizon A). Data extracted from medical charts included age, height, weight, body mass index [BMI], tanner stage, presence of gonads, age at gonadectomy, medications including type of HRT and age at treatment initiation, comorbidities, and laboratory or radiographic evaluation (serum estradiol concentrations, DXA, bone age results). Data collection was approved by the Institutional Review Board.

Z-scores for lumbar spine areal BMD (LBMD) and total body less head (TBLH) bone mineral content (BMC) were calculated using female normative data. Body composition results are presented as lean body mass and fat mass indices (LMI, FMI), which were calculated as kg of lean or fat mass respectively/m^2. Height, weight, body mass index (BMI), LMI, and FMI Z-scores were calculated using female normative data (28). Overweight and obesity were defined using the Centers for Disease Control (CDC) classification (29). Height and weight Z-scores were calculated using the CDC growth charts. Results are expressed as median and range (i.e., min-max). Z-scores within 1SD from population mean are considered normal.

Subjects

We identified six subjects with CAIS—one had intact gonads and five had history of gonadectomy early in life. Gonadectomy was performed between 2 and 11 months (median age 5.6 months). HRT was initiated at an age of 12 years (range 10.5–14.2) using estradiol as either a transdermal patch or oral pill. HRT was started at low doses but increased to achieve appropriate growth and acquisition of secondary sexual characteristics with a max of 0.1 mg/day (patch), 1 mg orally once daily (Estrace), or 0.9 mg orally once daily (Premarin) (**Table 1**). Induction of puberty was late in Subjects 4 and 5 because families had not fully disclosed the diagnosis and patients were lost to follow-up after gonadectomy before eventually returning to care. Compliance with HRT ranged from poor to good as evidenced by serum estradiol levels (**Figure 1**), which were obtained in those treated with transdermal estrogen for clinical surveillance. Height, weight, and BMI Z-scores are presented in **Table 1**. One subject (patient 2) was obese, and subjects 4 and 5 were overweight. Subjects 1, 3, and 6 had reached adult height at the time of the first DXA. The rest of the subjects were still growing when the first DXA was performed. Subject 2 had a growth rate of 5 cm/year and a bone age that was concomitant to chronologic age (bone age 12 years, chronologic age 12 years 1 month). Subject 4 had reached a near-adult height growing at 2.5 cm/year. Her bone age was 14 years at the chronologic age of 16 years 6 months. Subject 5 was growing at a rate of 7.2 cm/year. Her bone age was 12 years at the chronologic age of 13 years 8 months. Subjects 1, 2, 3, and 5 received vitamin D supplementation to optimize bone health. Subjects 1–3 achieved serum 25-OH-vitamin D concentrations >30 ng/ml. The 25OHD has not yet been obtained for subject 5. Subjects 4 and 6 have not started vitamin D. Information on calcium intake is not available.

DXA Results

Subjects With History of Gonadectomy

DXA scans were obtained at a median age of 15.6 years (range 12–16.8) after receiving HRT for a period of 2.5 years (range 0.8–4.6). Patients 1–3 had serial DXAs over time to assess response to HRT.

Median age-adjusted lumbar areal BMD-Z (LBMD-Z) was −1.8 (range −3.59 to 0.49) at the initial screen (**Table 1**) with two subjects (Cases 1, 5) having Z-scores < −2. The data from longitudinal follow-up are presented in **Figure 1** and show a decline in LBMD-Z over 2.8 years (range 1.7–3) of observation. Total body less head (TBLH) BMC was less affected at baseline (median Z = −0.46, range −2.39 to 0.26, **Table 1**); results varied during follow-up with subject 1 showing mild improvement. LMI-Z was normal in all subjects except 1 (Subject 1) with median LMI-Z +0.1 (range −1.39 to 0.7);

TABLE 1 | Baseline characteristics, type/timing of HRT, and results of first DXA scans for all six cases.

Subjects	Age at GND (mo)	Age HRT started (yr, mo)	Age 1st DXA (yr, mo)	Duration HRT at time of 1st DXA (yr, mo)	Weight Z	Height Z	BMI Z	Lumbar BMD Z	TBLH BMC Z	LBMI Z	FMI Z	HRT	Compliance
1	2	10, 11	15, 6	4, 7	+0.04	-0.12[A]	+0.1	-3.59	-2.39	-1.39	+0.22	E2 Patch: 14 mcg/24 h for 1 yr → 37.5 mcg/24 h for 1 yr → 50 mcg/24 h for 1 yr → 100 mcg/24 h for 1 yr (current therapy)	Intermittent
2	6	10, 5	12, 1	1, 8	+1.42	-0.03	+1.65	+0.49	+0.26	0.7	+1.03	E2 Patch: 14 mcg/24 h for 1 yr → 37.5 mcg/24 h for 6 mos → 50 mcg/24 h for 6 mos → 75 mcg/24 h for 6 mos → 100 mcg/24 h for 1 yr, 5 mos → Estrace 1 mg po daily (current therapy)	Poor
3*	11	11, 11	16, 0	4, 1	+1.05	+0.98[A]	+0.75	-1.1	-0.46	-0.6	+0.46	E2 Patch: 14 mcg/24 h for 1 yr → 50 mcg/24 h for 8 mos → 75 mcg/24 h for 7 mos → 100 mcg/24 h for 1 yr → stopped all estrogen therapy for 3 mos → Premarin 0.9 mg for 8 mos → E2 Patch: 50 mcg/24 h for 2 yrs → increased to 75 mcg/24 h (current therapy)	Intermittent
4	3	14, 2	16, 8	2, 6	+1.69	-0.17	+1.77	-1.8	-0.44	0.49	+1.49	Estrace: 0.25 mg daily for 3 mos → 0.50 mg daily for 7 mos → 0.75 mg daily for 1.4 yrs → 1.0 mg daily (current therapy)	Good
5	6	12, 10	13, 6	0, 8	+1.39	-0.27	+1.6	-2.65	-1.51	0.1	+1.29	E2 patch: 14 mcg/24 h for 7 mos → 25 mcg/24 h for 5 mos → 37.5 mcg/24 h for 16 mos → 50 mcg/24 h (current therapy)	Intermittent
6	N/A	N/A	16	N/A	-0.34	+0.83[A]	-0.96	-1.52	-1.26	-0.6	-1.52	N/A	N/A

*GND, gonadectomy; HRT, hormone replacement therapy; Z, Z-score; BMD, bone mineral density; TBLH, total body less head; TBLH BMC, total body less head bone mineral content; LBMI, lean body mass index; FMI, fat mass index; MS, Menostar transdermal patch (changed once weekly); VD, Vivelle-Dot transdermal patch (changed twice weekly); CL, Climara transdermal patch (changed once weekly). N/A, not applicable. *Subject 3 has Sickle Cell Anemia (Type SS). [A]Subject has reached adult height.*

results varied during follow-up. FMI-Z was elevated (greater than +1) in patients 2, 4, and 5 and normal in the rest of the subjects.

Subject With Intact Gonads

The adolescent (Case 6) was studied at age 16 years after undergoing spontaneous puberty. LBMD-Z and TBLH BMC Z-scores were −1.52 and −1.26, respectively. LMI-Z and FMI-Z were −0.6 and −1.52, respectively (**Table 1**).

DISCUSSION

Bone density data in children and adolescents with CAIS are sparse. In this report, we assessed BMD and body composition in a series of five adolescents with CAIS who underwent gonadectomy during infancy. We observed low lumbar BMD ($Z < -1$) in all but one adolescent at the time of the first DXA. Longitudinal follow-up in three subjects with intermittent compliance to estrogen replacement showed a decrease in lumbar BMD. Given our small numbers and the observational nature of this report, we cannot reach any firm conclusions about estrogen replacement and bone health in CAIS. However, our data raise concerns about a negative effect of poorly treated hypogonadism on the skeletal health of adolescents with CAIS. In this series, lean mass was grossly normal and fat mass was reflective of BMI. A single additional adolescent with intact gonads and spontaneous puberty also had low lumbar BMD and normal LMI-Z.

CAIS is a disease model that allows us to explore the role of androgens on the skeleton. Bone tissue is sexually dimorphic (30, 31). Estrogens limit periosteal bone expansion yet stimulate endosteal bone apposition in females, while androgens promote radial bone expansion in males (30). Both estrogen and testosterone play important roles in bone phenotype, and their combined effects lead to men generally having wider but not denser skeletons compared to women. Bone size in testicular feminized male rats is intermediate between male and female control animals (32). In pre-pubertal children with CAIS, alterations in bone mass are likely to reflect the role of androgens on the bone.

In adolescents and adults with CAIS, bone mass may be further affected by gonadectomy. Because of the risk for testicular germ cell tumors (GCTs), prophylactic gonadectomy has been recommended in CAIS patients although optimal timing for the procedure remains a topic of debate (6, 33, 34). Gonadectomy early in life at the time of diagnosis has been practiced for years. Since the risk for GCTs is low in children, gonadectomy after completion of spontaneous puberty *via* aromatization of endogenous testosterone has also been proposed by some groups (19, 33). More recently, some women with CAIS elect to defer gonadectomy to benefit from endogenous sex hormone secretion (6, 33). Once gonadectomy is performed, the individual becomes hypogonadal and is placed on estrogen replacement. Relevant to bone health, the ideal HRT regimen for

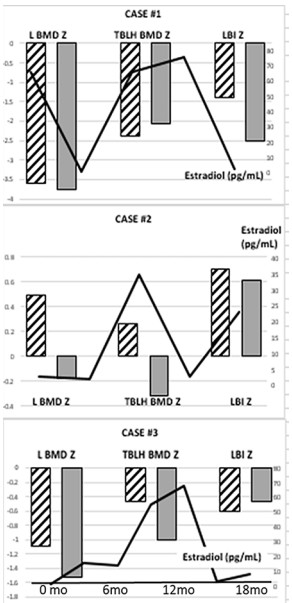

FIGURE 1 | Lumbar (L) and Total Body Less Head (TBLH) BMD Z-scores and LMI-Z in three adolescents with CAIS, history of early gonadectomy, and longitudinal DXA measurements. Hatched columns represent the results of the initial DXA study and gray columns represent the follow-up DXA results. Estradiol levels throughout the time of follow-up are shown as a solid line.

bone accrual and maintenance is not well established. Changes in bone mass in CAIS, therefore, should be interpreted keeping in mind whether gonadectomy has been performed, when such procedure took place, as well as the type of estrogen replacement regimen and patient adherence.

BMD data in CAIS are more recently expressed using female normative data. Whether this approach is appropriate is uncertain since fracture data in CAIS are not available. As BMD serves as a predictor of fractures, understanding bone fragility and relationship with BMD in CAIS may shed light on the most appropriate way to assess bone mass and optimal HRT in this population.

Reduced bone mass has been reported in adults with CAIS (**Table 2**). The information derives from retrospective, descriptive studies over the last decade (11–20). As a common theme, bone mass was affected primarily in the spine with BMD T-scores falling in the osteopenic range for most studies (11–20). Most of the published studies include women who were studied years after gonadectomy (**Table 2**); thus, their DXA results reflect the impact of the disease itself and sex hormone replacement on the skeleton. Poor compliance with HRT has been shown to be associated with worsening BMD in one of these studies (13), while transdermal therapy was found to be superior to oral estradiol in optimizing bone health in a study by Gava et al. (20). Longitudinal follow-up after gonadectomy yielded variable DXA results with estrogen replacement (18, 19). The reason for this diversity in HRT response is not fully understood. Furthermore, the specific estrogen regimens are not clearly detailed in all

TABLE 2 | BMD studies in adults with CAIS.

Reference	Gonadectomy				Intact Gonads			Comments
	N	Age at GND	Age at DXA	Lumbar BMD T	N	Age at DXA	Lumbar BMD	
Soule et al. (10)	4	15.8+12.9	32.7+10.4	-2.6+0.9	1	29	-1.5	
Mizumuma et al. (11)	N/A	N/A	N/A	N/A	2	19 & 28	-0.8 & -3.1	
Marcus et al. (12)	18	13.3 (<1-31)	41.7+9.1	-1.2+1.1	N/A	N/A	N/A	Poor compliance with E2 Rx was associated with greater lumbar BMD deficits. Six pts with CAIS had fractures. Lumbar BMD T in six additional PAIS gonadectomized adults was -0.54 (-1.95-1.3).
Sobel et al. (13)	10	23.4+8.6	35.2+14.3	-2.4+1.0	1	21	-3.0	Lumbar BMD T in six additional PAIS gonadectomized adults was -1.9+0.95.
Danilovic et al. (14)	3	16, 15.2, & 27.6	22, 25, & 24.3	-1.4, -1.65, & 0.4	2	24 & 21	-1.6 & -2.6	
Han et al. (15)	46	15.9+7.3	32.2+10.7	-1.29+1.2	N/A	N/A	N/A	Lumbar BMD was the same as in 18 46XY adults with GD and 25 46XX GD.
Taes et al. (16)	1	15	31	-3.4	N/A	N/A	N/A	After gonadectomy, E2 replacement resulted in a decreased endosteal circumference, increased cortical thickness and area, but unchanged periosteal circumference.
Bertelloni et al. (17)	43	NR	NR	Mean -1.9	10	NR	Mean -0.7	Fracture: n = 1/43 in the gonadectomized group only.
King et al. (18)	104	14.8 (13-16.5)	33.8 (31.4-36.2)	-1.34 (-1.55 to -1.33)	12	25.1* (18.3-52.3)	-1.2 (-4.2-1.0)	
Gava et al. (20)	32, 32 controls	12.3+7.9 (0-24)	34.5+10.4	-1.95+0.94	N/A	N/A	N/A	Transdermal estrogens were associated with better TB BMD. No subject had fractures.

GND, gonadectomy; NR, not reported; T, T-score; TB, total body; GD, gonadal dysgenesis; N/A, Not Available.
**refers to the age of gonadectomy with DXA performed shortly before gonadectomy.*

studies and vary among reports, so identifying an optimal estrogen regimen for bone health in CAIS based on the current literature is challenging.

Maintaining gonads after spontaneous puberty and until later in adult life has been proposed as a strategy to preserve bone health (6, 19). Current studies (**Table 2**) include a small group of subjects, mostly in their 20s and 30s with intact gonads. Although sample sizes are small to draw firm conclusions, collectively the data support a small BMD deficit in the lumbar spine. Comparisons between individuals with and without gonadectomy are provided in two studies; unfortunately, the results are conflicting. Bertelloni et al. observed significantly lower BMD-Z at the lumbar and femoral necks among those who underwent gonadectomy compared to those with intact gonads (18). In contrast, the study by King et al. showed that lumbar BMD-Z were similar in both gonadectomized and non-gonadectomized individuals and did not decrease after gonadectomy (19). Perhaps differences in estrogen replacement can explain the variable outcomes on bone health.

The pediatric experience using DXA to assess bone mass in CAIS is counted to less than 20 subjects (**Table 3**). The larger series of 10 children (seven with gonadectomy and three without) is dated back to 1998 and carries the inherent limitation of lack of large normative data to calculate BMD-Z (9). Nonetheless, in this series lumbar BMD-Z was reduced compared to controls. Three more recent cases of adolescents without gonadectomy observed a lumbar BMD-Z that ranged from −2.9 to +0.9 (10, 11, 14). Collectively, the data in adolescents and women with preserved gonads support some degree of bone mass deficit in CAIS, which can reflect the lack of androgen action on the growing skeleton. It is also possible that the endogenous estrogen concentrations, which are derived from testosterone conversion, are sufficient for breast development and growth but inadequate for optimal bone accrual. A bone mass deficit was also observed in our series. The variability in estrogen dose, regimens, and adherence and differences in body weight and fat mass may have contributed to our results. Given these limitations and the small sample size, we cannot delineate the individual effects of CAIS itself, surgical intervention, and adherence to estrogen therapy. Finally, DXA Z-scores may have been underestimated in our growing subjects with a delayed bone age (35, 36).

TABLE 3 | BMD reports in adolescents (<18 years) with CAIS.

Reference	Gonadectomy				Intact Gonads		
	N	Age at GND	Age at DXA	Lumbar BMD Z	N	Age at DXA	Lumbar BMD
Munoz Torres et al. (7)	N/A	N/A	N/A	N/A	1	17	-4.1
Bertelloni et al. (8)	7; 15 controls	15.4 +1.8	17.7 +2.2	-2.5+0.8	3	4,11 & 16	-2.9+1.1
Marcus et al. (12)	2	<2.5	14 & 11	-0.1 & -1.43	2	14 & 12	+0.0 & +0.9
Sobel et al. (13)	N/A	N/A	N/A	N/A	1	17	-2.9
Chin et al. (9)	N/A	N/A	N/A	N/A	1	15	-0.6

GND, gonadectomy; N/A, Not Available.

Our series provides the first lean mass data using DXA in CAIS. Fat mass has been reported normal or increased in a previous report (20) and was elevated (FMI-Z above 1 SD) in three subjects in this series. Both lean and fat mass were recently linked to bone mass, insulin sensitivity, and cardiometabolic health (21–23). Potential deficits may have an adverse impact in all these health parameters. Testosterone, acting through the androgen receptor, stimulates protein synthesis and hypertrophy of muscle fibers (26), while suppression of endogenous testosterone production in young men results in decreased protein synthesis and muscle strength and in increased adiposity (27). One would predict, therefore, a decrease a lean mass in CAIS. In this series, however, we observed normal lean mass in all but one subject. Lean mass was calculated using female normative data, and whether this is the most appropriate assessment in CAIS is uncertain. Our subject numbers are small, and potential differences in lean mass in CAIS compared to the general female population need to be further explored.

In conclusion, changes in adolescent bone mass and composition in CAIS are largely unexplored, and this case series adds some insight into these processes. Although our results on lean mass are reassuring, our cases raise concerns that bone health is compromised. Given the small number of subjects, we cannot reach any conclusions about the benefits of early *vs.* late gonadectomy on bone health. However, our data do support an adverse effect of poorly treated hypogonadism on BMD. Given the rarity of the syndrome, our data call for multicenter, natural history studies to understand the risk of bone fragility and its most appropriate management in order to formulate HRT regimens that will maximize bone accrual and other health outcomes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB of Children's Hospital of Philadelphia. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AM collected the data and provided the first draft. MM assisted with data collection. LP, TK, and AK contributed in the critical review and editing of the manuscript. MV developed the final draft and assisted with data analysis. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Impact of Diet and Physical Activity on Bone Health in Children and Adolescents

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There is growing recognition of the role of diet and physical activity in modulating bone mineral density, bone mineral content, and remodeling, which in turn can impact bone health later in life. Adequate nutrient composition could influence bone health and help to maximize peak bone mass. Therefore, children's nutrition may have lifelong consequences. Also, physical activity, adequate in volume or intensity, may have positive consequences on bone mineral content and density and may preserve bone loss in adulthood. Most of the literature that exists for children, about diet and physical activity on bone health, has been translated from studies conducted in adults. Thus, there are still many unanswered questions about what type of diet and physical activity may positively influence skeletal development. This review focuses on bone requirements in terms of nutrients and physical activity in childhood and adolescence to promote bone health. It explores the contemporary scientific literature that analyzes the impact of diet together with the typology and timing of physical activity that could be more appropriate depending on whether they are children and adolescents to assure an optimal skeleton formation. A description of the role of parathyroid hormone (PTH) and gut hormones (gastric inhibitory peptide (GIP), glucagon-like peptide (GLP)-1, and GLP-2) as potential candidates in this interaction to promote bone health is also presented.

Keywords: macronutrients, exercise, bone mass, gut peptides, hormones, children, adolescent

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INTRODUCTION

Bone tissue is a real organ in constant change, with both locomotive and supportive functions. It can be stiff but also flexible. These characteristics depend on the bone's material composition. The bone grows in length with endochondral ossification processes and in size with the modeling process of periosteal and endosteal surfaces (1). In particular, formation and resorption allow increasing bone mass and changing tissue density. The cartilaginous epiphyseal plate is the space where the bone grows in length as a result of chondrocyte proliferation and maturation, replacing the cartilage tissue with bone construction. In order to allow the complete ossification process, osteoclasts and osteoblasts are directed, by blood vessels, into the new cartilage tissue (2). This process runs constantly till the early twenties until the growth plate cartilage is replaced completely by new bone (3).

When the bone stops to grow in length, it keeps increasing in cross section in order to adapt to different mechanical loads and to compensate for bone loss. Osteoclasts and osteoblasts perform different tasks during the process of bone construction; the former are responsible of shaping the long bone's outer surface by building on circumferential lamellae. Osteoclasts are responsible for endosteal bone surface resorption and overall bone remodeling. The process of bone shaping and remodeling is sex and age dependent (4, 5), leading to the bone with an optimal size, shape, and architecture to withstand the normal physiological loads imposed on it. Failure to gain a sufficiently strong skeleton during growth may predispose to bone fragility later in life.

Thus, what are the key factors that may positively influence skeletal health in children and adolescents to maximize peak bone mass? Lifestyle, including diet and adequate physical activity, is an external factor of bone mass development that during growth and also during adulthood can help in the building of a strong adult skeleton (6, 7). Most of the literature that exists for children and adolescents about the effects of diet and physical activity on bone health is translated from studies conducted in adults. Thus, so far, relatively few studies have investigated the association between macronutrients or physical activity and bone mass depending on whether they are children or adolescents. Therefore, there are still many unanswered questions about what type of diet and physical activity may positively influence skeletal development. This review focuses on bone requirements in terms of nutrients and physical activity during childhood and adolescence. It explores the contemporary scientific literature that analyzes the impact of diet together with the typology and timing of physical activity that could be more appropriate depending on whether they are children and adolescents in order to assure an optimal skeleton formation. A description of the role of parathyroid hormone (PTH) and gut hormones (gastric inhibitory peptide (GIP), glucagon-like peptide (GLP)-1, and GLP-2) as potential candidates in this interaction to promote bone health is also presented.

THE INFLUENCE OF MACRONUTRIENTS AND MICRONUTRIENTS ON BONE HEALTH

Nutrition is an essential process for healthy growth and development of the skeleton. Diets are mainly composed of macronutrients (protein, fat, and carbohydrates) and also of micronutrients like dietary calcium, phosphorus, and vitamin D. Together, they are essential factors in promoting bone health and preventing bone loss. In the following paragraph, we will focus on studies that have investigated the influences of macronutrients and micronutrients on the bone to assure an optimal skeleton formation.

Proteins

Protein consumption exerts a beneficial effect on bone health in adults, while very little is known on short- or long-term effects of protein supplementation on bone turnover and bone development in children and adolescents. As far as we know, the recommended dietary allowance (RDA) in healthy children and adolescents is strictly derived from studies conducted in adults (8, 9) in which the recommended protein intake varies from 1.03 g/kg for all individuals to 0.97 g/kg per day for children aged 18–24 months.

In 2012, the EFSA Panel gave a scientific opinion on setting the dietary reference value (DRVs) for protein, which also takes into account protein quality. The daily amount of protein is calculated according to age and sex and set the population reference intake (PRI) that for adults goes from 0.66 to 0.8 g/kg body weight per day. In older adults, the PRI goes from 0.8 to 1.0 g/kg body weight per day in relation to low energy requirement of sedentary elderly or more energy dense protein for physically active groups. In infants and children, the EFSA Panel suggested a PRI from 1.65 to 1.1 g/kg body weight per day for the first year of life; between 1.15 and 0.9 g/kg body weight per day from 2- to 5-year-old children; and from 5 to 18 years old, the PRI goes from 1.1 to 0.8 g/kg body weight per day (10). Moreover, not much data are present for children above or under average weight and young athletes; and this might cause insufficient coverage in protein and energy requirements along children's growth (11) in order to assure an optimal skeleton formation (12).

Usually, in adult athlete population, protein RDA is defined by 1.2 and 1.7 g/kg/day (13), but a recent study affirms that protein intake in young boys before pubertal maturation should increase up to 2 g/kg/day in order to obtain higher bone acquisition as positive effect of physical activity because the positive effect on skeleton health is due to protein intake instead of calcium intake (12, 14). Thus, more research to clarify the point is necessary. Another open question is whether recommended protein intakes should be increased on the basis of the source of protein. Many conflicting studies have tried to examine the beneficial or detrimental role of protein intake on bone health, based on the source of protein (animal *vs.* vegetable) and the amount of protein ingested (high *vs.* low quantities) (15), namely, the sulfur content that may vary in sulfur-containing amino acids present in the protein source (animal or vegetable proteins). Therefore, a greater production of sulfuric acid could induce low- or mild-grade metabolic acidosis that, in turn, may have a negative effect on bone remodeling by enhancing bone resorption (16). However, when a balanced diet with adequate intake of calcium, vitamin D, fruits, vegetables, and protein of animal source is ingested, this does not exert a detrimental effect on the bone and improves bone health (17) On the contrary, in pubertal girls, when calcium intakes were less than 675 mg/day, the high-protein intake, especially from animal sources, had a negative effect on bone mass accrual deposition (18). It is quite clear, from literature, that sulfur amino acids play critical roles in metabolism and overall health maintenance. Animal-derived foods are a good source of sulfur-containing amino acids (19). Besides their role in protein synthesis, methionine and cysteine are precursors of important molecules (20). The most popular food for children and adolescents that are a good source of dietary sulfur-containing amino acids are chicken and beef, but also white eggs mainly present in products like omelet, frittata, crepes, mayonnaise,

biscuits, and cakes. So the positive effects of protein on the bone are exerted only if the energy requirements are satisfied by the carbohydrates and fats; otherwise, proteins are catabolized to sustain the energy demands. Thus, adequate intake of fat and carbohydrates to maintain bone health is essential (21, 22).

The protein needs of adult athletes are higher than those of non-athletes (23, 24). Thus, we do not know the quantity of acid formed, as sulfate may have a negative effect on the young skeleton. Thus, further studies about the impact of the source of protein origin (animal or vegetable) on the bone of child athletes or highly active children would be of interest. Moreover, this concept is closely linked to food habits. In fact, there is some evidence that suggests substantial differences in the consumption of food containing vegetable or animal proteins on the basis of age. Children ranging in age from 2 to 13 years (in both sexes) are used to consuming more red meat, poultry, or fish daily. Adolescents, from 14 to 16 years old, especially girls, have a marked decrease in animal protein consumption. However, the average intake of the meat was generally higher for males (100 g/day) than for females (80 g/day) (25).

Furthermore, it is important to underline that proteins may affect the bones at various levels: 1) they represent the major component of the bone matrix, and 2) they impact calcium excretion and absorption (26) and serum concentrations of insulin-like growth factor (IGF-1) (25). Therefore, adequate protein intake may be crucial for young athletes, for example, those involved in non-weight-bearing activities like swimming, who seem to be at increased risk for suboptimal peak bone mass development (27). Western diet often accounts for being responsible for osteoporosis or bone fracture due to the high protein content associated with hypercalciuria (21). Thus, as protein intake increases, there is an increase in urinary calcium excretion, with most subjects developing negative calcium balance as well as increased risk of fracture (28). Therefore, it is essential that the intake of protein should be adequate in order to fully realize the benefit of each nutrient on the bone. In fact, for example, proteins are able to modulate the IGF-1 levels that, in turn, would impact on both the skeletal muscle and bone, reducing fracture risk and increasing speed of recovery following a bone injury (29). In addition, there is evidence of a positive bone turnover response after protein intake shortly after intense exercise in adolescents. A study reveals a significant reduction in marker of bone resorption (CTX) after protein intake based on whey protein beverage following intense physical activity such as swimming. Thus, protein intake, after exercise, is important for the increase of bone mass during childhood, in particular if the subjects are active, or athletes (30). On the other hand, if protein intake is low (0.7–0.8 g/kg), the amount of PTH levels will increase in blood, while if moderate (1.0–1.5 g/kg), it is associated with normal calcium metabolism, without altering bone homeostasis. In gymnasts bone resorption was reduced by a high carbohydrate meal consumed 90 minutes before the training but not by a high protein meal (6). After all, any lifestyle stratsegy that can promote bone buildup in children, without affecting whole body homeostasis, is beneficial because it will drive towards higher peak bone mass and improvement in bone mineral density (BMD). Future research should focus on the long-term benefits of protein intake on blood markers of bone turnover in association with exercise especially in children and adolescents practicing low- and high-impact physical activity.

Fat and Carbohydrates

Many studies have explored the effects of fat and carbohydrates intake on bone health in children by focusing on calcium absorption rather than its direct effect on BMD, bone mineral content (BMC), and bone remodeling. Thus, we will first focus on it to briefly make the point on what is known and the importance with respect to the impact for bone growth in children. The studies have been carried out to understand whether or not fat or carbohydrate hindered calcium absorption in the intestine. Until now, calcium absorption has been most studied with respect to its impact on bone metabolism. As regards calcium absorption and fat, most of the studies were carried out on animal models fed with diets that contained a variable percentage of saturated fats (SFs) (5%, 14%, 28%, and 45%). The results have shown poor calcium absorption probably due to the formation of non-digestible calcium and saturated fatty acids (SFA) complexes in the intestine. In particular, a reduction in calcium absorption started when the diet administered contained up to 28% of fat, while there was a dramatic decrease in intestinal absorption of calcium when it reached 45% of fat. As a consequence, high-fat diet consumption in animals and humans is associated with reduction of BMD and bone strength (31). Actually, adverse microstructure changes occur in the cancellous bone compartment. Corwin and collaborators based on data from the Third National Health and Nutrition Examination Survey (NHANES III) conducted a study on 14,850 subjects, confirming a negative association between SF intake and BMD in both men and women (32).

Regarding the impact of carbohydrate intake on calcium absorption, numerous studies investigated the role of monosaccharide (particular glucose) and disaccharides (particular sucrose), showing an effect on the renal metabolism at the level of the distal tubule region of the nephron, therefore influencing the reabsorption of calcium (28, 33–35). In particular, there will be a large increase in renal excretion following glucose ingestion. For example, Ericsson et al. in human studies pointed out an exaggerated loss of calcium through the urine following the intake of a solution of glucose. The lactic acid formed by the osteoclast following the increase in glucose intake could induce the dissolution of calcium and magnesium from the bone surfaces, with consequent increase of urine calcium excretion with respect to the normal range. Other studies on both human and animal models showed that the insulin spike triggered by the ingestion of a high amount of glucose was directly proportional to the urinary levels of calcium excretion (36). These data suggest that more attention should be paid to the children's diet. The question is why reduction of calcium absorption induced by fat and sugar could affect the growing of a healthy bone in children. Because as they grow, there is a decrease in the consumption of milk and a concomitant increase in the consumption of unhealthy food rich in SF and

soft drinks, often attributed to gaining independence in choosing what to drink. In particular, data from the Continuing Survey of Food Intakes report (CSFII 1994–1996) suggest that with aging, there is a reduction in milk intake with a ratio of about 30 ml with a concomitant increase of approximately 126 ml in sweetened drinks. This is associated with an increase in caloric intake, of approximately 30 kcal, and a concomitant reduction in calcium consumption of 34 mg for each 30 ml of milk displaced (16). In young children, both athletes or not, the increased intake of soft drinks or the consumption of food rich in SF could have a negative impact on bone health and performance due to the impact of these on calcium absorption. This is the reason why it may be important to suggest using unsweetened drinks (water and milk) or orange juice, or sports drinks but only with small amounts of carbohydrate (<2%) and moderate amounts of sodium (37) and fortified with calcium in order to obtain rehydration and reduce or prevent loss of BMD. Furthermore, following a healthy diet rich in unsaturated fat is also suggested. For example, animal studies have shown that polyunsaturated fatty acids (PUFAs) such as omega-3s have been shown to reduce bone resorption and increase bone formation (38). The positive effect of a diet high in PUFA was confirmed in humans. The subjects involved in the study were assigned to three different groups of intakes consisting of 8%–13% of SFA, 12%–13% of monounsaturated fatty acids (MUFA), and 9%–17% of PUFA for a period of 6 weeks. There was no change in levels of bone-specific alkaline phosphatase (BSAP), selected as a marker of bone formation, across the three diets, while there was a reduction of bone resorption following the PUFA-enriched diet (39). The results indicated that dietary PUFA may have a protective effect on bone metabolism. Studies are necessary to see the impact of unsaturated fat on bone remodeling in children. Carbohydrates are present in fruits and vegetables and influence the absorption of calcium and therefore might influence bone growth. For example, chicory and artichokes have a high content of non-digestible carbohydrates (they are not digested by mammalian enzymes) called fructans such as inulin, which increases the absorption of calcium (40, 41). In fact, a study conducted on 9- to 13-year-old boys and girls, for a total of 12 months, with 8 g/day supplementation of inulin-type fructan, showed that calcium absorption, BMC, and BMD were significantly higher in the inulin-type fructan-supplemented group than in the placebo (supplemented with maltodextrin) control group (42). As far as the mechanism of action is concerned, it was suggested that these types of molecules, which are not digested, will reach the colon where they will be fermented, producing organic acids capable of reducing the pH by increasing the solubility and availability of calcium (43). The daily intake of carbohydrates should not go down 50%–55% of the diet. Adam-Perrot et al. confirmed that consumption of a low-CHO diet leads to an increase in urinary calcium loss and a decrease in markers for bone formation. Moreover, in adults, low-CHO diets lead to an increased consumption of animal protein, generating an acidosis that promotes calcium mobilization from the bone, finally leading to an increase of urinary calcium (44). The lack of a consistent definition for "low carbohydrate diets" complicates efforts to compare the results of the studies already published in this field. While it is known in the adult population that when we refer to "very low carbohydrate diets," we are talking about less than 70 g/day based on the proportion of energy intake; a diet containing 200 g of carbohydrate might be classified as moderately low for a 2.000 calorie intake, moderate carbohydrate for 1.500 calories, and high carbohydrate for 1.200 calories (45).

In conclusion, there is a need for studies about the effects of carbohydrates and fat on bone metabolism in children to see their impact on bone growth. Future studies should focus on both the short- and long-term benefits of carbohydrate and unsaturated fat consumption/supplementation on bone remodeling and BMD in children and adolescents, by looking also to those children participating in intensive training.

Micronutrients

Vitamin D and calcium are known to play key roles in bone health. Optimal calcium intake is estimated to be 400 mg/day from birth to 6 months, 600 mg/day in infants (6 to 12 months), 800 mg/day in young children (1–5 years) and 800–1,200 mg/day for older children (6–10 years), and 1,200–1,500 mg/day for adolescents and young adults (11–24 years) (46, 47). Milk and milk products such as 125 g of yogurt or 50 g of cheese allow the intake of about 300 mg of calcium. However, alternative calcium sources are orange juice, some vegetables such as cabbage family or large leafy vegetables, spinach, legumes, and some cereals, which contribute to the daily calcium intake for about 10%. However, vegetables having high calcium content have reduced calcium bioavailability due to the high concentration of oxalates (48). In general, reducing the intake of dairy products below the recommended daily doses can have a negative impact on the bone not only due to the lack of calcium but also due to other micronutrients such as phosphorus, potassium magnesium, or vitamins (B2 and B12, A, and D) (49).

The effects of calcium alone and together with vitamin D supplementation on the bone health of children have been investigated by different trials using also twin children. Johnston and collaborators showed that in prepubertal twins supplemented with 1,612 mg of calcium daily for 3 years, there was an increase of BMD with respect to the twin used as a control and supplemented with 908 mg of calcium in spite of the same intake of all nutrients and the equivalent level of physical activity (50). In a double-blind randomized control trial, it was investigated whether there was a differential response to calcium supplementation in elite prepubertal gymnasts and schoolchild controls. It was found that 1,250 mg daily of calcium supplementation, with a low level of physical activity, had only a small positive change in tibia trabecular volumetric BMD in the control group. Moreover, it was found that there was no beneficial effect of additional calcium in prepubertal gymnasts who already consume their recommended nutrient intake of calcium (51). In a randomized trial, adolescent girls, aged 12 years, were enrolled and supplemented with daily calcium, 800 mg of calcium carbonate and 400 IU vitamin D for 12 months. Daily calcium and vitamin D supplementation promoted greater

trabecular BMC and volumetric BMD acquisition in these preadolescent girls (52). Accordingly, Greene and collaborators confirmed that 800 mg of calcium and 400 UI of vitamin D supplementation, every day for 6 months, increased trabecular density and strength strain index and increased the tibial cortical area in female identical twins, aged 9 to 13 years (53). However, a meta-analysis study showed that increased dietary calcium/dairy products, with and without vitamin D, significantly increased total body and lumbar spine BMC in children with low baseline calcium intakes (54). So it seems that calcium and vitamin D supplementation above the recommended nutrient intake has a modest influence on the bone especially in active prepubertal children. On the other hand, calcium supplementation seems to be beneficial for the child population with low daily intake.

Children aged between 3 and 17 years seem to take more than 50% of the recommended daily dose of calcium through dairy products as confirmed by studies carried out in France (2005–2007) and the United States (49). The latter pointed out that children aged between 2 and 18 years take about 950 mg/day through dairy products (especially milk and cheese), which represents the main source (55). The optimal quantity of phosphorus to take is in a ratio of 2:1 to calcium (in favor of the latter) since it could have the opposite effect. Sodium may also have an effect on urinary calcium excretion, as both sodium and calcium compete for reabsorption in the renal tubules. It is estimated that for every 2,300 mg of sodium excreted, approximately 50 mg of calcium is lost in women (56). The influence of short-term calcium supplementation in adult athletes during exercise on bone remodeling has been investigated. Short-term supplementation with calcium, 60 min before physical activity, did not appear to affect bone resorption (57), but this could be due to the quantity of supplementation or the time of consumption before the trial. In fact, a calcium supplement of 1,000 mg, taken 30 min before exercise, did not affect bone resorption in competitive adult male cyclists, while 1,350 mg of calcium taken 90 min before the exercise reduced bone resorption in competitive adult female cyclists (58). As regards vitamin D, the general recommended daily dose is 200 IU/day for children. Specifically, the RDA for children up to 1 year is 400 IU/day, which increases up to 600 IU for children aged 1 year or older. Also in this case, the upper limits are set beyond which it could have a detrimental effect, specifically for children up to 6 months with daily dose of 1,000 IU, from 6 to 12 months of age with daily dose of 1,500 IU, from 1 to 3 years of age with daily dose of 2,500 IU, from 4 to 8 years of age with daily dose of 3,000 IU, and from 9 and 18 years with daily dose of 4,000 IU (48). However, it should be kept in mind that in addition to calcium intake, there are also other factors that affect bone health such as hereditary and environmental factors.

DIETARY COMPOSITION

Bone health could be more influenced by dietary long- or short-term changes rather than specific nutrients, although there are insufficient studies to prove it.

The HELENA study failed to show, in Spanish adolescents, the association between Mediterranean diet and BMC (59) as well as a study of Monjardino et al. (60), which found no association between forearm BMD and different dietary patterns. The work of Shin et al. observed that adolescents in the highest tertile with a dietary pattern rich in cereal and milk score significantly a reduced chance of having low BMD than do those in the lowest tertile (61). The association among dietary patterns, physical activity BMC, and BMD needs to be further explored especially in children. In relation to this issue, Muñoz-Hernandez et al. showed that moderate-to-vigorous physical activity and less time for sedentary behavior seem to improve bone health in overweight or obese children with related poor adherence to the Mediterranean dietary pattern (62).

In the review of Mariotti and collaborators, they indicated that classic vegetarian diets provide more than adequate protein and amino acids with respect to the DRVs; furthermore, children who are consuming sufficient energy to cover their necessities for growth should automatically reach sufficient protein intake and protein variety from vegetarian diets (63). Taking into account that an extreme dietary position such as a vegan diet or a vegan who consumed only uncooked and unprocessed plant-based food might show lacking micronutrient concentration, for example, calcium. Vegans require calcium-fortified foods that in combination and variety may help meet their daily calcium needs (64). However, in adults, as well as in children, it is not clear enough the mechanism behind bone health and dietary intake; therefore, further studies are needed to clarify this complex mechanism.

THE INFLUENCE OF PHYSICAL ACTIVITY ON BONE HEALTH

Regular physical activity during growth seems to be one of the most important factors influencing peak bone mass. According to the International Osteoporosis Foundation (IOF), about 22% of men and 46% of women aged 50 years will experience osteoporotic fractures during the remainder of their lives (65). Thus, what should be done to optimize peak bone mass is to maximize the increase in BMD during the first 25 years by appropriate physical activity and to minimize the decrease in BMD after 40 years due to endocrine changes related to aging by regular physical activity. This easy strategy would reduce the occurrence in fractures later in life. However, so far, an exercise program for children and adolescents that will optimize peak bone mass in detail has not yet been defined.

The WHO has suggested that physical activity confers benefits for bone health in children and adolescents (66). Evidence comes from randomized small trials, which have some limits like confounding factors inherent to the cross-sectional studies (e.g., type of the exercise training, duration of the intervention, and group of intervention), which makes it difficult to figure out the osteogenic effects of physical activities. There are many systematic reviews that focus on bone strength in children and adolescents of both sexes in relation to physical

activity (67). Some of them reported changes in bone structure rather than bone mass linked to enhanced bone strength.

In general, moderate-to-vigorous physical activity was linked to positive bone outcome especially in males, although discrepancy in the methodology assessment made it difficult to establish the amount and type of physical activity that might lead to favorable bone outcomes and therefore might exert the osteogenic action (68). What is recognized to exert an osteogenic action are interventions that must include high-intensity exercise of enough ground reaction forces (GRFs), to significantly increase bone mineralization and prevent osteoporosis and fragility fracture later in life. In fact, it was shown that high-impact jumping activities with GRFs from at least 3.5 and 8.8 × BW (10 min for two to three times per week) are effective in increasing BMD and/or BMC in children and adolescents, indicating that the most important factor is the intensity and not the duration of the stimulus (69). Usually, the intensity of an osteogenic exercise was expressed as GRFs, the combination of magnitude of force and speed by which it is applied (69, 70). It was demonstrated that activities with the most osteogenic potential have GRFs greater than 3.5 times BW (per leg) with peak force occurring in less than 0.1 s (71). Worthy of note is also a recent article about a project that assessed whether early elementary school children participating in 20 min of vigorous activity 3 to 5 days per week with 5 min of jumping component (GRFs between four and seven times' body weight) would improve bone quality and muscular strength (70). The study confirmed the effectiveness of the program. Thus, a higher intensity level of physical activity achieves positive effects on BMC, BMD, and accretion (72). The importance of vigorous exercises as a favorable predictor of bone strength was further confirmed. In particular, 1 h per day of moderate-to-vigorous physical activity was able to improve bone strength in 6-year-old subjects (73). Also, participation in unstructured weight-bearing physical activity had strong and consistent positive effects on bone development (71, 74), further confirming that the high level of physical activity intensity is associated with higher modification in bone parameters during childhood (75, 76).

An evaluation study conducted from middle childhood to middle adolescence in more than 300 boys and girls in a 10-year longitudinal study confirmed that high participation in moderate-to-vigorous physical activity during childhood led to bone strength benefits in late puberty and that to improve bone BMC is necessary for vigorous physical activity interventions (77). Thus, vigorous physical activity was associated with BMC at skeletal sites from childhood to adolescence, and the effect was not modified by maturity or age (77). In fact, both engaging and maintaining high levels of vigorous physical activity participation during puberty in boys are associated with greater gains in bone mass and density (78). This is because at the age of 11–13 years, the growing bones of adolescents are more sensitive to mechanical loading than adult bones. Regarding the time spent on physical activity to achieve an osteogenic response, it was suggested that in adolescents, about 30 min per day of vigorous physical activity has shown benefits for the femoral neck BMD (79, 80), while 3 h per week could be enough to elicit an increase in bone mass (81). In fact, bone development is influenced by volume and intensity. Male adolescents (11–13 years) who participated in vigorous physical activity were positively correlated with increased whole body and lumbar spine BMC than sedentary, light, and moderate physical activity. Furthermore, the best benefits on BMC appear when 15 consecutive minutes of vigorous activity (like running) with respect to those who did it from 5 to 15 min and those who did it at less than 5 min. Therefore, with the same intensity of stimulus (vigorous activity), those who have carried out a longer duration showed better BMD than the rest of the subjects (82). However, it is important to note that not all activities have the same osteogenic effect on bone mass in children or adolescents. Such as in boys, long-term soccer participation, starting at a prepubertal age (Tanner stage 1–2), results in greater acquisition of bone mass and a lower accumulation of body fat (83). Bone acquisition is higher also in adolescent male footballers compared with swimmers and cyclists (84, 85). This suggests that weight-bearing activities should be incorporated into training of swimmers in order to develop a stronger skeleton during adolescence. This would allow to optimize peak bone mass and reduce low bone status later in life. In addition, GRFs during impact exercises, especially during pubertal growth, play an important role in maximizing bone mineral gain (71). Among these activities, gymnastics has been shown to be particularly osteogenic for bone development in children because it is a high-impact activity and involves the subject at an early age during growth (86). However, intense athletic activity in growing and maturing gymnasts is often associated with inadequate dietary intake, which leads to relatively low fat mass (87) and consequently possible alteration of endocrine function (80). However, two major systemic reviews showed that prepubertal gymnasts have higher BMD and BMC than age-matched untrained controls. Therefore, gymnastics activities seem to be the one of most effective exercises for improving bone mineral gain in growing and maturing children (88, 89). Thus, the positive effect on bone accumulation seems to outweigh the possible negative influence of other characteristics.

What is important is to underline that optimal timing of a physical activity intervention differs depending on the stage of maturity of the pediatric population. In fact, BMC increases linearly, with no sex differences until the onset of the pubertal growth spurt (90, 91). Therefore, there are no differences in BMC and BMD between 6-year-old boys and girls (4) or younger subjects (3–5 years old) (5). However, some difference can be appreciated from the puberty phase onward where at the tibial diaphysis the cortical bone increases in size more for boys than for girls (92); therefore, the BMD varies considerably for the same chronological age among boys and girls. The peak of growth rate occurred earlier but is smaller in females than in males, and the female growth curve flattens before and with lower peak than men (93). Thus, males have a longer prepubertal period of growth because their pubertal growth spurt occurs 1 or 2 years later than in girls (94–96). However, this can cause a transient period of increased porosity at the cortex during

periods of rapid growth. Particularly, boys demonstrate greater porosity than girls in this period (97). Bone thickness remains relatively stable until late puberty, as endosteal apposition is unable to keep pace with the rapid periosteal resorption that dominates the process of metaphyseal investing during periods of rapid longitudinal growth (98). The lag of bone thickness growth may contribute to increased bone fragility, and it could be a direct result of increased calcium demands, resulting in higher rates of intracortical bone turnover and increased porosity due to incomplete consolidation of bone (99).

Looking inside the sexual difference in BMC during adulthood, Baxter-Jones and collaborators analyzed 30 years later, in a prospective study, 82 female and 72 male children (age range 8 to 15 years) both more and less physically active (100). Participants returned for follow-up at age 23 to 30 years (2002–2006), and the cohorts were divided into active, average, and inactive groups. When compared with the inactive group, active females and males had greater adjusted BMC. In young adulthood, the male and female adolescent active groups were still more active than their peers and had still greater adjusted BMC. It was concluded that the skeletal benefits of physical activity in adolescents are maintained into young adulthood.

In conclusion, physical activity with certain characteristics seems to be better than others to improve bone mass. It should be dynamic (101) and vigorous (73, 75–78) with impact and load (71) to have a strong and consistent positive effect on bone development. However, further research to better clarify the modulatory role of the different activities on bone health in children and adolescents is necessary to improve and maintain bone health.

POTENTIAL EXPLANATORY MECHANISM: PTH AND GUT PEPTIDES

Bone metabolism is influenced not only by hormones, such as the PTH, which is essential for the maintenance of calcium homeostasis, but also by hormones produced by other peripheral districts like the gastrointestinal tract (**Figure 1**). What is known about the influences of these factors on the bone and how are these affected by nutrients and physical activity?

The PTH has multiple effects on the skeleton. PTH stimulates osteocytes, osteoblasts, and their precursors. The physiological function of PTH is to maintain extracellular fluid calcium concentration and to prevent hypocalcemia. Therefore, with a feedback mechanism, PTH production is closely regulated by serum calcium concentration: PTH secretion increases whenever calcium concentration falls below normal, and the hormone is accountable for difference in blood calcium concentration, directly influencing the bone rather than kidney metabolism.

The hormone can prevent hypocalcemia at the cost of progressive bone destruction and loss of bone mineral (102).

Despite the role of PTH on bone homeostasis and remodeling, there are few studies about changes in PTH secretion and actions in healthy young subjects. These studies are not recent and did not focus on the effects of macronutrients

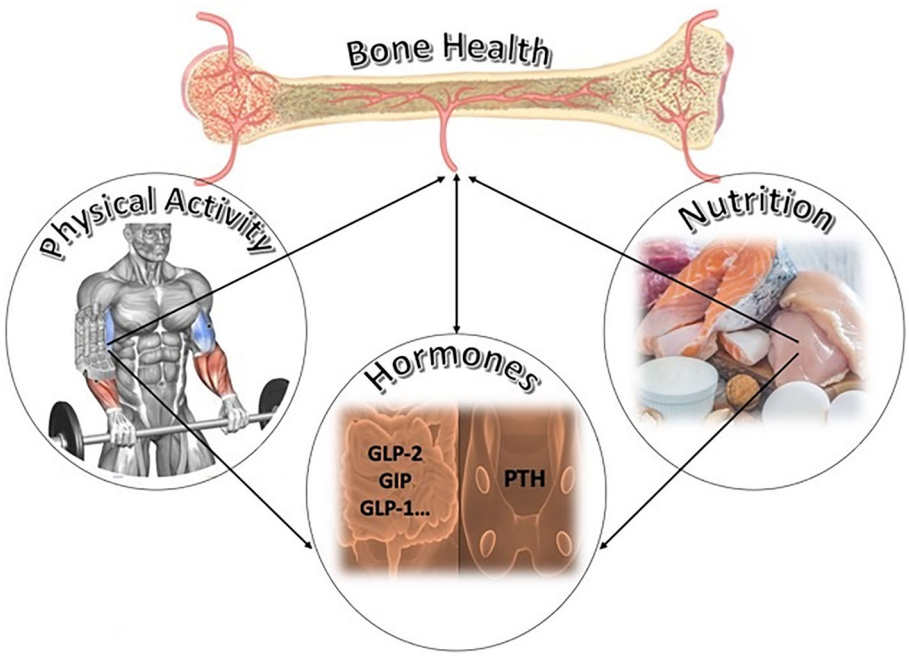

FIGURE 1 | Potential key factors of bone homeostasis and remodeling: the bone is modulated by nutrition and by physical activity. Nutrition influences the release of hormones like gut peptides (GLP-1, GLP-2, and GIP) and modulates the secretion of parathyroid hormone (PTH). Both in turn influence bone remodeling. Physical activity, adequate in volume and intensity, could impact bone homeostasis by influencing hormones released by peripheral organs. This figure is our own creation.

and physical activity on PTH secretion. One of these studies shows the opposite variations of PTH and of 25-hydroxyvitamin D in a group of 42 children living in South Argentina (103). The association between a high level of PTH, a low level of vitamin D, and reduced bone mass was confirmed in a letter study conducted in pubertal and prepubertal Finnish girls (104). The secretion of PTH is modulated by vitamin D (105), suggesting that micronutrients could affect PTH secretion. Thus, also macronutrients could modulate PTH secretion in adolescence. For example, lower PTH concentrations and beneficial effects on bone size were observed in early pubertal children who have high fruit and vegetable intakes (106). This strongly suggests that nutrient supplementation influences PTH release in children and consequently bone homeostasis.

Physical exercise influences PTH release and increases PTH production, suggesting a possible key role in bone formation and adaptation to its mechanical features (107). However, research into the effects of exercise on PTH expression and secretion is still limited, and the study was conducted in adults. However, what was observed is that the increase in systemic PTH levels seems to depend on the type, intensity, and duration of exercise (108, 109). It was demonstrated that bone adaptation during exercise is not only a function of the dynamic loading but also PTH release and that PTH signaling contributes differently at structural and tissue levels (101). During exercise, there is an increase in calcium demand by the active muscle, but this increase is at the expense of bone mineralization at the periosteum (110). In fact, calcium supplementation during exercise may reduce bone resorption markers in adult female cyclists (58) and in general lessen the increase in PTH (57). In postmenopausal women, the time of calcium supplementation also seems to be important on the PTH release (111). In fact, the excess of calcium during exercise may impair systemic PTH release with a feedback mechanism (58). Further research is necessary to determine the effects of macronutrients and exercise alone and together on PTH secretion in children and adolescents to determine how this influences bone homeostasis and skeletal adaptations during growth. The gastrointestinal hormones have recently been seen to influence bone metabolism. The relationship between hormones secreted by the gut and bone is a recent study and opens the way to new interesting fields of research.

The bone and gut are strictly connected, and gut hormones respond to food intake, triggering bone resorption. Bone resorption is increased during the night compared with day, and diurnal suppression is eliminated by fasting, confirming the role of gastrointestinal hormones in controlling bone homeostasis (112)

Among the gut hormones, GIP, GLP-1, and GLP-2 are known to be involved in the regulation of bone turnover. These hormones have been extensively studied for their effects on glucose and lipid metabolism (113–118) and are of interest in the light of the interplay between the bone and glucose metabolism, which seems to be compromised in diabetes (119). The exact mechanism of action of these peptides on the bone is unclear. The GLP-1 and GLP-2 receptors are expressed in the immature human osteoblast cell lines MG-63 and TE-85 (120), but GLP-2 has not been identified in human osteoclasts or in any other bone-related cell types despite the impact of GLP-2 on osteoclast activity. GLP-2 is co-secreted with GLP-1 by L cells in the small and large intestine and was initially studied for its ability to stimulate mucosal growth and nutrient absorption in the intestine (121). GLP-2 treatment is associated with the reduction of serum and urinary markers of bone resorption in postmenopausal women, while bone formation appears not to be affected (122, 123). The GIP receptor is expressed in both osteoblast- and osteoclast-derived cell lines and increases the expression of type 1 collagen. It maintains osteoblast homeostasis with an anabolic effect on the bone. It also has an inhibitory effect on the bone resorption activity of PTH (124).

This information suggests that the gut hormones could impact bone health and affect bone quality. Thus, more studies are required to investigate the effects of diet and physical activity on secretion of gut peptides in children and the modulatory role, if any, to assure an optimal skeleton formation.

CONCLUSION

There are several studies that have investigated in adulthood the best strategies in order to maintain good lean mass and bone mass by modification of diet and physical activity (125–130). Most of the adult studies have focused on osteoporosis risk subjects, postmenopausal women, and have analyzed the effect of minerals (mainly calcium), vitamin supplementation (mainly vitamin D), and nutrients, especially protein supplementation (131). There are few studies in childhood, and there are many questions to be answered. In fact, the skeleton is not an inert structure playing a supporting role for muscles and a protective role for inner organs like the brain. It is able not only to regulate its own physiology but also to influence energy metabolism by continuum interplay with the peripheral organs like the adipose tissue-derived hormones, the gastrointestinal tract-derived peptides, and insulin and by also producing the bone-derived peptides like osteocalcin and lipocalin-2. Nutrition and physical activity may influence the release of these factors in children. How this could impact bone health in children and adolescents is not completely known. Thus, studies that will address questions about the complex interplay between the bone, gut, white and brown adipose tissue, nutrition, and physical activity are required to provide in the near future new insight into this fascinating topic of metabolic endocrinology.

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Lower Limb Deformity and Gait Deviations Among Adolescents and Adults With X-Linked Hypophosphatemia

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Background: X-linked hypophosphatemia (XLH) is a rare genetic disorder characterized by lower limb deformity, gait and joint problems, and pain. Hence, quality of life is substantially impaired. This study aimed to assess lower limb deformity, specific radiographic changes, and gait deviations among adolescents and adults with XLH.

Design: Data on laboratory examination and gait analysis results were analyzed retrospectively. Deformities, osteoarthritis, pseudofractures, and enthesopathies on lower limb radiographs were investigated. Gait analysis findings were compared between the XLH group and the control group comprising healthy adults.

Patients and Controls: Radiographic outcomes were assessed retrospectively in 43 patients with XLH (28 female, 15 male). Gait analysis data was available in 29 patients with confirmed XLH and compared to a healthy reference cohort (n=76).

Results: Patients with XLH had a lower gait quality compared to healthy controls (Gait deviation index GDI 65.9% +/- 16.2). About 48.3% of the study population presented with a greater lateral trunk lean, commonly referred to as waddling gait. A higher BMI and mechanical axis deviation of the lower limbs were associated with lower gait scores and greater lateral trunk lean. Patients with radiologic signs of enthesopathies had a lower GDI.

Conclusions: This study showed for the first time that lower limb deformity, BMI, and typical features of XLH such as enthesopathies negatively affected gait quality among adolescents and adults with XLH.

Keywords: XLH, gait analysis, gait deviations, hypophosphatemia, deformity, pseudofracture, enthesopathy, BMI

INTRODUCTION

X-linked hypophosphatemia (XLH, OMIM 307800) is a rare disorder of mineral metabolism associated with progressive rickets, profound deformities, and osteomalacia. Due to loss-of-function of mutation in the phosphate-regulating gene with homology to endopeptidases in the X chromosome (PHEX), dysregulation of fibroblast-like growth factor 23 (FGF23) leads to chronic renal phosphate wasting and impaired activation of 1,25 dihydroxyvitamin D [1,25(OH)2D] (1).

Patients with XLH can develop rachitic deformities of the lower limbs and short stature due to chronic phosphate depletion and associated growth plate pathologies. Musculoskeletal pain, dental abscesses, and fatigue further substantially impair quality of life (QoL) among patients with XLH (2). Therefore, patients of all ages with XLH should be treated in a multidisciplinary setting (3, 4).

Most patients with XLH report gait and joint problems, which are burdensome (5, 6). Thus, multiple surgical corrections of XLH-associated long bone deformities with bowing and maltorsion of the lower limbs are frequently required (7, 8).

Gait abnormalities have been a common feature among adults with XLH (6). In children with XLH who had no prior surgical interventions, a detailed analysis showed femoral and tibial malrotation and maltorsion, varus and valgus deformity of the lower limbs, and compensation mechanisms during gait (9).

Gait characteristics in adults have so far only been reported in a single study that only included a heterogenous group of nine non-surgically and surgically treated adults with XLH (10). However, a detailed and standardized analysis of lower limb deformity was not performed in this study.

Furthermore, a previous study has reported the radiographic features of adults with XLH (11). Moreover, some authors have shown a high incidence of lower limb deformities (12, 13). However, a comprehensive analysis of lower limb deformity and gait was not performed.

Thus, the current study aimed to analyze lower limb deformity, specific radiographic changes, and gait deviations among adolescents and adults with XLH who received prior surgical interventions and those who did not. Moreover, factors influencing gait quality were identified.

PATIENTS AND METHODS

This was a single-center study of adolescent and adult patients with XLH with and without previous surgical intervention performed at Orthopaedic Hospital Speising, Vienna. Data on patients with XLH aged older than 16 years were retrospectively analyzed using the dataset of our gait laboratory registry Furthermore, participants were actively recruited *via* the national XLH alliance "Phosphatdiabetes Austria". The study was approved by the local ethics committee (EK37/2020).

Inclusion/Exclusion Criteria

The inclusion criteria included patients aged above 16 years who have a genetically verified XLH and who presented with fused growth plates of the lower limbs at the time of examination. The exclusion criteria were patients with other types of hypophosphatemia and those with incomplete radiographic data.

Data Acquisition

Laboratory examination and gait analysis results from maximum 6 months before and after radiographic examination were included in the analysis.

Full length anteroposterior (ap) radiographs (hip to ankle) of both lower limbs in standing position with centralized patella and with a calibration ball were obtained to analyze frontal lower limb deformity.

Deformity analysis of ap radiograph images (hip to ankle) was performed by one examiner (A.S.) using the TraumaCad software (Brainlab AG, Munich, Germany) according to standard measurements (14, 15) (**Figure 1**). Long leg films were standardized with a centralized patella to minimize rotational effects.

Mechanical axis deviation (MAD), leg length discrepancy (LLD), mechanical lateral proximal femoral angle (mLPFA), mechanical lateral distal femoral angle (mLDFA), mechanical medial proximal tibial angle (mMPTA), and mechanical lateral distal tibial angle (mLDTA) were assessed in ap radiographs. On lateral radiograph, the femoral and tibial diaphyseal procurvatum angles were analyzed. Radiographic angles were compared with published values (14). Pseudofractures and enthesopathies of the lower limb were evaluated. Furthermore, radiographic scores were used to describe osteoarthritis of the hip (16), knee (17), and ankle (18).

Gait Analysis

The modified Cleveland model for movement of the lower extremity and the Plug in Gait model for movement of the upper extremity marker set were applied. The Vicon motion capture system (Vicon, Oxford, the United Kingdom) (19, 20) was used for gait analysis.

To obtain kinematic data from a minimum of five force plate (AMTI Advanced Mechanical Technology Inc., Watertown, Massachusetts) strikes per foot, patients were instructed to walk a 12-meter walkway at a self-selected speed. A custom Matlab script (The MathWorks, Natick, Massachusetts, Version 2019a) was used to graph and compare data between groups. The Gait Deviation Index (GDI) was calculated according to the study of Schwartz and Rozumalski (21). A historic gait laboratory cohort of healthy adults was used.

Internal foot progression during the single support phase was referred to as intoeing. A lower range of motion (ROM) of the hip, knee, and ankle, as well as external rotation of the hip and knee were defined as values exceeding two standard deviations from that of the control group. A higher lateral trunk lean was defined as an increase in the medio-lateral ROM of the thorax exceeding two standard deviations from that of the control group.

Statistics

The Kolmogorov–Smirnov test for testing normal distribution, independent *t*-test (data with normal distribution), and Mann–Whitney U test (data with non-normal distribution) were used to

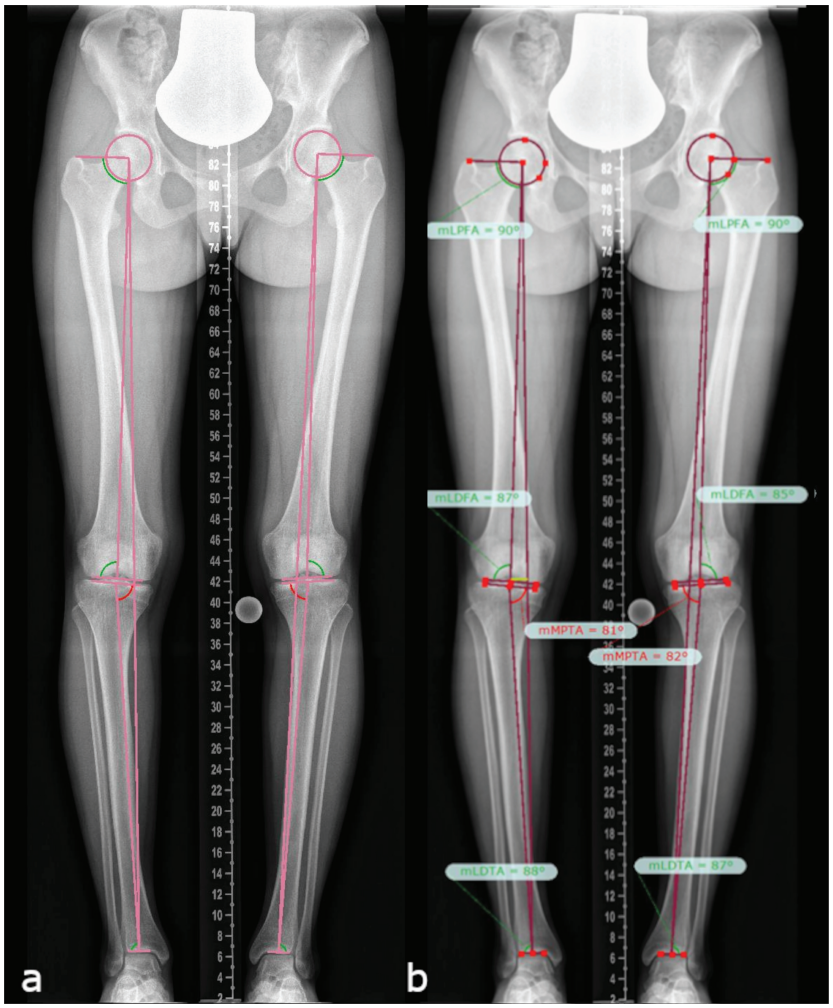

FIGURE 1 | Lower limb deformity measurements on full-length anteroposterior radiograph images (hip to ankle) of both lower limbs in standing position **(A)**, as assessed using the TraumaCAD software according to standard measurement protocols **(B)** (14, 15), in a patient with mild XLH (18 y/o, female, BMI of 21.6 kg/m^2, GDI of 89.8, no surgeries, receiving conventional treatment since childhood, and mild bilateral varus deformity proximal tibia).

perform statistical analysis for the comparison of gait parameters between patients with XLH and age-matched controls. Statistical parametric mapping was used to assess waveforms (22). The statistical value of gait parameters was assessed using Matlab and SPM in Python (Statistical Parametric Mapping. Retrieved from www.spm1d.org). The least squares approach was utilized to quantify the strength of the relationship between BMI and GDI or lateral trunk lean in the linear regression models. Strength was evaluated using R^2, and the F-test was applied to evaluate statistical significance. For parameters with normal and non-normal distribution, Pearson's or Spearman's correlations were calculated, respectively. Data with normal distribution were assessed using the Shapiro–Wilk test. A p value of ≤ 0.05 was considered statistically significant. Data were processed using Jamovi version 1.1.19 (The Jamovi Project, 2019. Retrieved from https://www.jamovi.org; R Core Team 2018. R: Retrieved from https://cran.r-project.org/).

RESULTS

Study Population

During the study period from January 2010 to April 2021, data on 51 adolescents and adults with XLH were eligible from the database. Eight patients were excluded due to incomplete radiographic data. Finally, 43 adolescents and adults with XLH (86 legs; 28 women, 15 men; mean age: 29 years) who underwent complete radiographic examination and 29 patients (20 women, 9 men; mean age: 32 years) with gait analysis data were included in this study.

The gait analysis data between the XLH group (n = 29, 58 limbs) and the control group (n = 76 healthy adults, 152 limbs) were compared. The gait parameters of patients with XLH treated non-surgically (n = 7) and those treated surgically (n = 22) were further analyzed in subgroups. The control group included 37 men and 39 women, with a mean age of 28.3 (range: 21–50) years, and

data were obtained from our gait analysis database. The average weight of the control group was 69.0 (range: 47.4–101.7) kg; average height, 174.3cm (range: 155–198.0); and average BMI, 22.6 (17.6–29.1) kg/m^2.

In total, 4 (16%) of 25 patients with available medical records received conventional medical treatment during childhood and at the time of radiographic examination. Meanwhile, 11 (42%) of 26 patients with available medical records did not receive any pharmacologic treatment during childhood. One patient was treated with burosumab, a monoclonal antibody to FGF23, starting at 3 weeks prior to gait analysis.

In total, 35 (81%) of 43 patients underwent limb alignment surgeries prior to this study. 23 patients (53%) had more than five multiple bony surgeries using guided growth, osteotomies, hexapod fixators, or intramedullary nails. Two patients (aged 60 and 71 years) had three arthroplasties (1 hip, 2 knees) at the time of examination.

Mean height of XLH patients (n = 40) was reduced and BMI elevated (**Table 1**), in particular 16 (40%) patients were < 150 cm tall. Moreover, 26 (65%) patients had an increased BMI, with 14 (35%) overweight (BMI: > 25 kg/m^2) and 12 (30%), obese (BMI: > 30 kg/m^2) classification.

TABLE 1 | Demographic data of the study population including mean age, weight, height, and BMI and ranges (min–max) inside the brackets.

Study population (n = 43 patients)	**Female Male**	**n = 28 n = 15**
	Age	29 (16–71) years
	Weight	66.5 (46.9–96) kg
	Height	153.2 (138–167) cm
	BMI	28.6 (18.4–43.8) kg/m^2
Radiographic analysis	**Valgus (MAD > 15 mm)**	21 (15–25) mm n = 6 legs
	Varus (MAD > 10 mm)	50 (13–164) mm n = 39 legs
	JLCA	2° (0°–10°) n = 86/86 legs
	mLPFA	100.2° (68°–123°) n = 86/86 legs
	mLDFA	94.1° (79°–122°) n = 86/86 legs
	mMPTA	84.9° (30°–98°) n = 86/86 legs
	mLDTA	90.5° (31°–114°) n = 86/86 legs
	LLD	9.4 mm (0°–52°) n = 41/43 patients
	Femoral bowing	18.9° (-5° to 83°) n = 48/86 legs
	Tibial bowing	6.7° (-9° to 39°) n = 45/86 legs
Laboratory values (n = 23 patients)	**Calcium**	2.37 (SD: 0.14) mmol/L
	Phosphate	0.68 (SD: 0.21) mmol/L
	ALP	125.85 (SD: 47.40) U/L
	PTH	80.42 (SD: 45.60) ng/L
	25OH-Vitamin D	50.92 (SD: 28.51) nmol/L

*Radiographic analysis of mean angles measured using the TraumaCad software (Brainlab AG, Munich, Germany) according to standard protocols (15), mean femoral and tibial bowing angle, and mean lower limb discrepancy. Cases of varus and valgus, defined as MAD of > 15 mm and > 10 mm, respectively, are listed above. Number of legs and patients, marked with *. The mean laboratory values are listed above.*

Radiographic Findings of the Lower Limbs

Adolescents and adults with XLH had different types and degrees of lower limb deformity. Valgus and varus deformities were observed at varying levels (hip, knee, and ankle) (**Table 2**).

The radiographic outcomes (n = 43 patients, 86 limbs) were frontal lower limb malalignment (genu varum: MAD, > 15 mm medial; genu valgum: MAD, > 10 mm lateral) in 52.3% (n = 45) of limbs (mean: 50 mm medial to 21 mm lateral). Knee valgus deformity was detected in 6 (7.0%) and knee varus deformity in 39 (45.3%) of 86 limbs. In total, 76 (88.4%) of 86 hips had proximal femoral varus deformity (mLPFA: > 90°). Lateral radiograph showed a mean anterior femoral bowing angle of 18.9° (n = 48 femora) and tibial bowing angle of 6.7° (n = 45). Patients without prior surgery (n = 7) presented with mild to severe frontal axis deviation (MAD = 25 mm lateral/valgus to 164 mm medial/varus). In total, 13 (30.2%) patients aged 17–71 years presented with pseudofractures (25 in 19 femora, 9 in 8 tibiae, and 5 in 4 fibulae). Among them, nine (69.2%) were aged < 30 years.

The radiographic scores indicated osteoarthritis in 68 (79.1%) of 86 hips, 56 (65.1%) of 86 knees, and 45 (52.3%) of 86 ankles. Moreover, 27 (69.2%) of 39 feet presented with degenerative changes in the talonavicular joint. In total, 35 (81.4%) patients had radiographic signs of enthesopathies, mainly at the lesser (n = 28/86 limbs) and the greater (n = 27/86 limbs) trochanter.

Gait Deviations

Patients with XLH had a lower walking speed ($p < 0.001$) and broader step width ($p < 0.001$). Adolescents and adults with XLH had a low gait quality based on GDI, which is a global measure of gait quality (Mean 65.9%, SD 16.2%, range 27.6 - 92.2).

Regarding the sagittal profile, patients with XLH presented with a low hip, knee, and ankle ROM (**Figures 2A, B**). The main decrease in the sagittal ROM was observed in 27 (46.6%) of 58 knees (reference value = norm + 2 SD = 54.7°), 13 (22.4%) of 58 ankles (reference value = norm + 2 SD = 23.3°), and 12 (20.7%) of 58 hips (reference value = norm + 2SD = 35.8°).

Gait analysis showed a significantly greater external femoral rotation as well as a lesser tibial external rotation in the XLH group ($p < 0.001$, **Figures 3A, B**). The surgically treated group had lower rotation deviations of the femur ($p = 0.001$) and the tibia ($p = 0.001$) than the non-surgically treated group. In total, 15 (51.7%) of 29 patients with XLH (6 of 9 who did not undergo surgery) presented with intoeing.

TABLE 2 | Radiographic findings of patients with XLH who presented with varus and valgus deformity at the hip, knee, and ankle.

Deformity analysis ***n = 86 legs***	**Varus**	**Normal**	**Valgus**
Hip (LPFA: 85–90)	88.4% (76)	8.1% (7)	3.5% (3)
Knee/femur (LDFA: 85–90)	52.3% (45)	40.7% (35)	7.0% (6)
Knee/tibia (MPTA: 85–90)	32.5% (28)	51.2% (44)	16.3% (14)
Ankle (LDTA: 86–92)	33.7% (29)	51.2% (44)	15.1% (13)

Percentages are listed inside the brackets. The standard angle measurements according to Paley et al. (14, 15) were used to analyze the full-length anteroposterior radiographs of both legs in standing position using the TraumaCad software (Brainlab AG, Munich, Germany).

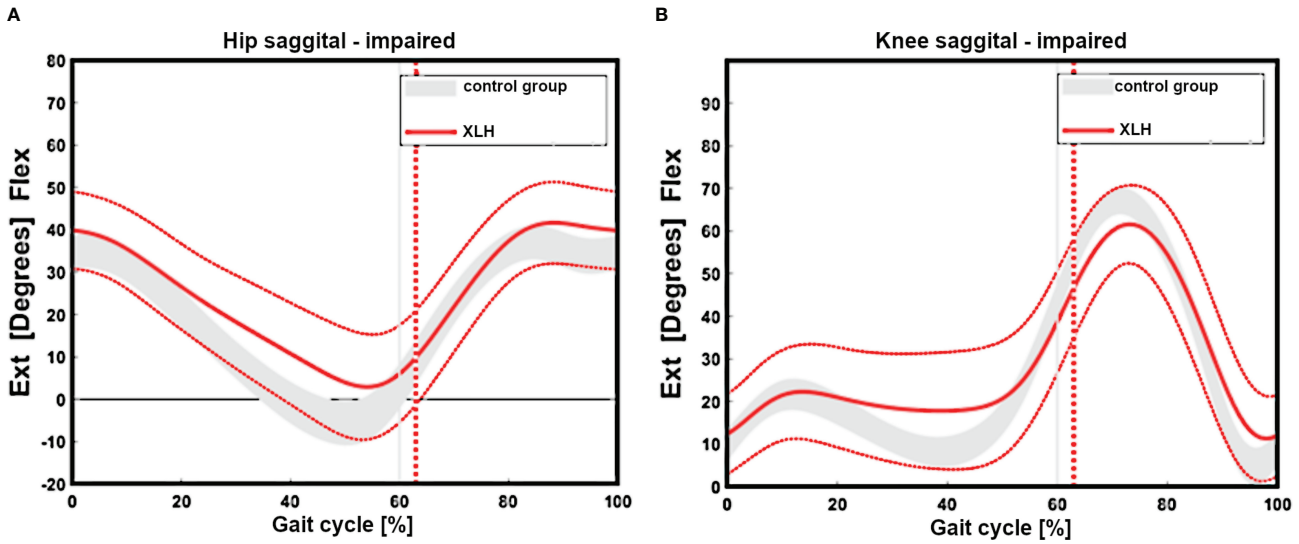

FIGURE 2 | Gait cycle analysis showing overall impaired sagittal range of motion of the hip (n = 29 patients) (reduced extension, **(A)**. Reduced extension during stance and flexion during swinging of the group with XLH (**B**, red line + 1 SD) *versus* the control group grey curved area.

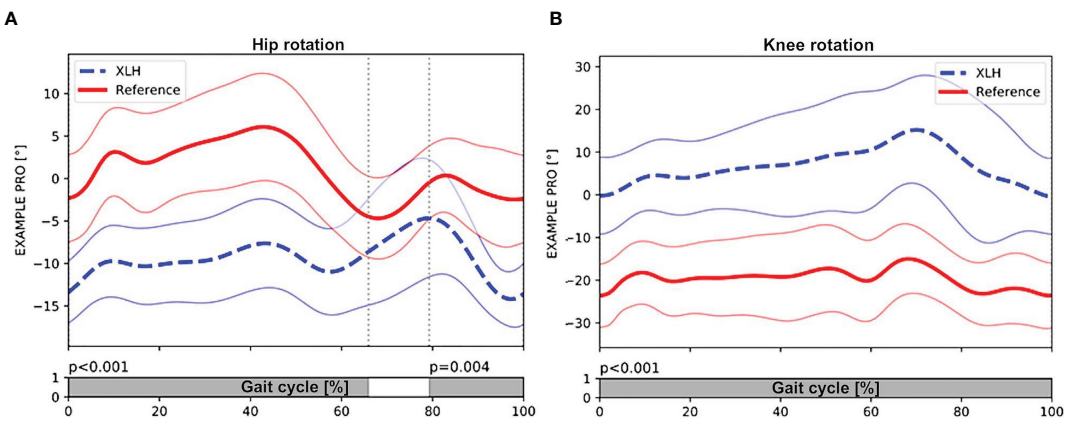

FIGURE 3 | Patients with XLH (n = 29 patients) (dotted blue line, + 1SD) had significant external rotation of the hip during stance ($p < 0.001$) and swing phase ($p = 0.004$) compared with the control group (solid red line, + 1 SD) **(A)**. **(B)** also showing significant internal rotation of the knee during the whole gait cycle ($p < 0.001$). Gray bar indicates the level of significance ($p < 0.005$) between the group with XLH and the control group during the gait cycle. Meanwhile, the white bar indicated non-significance ($p > 0.005$).

In total, 14 (48.3%) of 29 patients had a greater lateral trunk lean (waddling gait), defined as a frontal thorax ROM of > 7°. Patients with a higher BMI ($R^2 = 0.41$, $p < 0.001$) and those with varus knee deformity ($p = 0.002$, **Figure 4**) presented with a greater lateral trunk leaning. The proximal femoral hip angle (mLPFA) was not correlated with lateral trunk lean ($R^2 = 0.17$, $p = 0.241$). Furthermore, there was no association between thorax length in relation to leg length and lateral trunk lean ($p > 0.05$).

The tibial anterior bowing angle (procurvatum) was associated with a lower knee and ankle ROM (n=42 limbs, knee: $R^2 = 0.37$, $p < 0.001$; ankle: $R^2 = 0.21$, $p = 0.011$) and thus, gait quality (n = 21 patients, $R^2 = 0.27$, $p = 0.004$). However, the femoral anterior bowing angle (procurvatum) did not influence hip/knee motion or GDI significantly (n = 32, $p = 0.869$).

Patients with enthesopathies on anteroposterior radiographs of the lower limb had a low gait quality based on GDI ($p = 0.022$, **Figure 5**). A high BMI was associated with a low gait score (GDI: $R^2 = 0.32$, $p = 0.002$) and a greater lateral trunk lean ($R^2 = 0.17$, $p = 0.002$).

Standard laboratory parameters (n = 23) including serum calcium (Ca), phosphate, total alkaline phosphatase, and 25-hydroxy vitamin D [25(OH)D] levels were not associated with

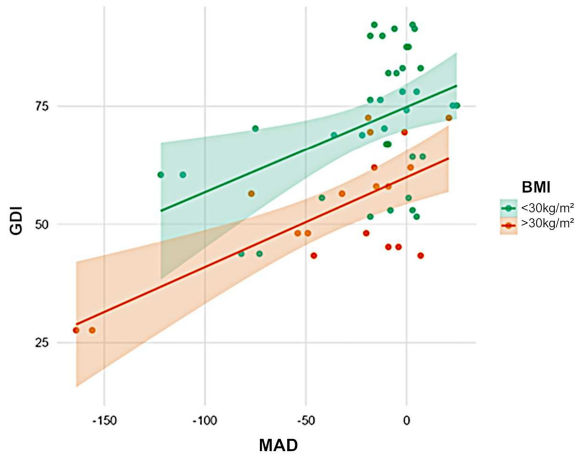

FIGURE 4 | Influence of weight and axial deviation on gait scores. Obesity (orange) was associated with low GDI scores (green). Furthermore, varus was negatively correlated with GDI. Obese patients with varus deformity had the lowest scores.

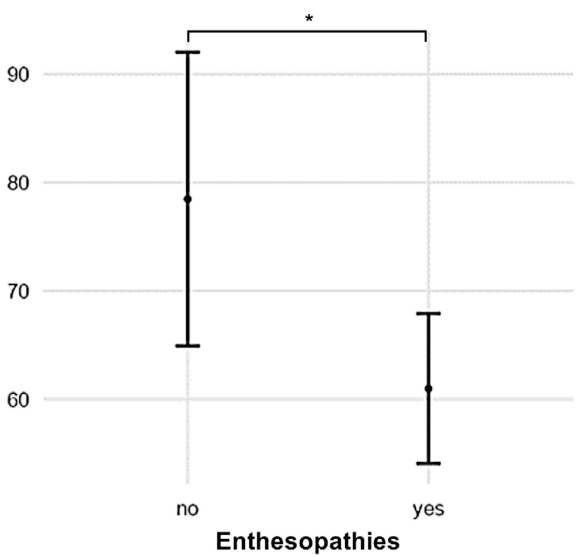

FIGURE 5 | Patients with radiographic signs of enthesopathies had low GDI scores compared with those without (p = 0.022). (* = p > 0.05).

GDI. Patients with a high BMI had a low 25(OH)D level. However, there was no difference in Ca or PTH levels.

DISCUSSION

The current study showed that adolescents and adults with XLH presented with a significantly low gait quality based on GDI as well as lateral trunk lean ("waddling"). Common features were complex lower limb deformities and signs of osteoarthritis, pseudofractures, and enthesopathy.

Lower limb deformity and gait impairment can substantially reduce QoL among adults with XLH (6). Aside from pain, fear of falling is common among adults with XLH, thereby resulting in reduced participation in social and public activities (23) and contributing to the burden of disease. Therefore, further studies that aim to not only identify and quantify contributing factors but also improve the multidisciplinary management of patients with XLH are warranted (6).

Lower limb alignment is important in biomechanical function, thus a normal gait. The characterization of biomechanical alterations in the lower limb among patients with XLH represents an essential prerequisite to assess the effects of medical or nonmedical interventions on factors contributing to burden of disease (24).

Although there are studies showing extremely high rates of lower limb deformity (12, 13, 25), there is currently no detailed measurement data regarding lower limb deformities among adults with XLH. Therefore, lower limb deformity among adults with XLH cannot be described and quantified. Zhang et al. (12) revealed that about 95.9% of patients with XLH (n = 217) presented with lower limb deformities. These were categorized as genu varum, genu valgum, and complex deformity. In XLH, lower limb deformity was defined as a complex deformity caused by additional anteroposterior (procurvatum) or torsional deformity, which was not described in previous studies (12, 13).

In our patient group, the severity of lower limb alignment deviations ranged from normal to severely pathologic (**Figures 6A–D** and **7A–D**). Compared to children with XLH who did not receive prior surgical intervention (9), adults had a lower incidence of valgus deformity and a higher incidence of varus deformity of the lower limb. However, similar rotational abnormalities with signs of a higher external femoral torsion and a lower external tibial torsion were observed during gait.

According to Horn et al. (7), patients who underwent frontal plane alignment correction and who had a neutral mechanical axis did not complain of significant residual torsional malalignment even though diaphyseal bowing was not completely corrected. Our data partly contradict these findings. Rotational deformity, including a high rate of intoeing, might have significantly contributed to gait deviations in adults with XLH, thereby causing burden of disease.

The pseudofracture rate (30%) in our study group was similar to that of other populations (13). Looser zones (pseudofractures) were found in adults with XLH, increasing with age It occurred in 28% of patients aged under 30 years. However, none of the patients aged under 18 years presented with pseudofractures (11). The youngest participant with pseudofractures on radiographic analysis was aged 17 years, and this patient underwent surgical intervention.

Anterior bowing of the tibia and femur (procurvatum), which is a sagittal plane deformity parameter, was not correlated with pseudofracture. Thus, this disease-specific radiographic finding may originate from other pathomechanisms compared with deformities and associated biomechanical strains only. Furthermore, our study did not show a correlation between the number of pseudofractures and gait quality. A previous study has shown the benefits of

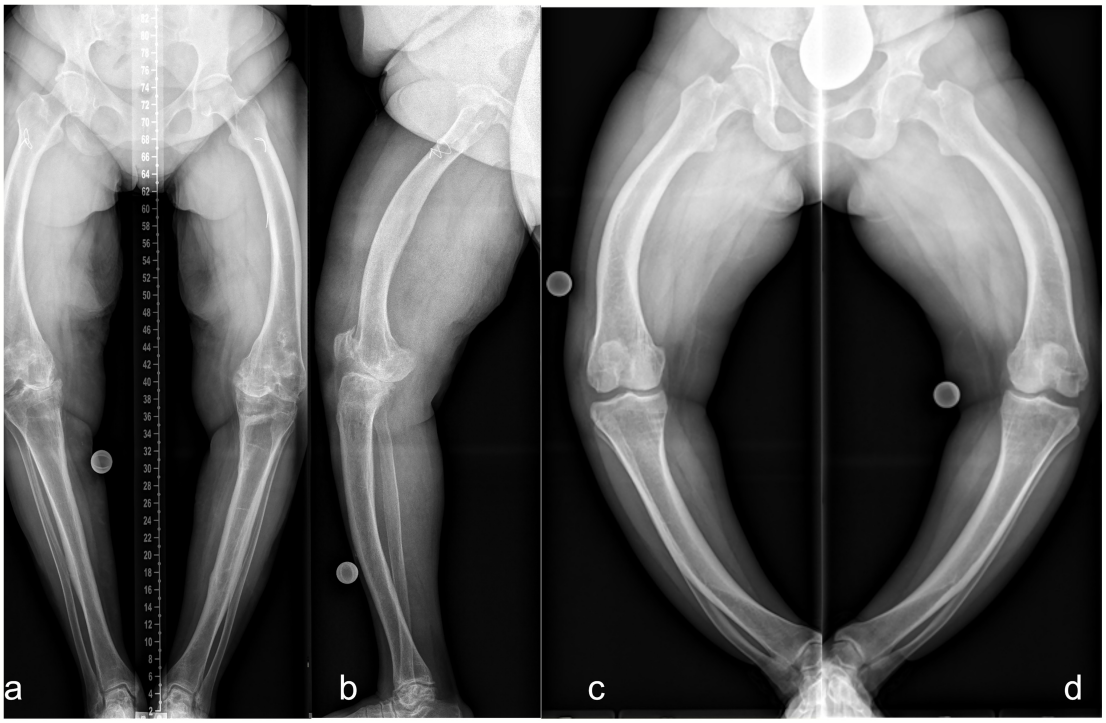

FIGURE 6 | Lower limb deformity of patients prior to surgical frontal plane correction at our hospital. Left (**A** frontal, **B** sagittal): 50 y/o, female, five prior surgeries, GDI: 43.7, BMI: 28.7 kg/m^2, conventional oral medication. Right: 33 y/o, female, no previous surgeries prior to presentation, GDI: 27.6, BMI: 37.6 kg/m^2. Full-length anteroposterior radiograph (hip to ankle) of both lower limbs in standing position was not performed due to severe varus deformity. Thus, radiograph image of each leg was taken individually **(C, D)**.

conventional treatment for the quality and quantity of bone mineralization among adult patients with XLH (26).

Enthesopathies and pseudofractures can significantly contribute to burden of disease (6). However, a reliable method for quantifying enthesopathies is yet to be developed (24). In this study, the negative influence of enthesopathies on gait quality confirmed the importance of this parameter among adults with XLH.

A murine model showed a higher occurrence of enthesopathies and joint structural alterations over time. Furthermore, all Hyp mice developed peripheral enthesopathies, thereby showing the mineralization of fibrochondrocytes at the achilles tendon and plantar fascia ligament insertions of the calcaneal tuberosity at the 12-month follow-up (27). Our study group showed comparable findings on detailed radiographic analysis. Thus, further research focusing on foot and ankle deformity among adults with XLH must be performed.

Enthesopathies may not respond adequately to conventional medical therapy among adults with XLH (28). Consequently, further research should be conducted to prevent a decrease in gait quality caused by this radiographic change.

The development of early and severe osteoarthritis is another highly relevant factor in the skeletal assessment of adults with XLH (6). Our cohort presented with a high rate of degenerative changes in the hip, knee, and ankle joints. However, only two patients underwent prior joint replacement surgeries.

Joint replacement has been effective in treating osteoarthritis among adults with XLH (29, 30). The surgical intervention was technically challenging due to severe deformity and poor bone quality (29).

Compared with the abovementioned studies, joint replacements in the current research occurred at a later age with consideration of the overall lower mean age of our study population. Joint replacement surgery may be postponed in patients with symptomatic osteoarthritis due to severe lower limb deformity. Hence, such a notion should be considered. In our study group, this finding was observed in at least three patients.

Steele et al. (10) comprehensively described the skeletal affection of the upper and lower extremities and spine in adults (n = 9). Degenerative changes in the entire skeletal apparatus were high. The gait characteristics (higher lateral trunk lean, lower hip extension and knee extension during stance) were similar to those reported in our study. However, an analysis of lower limb deformity was not performed, and there were no details regarding rotational and torsional deformities.

Lower limb alignment influences thorax movement among patients with XLH (9). Waddling is described as a disease-specific gait deviation in patients with XLH (9, 10, 31). However, this term has not been clearly defined, and its use might be inappropriate when considering patients' demands in the context of the psychosocial aspects of burden of disease. The term waddling was

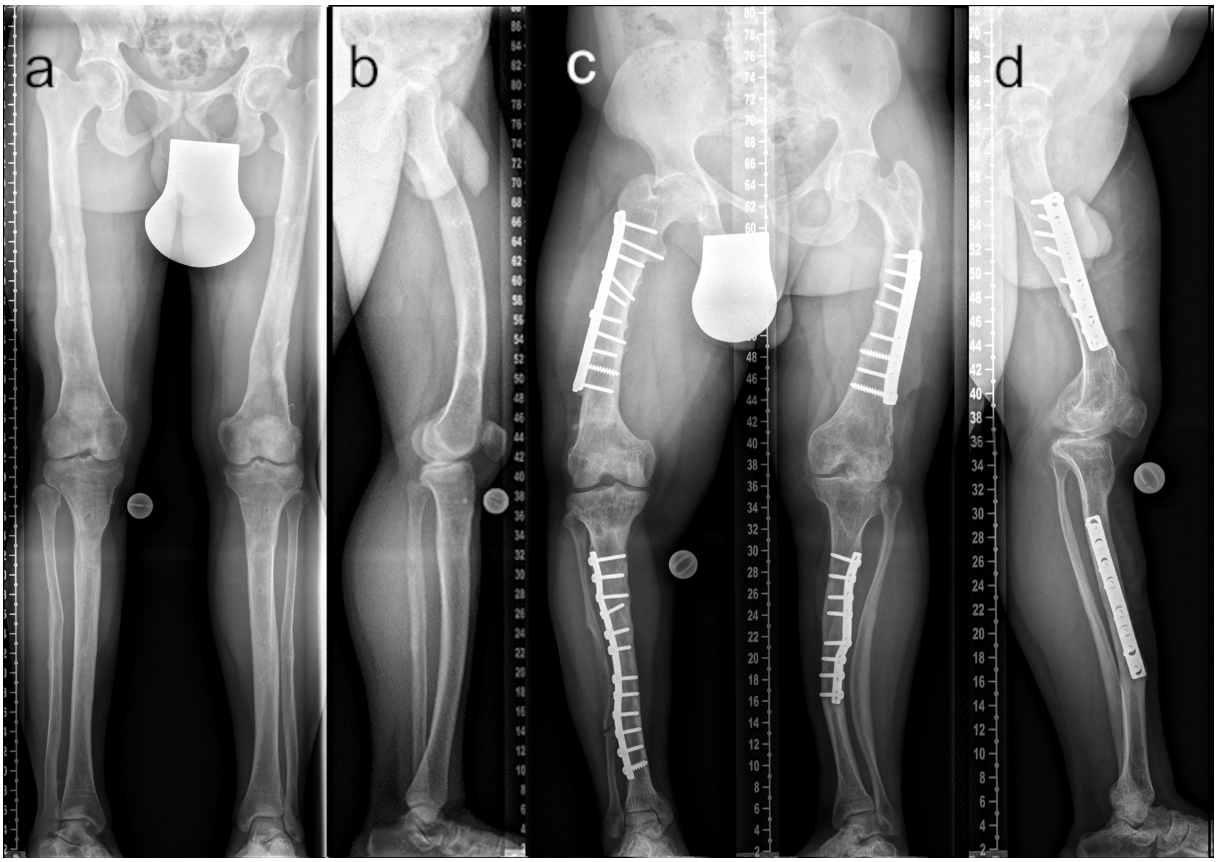

FIGURE 7 | Comparison of adequate (**A** ap; **B** lateral) and insufficient (**C** ap; **D** lateral) surgical frontal and sagittal plane correction. The patient depicted in **(A, B)** underwent multilevel lower limb correction at our hospital (28 y/o, male, GDI: 62.0, BMI: 38.3 kg/m^2, conventional oral medication). The patient depicted in **(C, D)** presented with multiple prior surgeries performed elsewhere (37 y/o, male, GDI: 43.3, BMI: 33.3 kg/m^2, conventional oral medication).

replaced with increased lateral trunk lean and found similarly high rates in adults compared to children with XLH (9). The increased lateral trunk lean as the compensation mechanism of varus limb deformity could again be observed in adults similar to prior reports of children with XLH (9). We hypothesized that patients with a longer thorax (in relation to leg length) were more likely to have a greater lateral trunk lean during gait, which was not the case in our series. LPFA, which is an indicator of frontal hip deformity (varus/valgus), was not correlated with lateral thorax motion. However, it might influence hip abductor moment due to a change in lever arm. Therefore, gluteal insufficiency and proximal femoral varus deformity may not be the main causes of an increased lateral trunk lean among patients with XLH.

The current study first revealed the impact of a high BMI on gait quality among adults with XLH. Patients with a high BMI presented with lower gait scores and a higher incidence of increased lateral trunk lean. While the value of BMI measurement in populations with short stature is discussed controversially, these data contribute to characterize the role of metabolic control in XLH. In line, previous studies have shown a high prevalence of overweight in adult XLH populations (32) and the correlation between a high BMI and a lower gait quality among children with XLH (9).

Most adults with XLH had joint stiffness (5), which significantly contribute to disease burden. The current study showed a significant decrease in hip, knee, and ankle joint ROM during gait. Thus, stiffness is a clinically relevant and measurable parameter. Interestingly, procurvatum of the tibia (anterior bending) was associated with a decreased ankle and knee ROM and overall gait quality (gait deviation index). Thus, joint stiffness, which is a typical symptom of XLH, might be aggravated by sagittal plane deformity.

This study shows the importance of lower limb deformity correction in improving gait among patients with XLH. Femoral and tibial varus deformity, torsional deformity, and tibial procurvatum deformity and a higher BMI may contribute to a lower gait quality. Patients with prior alignment surgeries are more likely to present with lower rotational deviations. However, due to group heterogeneity, further comparison was not performed.

Limitations

This study had several limitations. Included patients received regular medical care at multiple medical facilities across Austria

and Europe, as reflected by extremely diverse pharmacological treatment regimens at the time of examination as well as extensive heterogeneous histories of medical treatment. Most patients were in the crucial phase of linear growth prior to the development of current treatment standards and multidisciplinary approaches for rare bone disorders. The surgically treated patients underwent various and multiple surgical procedures, thereby reflecting the high osteotomy rates within the last decades, which further contributed to group heterogeneity. Although the diversity of medical and surgical history reflects real-life clinical settings in XLH care, the outcomes of this study might be blurred by inhomogeneity. Nevertheless, the finding of several specific characteristics in this cohort underline the relevance and applicability of our outcomes in defiance of phenotypic and therapeutic variability in XLH.

Whilst symptoms and burden of disease were assessed retrospectively in this study, functional or QoL scoring was not available in the analysis. Complete (preoperative) lower limb deformity assessment includes lateral full leg radiography and torsional magnetic resonance imaging or computed tomography scan. However, these additional examinations were not accessible to all patients in this study. Gait analysis can examine the rotational and torsional components of lower limb deformity (9, 33). To detect the possible influence of spinal degeneration on gait, spine radiograph might be necessary. Furthermore, future studies of a spine motion marker should be conducted to assess spine movement among adult patients with XLH.

To minimize ionizing radiation on the gonads, all standardized long leg radiography images were obtained using a gonad shield based on the local standards of care. However, this protective measure inhibits the detection of changes in the sacroiliac joint and enthesopathies of the sacrospinous ligament.

Due to disease-specific maltorsion, lateral radiographic angles were challenging to measure in some cases; therefore, only the procurvatum shaft angle was evaluated.

CONCLUSION

Adolescents and adults with XLH presented with an increased lateral trunk lean, lower gait scores, osteoarthritis, enthesopathies, and pseudofractures. This study for the first time showed that lower limb deformity (such as MAD, lateral bending, and malrotation), BMI, and features specific in XLH (such as enthesopathies) negatively affected gait quality among adolescents and adults with XLH. Therefore, patients with XLH require a comprehensive and adequate treatment in a multidisciplinary setting. Assessment of lower limb deformities and gait deviations should be implemented in future diagnostic and therapeutic studies to address this important aspect of disease burden in XLH patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethikkommission der Wiener Krankenhäuser der Vinzenz Gruppe Gumpendorferstraße 108 1060 Wien ethikkommission.wien@vinzenzgruppe.at (EK37/2020). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

GM, AK, AS, RK, AR, RG, CR, and GH contributed to conception and design of the study. GM and AS wrote first draft of manuscript. AR wrote sections of the manuscript. GM, AS, RK, GH, and AR did clinical data collection. AK and AR performed statistical analysis. AK collected gait data. GM, AK, AS, and AR performed data analysis. All authors contributed to the article and approved the submitted version.

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Vitamin D Status in Children With Short Stature: Accurate Determination of Serum Vitamin D Components Using High-Performance Liquid Chromatography–Tandem Mass Spectrometry

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Objective: Vitamin D is critical for calcium and bone metabolism. Vitamin D insufficiency impairs skeletal mineralization and bone growth rate during childhood, thus affecting height and health. Vitamin D status in children with short stature is sparsely reported. The purpose of the current study was to investigate various vitamin D components by high-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) to better explore vitamin D storage of short-stature children *in vivo*.

Methods: Serum circulating levels of 25-hydroxyvitamin D2 [25(OH)D2], 25-hydroxyvitamin D3 [25(OH)D3], and 3-epi-25-hydroxyvitamin D3 [3-epi-25(OH)D3, C3-epi] were accurately computed using the LC-MS/MS method. Total 25(OH)D [t-25(OH)D] and ratios of 25(OH)D2/25(OH)D3 and C3-epi/25(OH)D3 were then respectively calculated. Free 25(OH)D [f-25(OH)D] was also measured.

Results: 25(OH)D3 and f-25(OH)D levels in short-stature subgroups 2 (school age: 7~12 years old) and 3 (adolescence: 13~18 years old) were significantly lower compared with those of healthy controls. By contrast, C3-epi levels and C3-epi/25(OH)D3 ratios in all the three short-stature subgroups were markedly higher than the corresponding healthy cases. Based on cutoff values developed by Endocrine Society Recommendation (but not suitable for methods 2 and 3), sufficient storage capacities of vitamin D in short-stature subgroups 1, 2, and 3 were 42.8%, 23.8%, and 9.0% as determined by Method 3 [25(OH)D2/3+25(OH)D3], which were lower than those of 57.1%, 28.6%, and 18.2% as determined by Method 1 [25(OH)D2+25(OH)D3+C3-epi] and 45.7%, 28.5%, and 13.6% as determined by Method 2 [25(OH)D2/3+25(OH)D3+C3-epi]. Levels of 25(OH)D2 were found to be weakly negatively correlated with those of 25(OH)D3, and higher 25(OH)D3

levels were positively correlated with higher levels of C3-epi in both short-stature and healthy control cohorts. Furthermore, f-25(OH)D levels were positively associated with 25 (OH)D3 and C3-epi levels in children.

Conclusions: The current LC-MS/MS technique can not only separate 25(OH)D2 from 25(OH)D3 but also distinguish C3-epi from 25(OH)D3. Measurement of t-25(OH)D [25(OH) D2+25(OH)D3] alone may overestimate vitamin D storage in children, and short-stature children had lower vitamin D levels compared with healthy subjects. Ratios of C3-epi/25 (OH)D3 and 25(OH)D2/25(OH)D3 might be alternative markers for vitamin D catabolism/ storage in short-stature children. Further studies are needed to explore the relationships and physiological roles of various vitamin D metabolites.

Keywords: short stature, 25-hydroxyvitamin D2, 25-hydroxyvitamin D3, 3-epi-25(OH)D3, liquid chromatography–tandem mass spectrometry (LC-MS/MS)

INTRODUCTION

Short stature is a global public health problem (1). It is defined statistically as height less than 2 standard deviations (SD) of age- and sex-matched population (1, 2). Short stature with general health can lead to several physical or psychological concerns in modern society. Severe short stature is vulnerably linked with diverse developmental, educational, and social problems especially for children (3).

Stature is hereditary trait regulated by both genetic and environmental factors. Manipulation of environmental factors may be an effective strategy to maximize the growth potential of children (4). Short stature is associated with various underlying environmental factors, including inadequate dietary intake (4), essential nutrition or trace element deficiency (5), and exposure to environmental pollutants (6). Vitamin D plays essential roles in function and maintenance of bone health by regulating calcium and phosphate homeostasis throughout life (7). Previous studies established that vitamin D deficiency reduces skeletal mineralization and bone growth rate (8). Infants and young children are special risk groups of vitamin D deficiency due to their rapid growth with high nutritional requirements. However, there is paucity of data on levels of vitamin D status in short-stature children. Therefore, there is a need to explore the relationships between vitamin D status and patients with short stature.

Circulating 25-hydroxyvitamin D [25(OH)D] is currently widely used as a functional indicator for vitamin D status (9), which mainly comprises two biologically inactive precursors including 25-hydroxyvitamin D2 [25(OH)D2] and 25-hydroxyvitamin D3 [25(OH)D3] (10, 11). 25(OH)D2 is mainly sourced from plants and only enters body *via* diet, whereas 25 (OH)D3 is endogenously synthesized in skin *via* UV irradiation of 7-dehydrocholesterol (12). Several studies had considered that 25(OH)D2 is as effective as 25(OH)D3 in improving bone health (13), while others averred that 25(OH)D3 is more potent than 25 (OH)D2 in maintaining 25(OH)D levels, with a differential potency of at least 3-fold (14). Both 25(OH)D2 and 25(OH)D3 should be tested simultaneously to comprehensively assess vitamin D status. So far, few studies have quantified both 25 (OH)D2 and 25(OH)D3 levels in children with short stature. Thus, there is urgent need to explore the internal relationships between 25(OH)D2 and 25(OH)D3 status and short stature disease.

Recent studies reported that vitamin D3 metabolites are further metabolized through the C3-epimerization pathway (15). 25(OH)D3 undergoes epimerization in the liver to produce 3-epi-25-hydroxyvitamin D3 [3-epi-25(OH)D3, C3-epi] (the hydroxyl group in the C-3 position of A-ring changes from α to β orientation) (16). Although the physiological role of C3-epi is still obscure, previous studies have reported elevated C3-epi proportions in mothers and newborns, indicating importance of epimers in pregnancy and early development (17, 18). However, C3-epi presumably does not function as a storage pool because 3-epimerization is irreversible. Some studies showed that C3-epi has weaker calcemic regulatory effects compared with its non-epimeric form (19). It induces phospholipid synthesis in pulmonary alveolar type II cells and suppresses parathyroid hormone secretion with comparable amounts with non-epimeric metabolites (20, 21). Conversely, its conversion product in kidneys, namely, 3-epi-1α,25(OH)2D3, performs stronger differentiation or anti-proliferative activities than non-epimeric compounds *in vitro* (22). Additionally, it has greater metabolic stability compared with 1α,25(OH)2D3 despite having inequivalent binding strength to vitamin D receptors (VDR). This allows 3-epi-1α,25(OH)2D3 to remain in free form and hence participate in physiological processes (22). Numerous studies have found that the identical molecular weight and molecular physical–chemical property of C3-epi may lead to inaccuracies in 25(OH)D3 measurements. These findings render the necessity for specific separate detection of 25(OH)D3 and C3-epi (23). Liquid chromatography–tandem mass spectrometry (LC-MS/MS) technology can quantify 25(OH)D2 and 25(OH)D3 and distinguish C3-epi from 25(OH)D3 simultaneously. The purpose of the current study was to explore levels of 25(OH)D2, 25(OH)D3, and C3-epi in short-stature children using the LC-MS/MS method. Values of total 25 (OH)D [t-25(OH)D] as well as ratios of C3-epi/25(OH)D3 and

25(OH)D2/25(OH)D3 were then computed. The results of the current study are expected to provide a scientific bearing for the diagnosis, treatment, and prognosis evaluation of children with short stature.

PARTICIPANTS AND METHODS

Study Participants

The current study recruited patients who visited the child healthcare department for short-stature problems between January 2017 and January 2021 in Mianyang Central Hospital, Sichuan Province, China, as study participants. Clinically, individual diagnostic categories are often indistinguishable, and the demarcation of diagnoses often leads to joint diagnoses. Therefore, the current study summarized all subtypes under general term and boundary definition of short stature but excluded those caused by genetic, syndromic, organic, and psychosocial conditions.

The diagnosis of short stature was based on a previous diagnostic guideline (24). The height of the children was determined in relevance to their age, health status, family, and history of development. Their physical parameters (weight, height of sitting posture) and external signs of genetic conditions were also recorded. The bone age of each child was determined from X-ray images of the hands and wrists. Those with heights exceeding two standard deviations (SD) below average height of the corresponding gender and age, as stipulated in the standards for Chinese children and adolescents, were included in the study.

Exclusion criteria included children with other conditions such as growth hormone deficiency, multiple pituitary hormone deficiency, hypothyroidism, skeletal development disorder, intracranial tumor, chromosomal disease, chronic systemic disease, familial short stature, physical puberty delay, severe malnutrition, and other known causes of short stature. Participants who had received growth hormone, gonadotropin releasing hormone, or antihypertensive treatment were also excluded by a qualified pediatrician.

Healthy participants were assigned into the control group. The current study was approved by the Medical Ethics Committee of Mianyang Central Hospital.

Collection of Blood Samples

Venous blood was collected between 6:00 and 10:00 a.m. after overnight fasting to eliminate the influence of diet on serum measurements. The blood was centrifuged at 3,000 rpm for 15 min to obtain serum.

Determination of 25(OH)D2, 25(OH)D3, and 3-epi-25(OH)D3 by LC-MS/MS

This was undertaken based on our previously described study (25). Briefly, 10 µl of mixed internal standard was added to 200 µl of serum samples and then mixed with 1,000 µl of extraction solution (tert-butyl methyl ether). The supernatant was collected after vortex and centrifugation. Resulting solutions were dried under nitrogen gas and redissolved in 125 µl of methanol with 0.1% formic acid. The mixture was then vortex-mixed and centrifuged at 13,000 rpm for 5 min, and the resulting supernatant was transferred to a 96-well sample plate, which was then sealed and transferred to an autosampler. Calibrators and quality controls were prepared based on the same procedure.

Chromatographic analysis was performed on a Shimadzu LC-30AD UHPLC system equipped with a Kinetex 2.6 µm C8 100A column. Mobile phase A consisted of water with 0.1% acetic acid, and mobile phase B consisted of methanol with 0.1% acetic acid. Fifteen microliters of the sample solutions was injected into the LC system using a column temperature of 45°C and a flow rate of 0.6 ml/min. Mass spectrometer detection and quantification were undertaken in positive mode using multiple reaction monitoring (MRM) mode. Optimized parameters for mass detection were as follows: curtain gas was 35 psi; temperature was 550°C; ion spray voltage was 5,500 V; gas 1 and gas 2 (nitrogen) were both set at 60 psi; and the dwell time was 100 ms. Analyst® MD software (version number: 1.6.3) was used for chromatogram output, and MultiQuant™ MD software (version number: 3.0.2) was performed for data processing.

Detection of f-25(OH)D

The free 25(OH)D ELISA kit was obtained from DIAsource ImmunoAssays SA (Belgium) to detect f-25(OH)D levels. The assay was calibrated against Rate Dialysis, which is the gold standard method for the determination of free hormones. Final concentrations were analyzed using the RT-6100 enzyme label analyzer (Redu Life Science Co., Ltd., Shenzhen, China) based on kit instructions.

Evaluation of Vitamin D Nutritional Status

The capacity of vitamin D3 to store vitamin D is two to three times higher compared with that of vitamin D2. To provide alternative methods for accurate or sufficient vitamin D storage converted into active vitamin D [$1,25(OH)_2D$], three different computation methods were applied, including 25(OH)D2+25(OH)D3+C3-epi (Method 1), 25(OH)D2/3+25(OH)D3+C3-epi (Method 2), and 25(OH)D2/3+25(OH)D3 (Method 3) (25). Method 1 is most widely applied to determine t-25(OH)D values using various immunological assays, whereas Methods 2 and 3 may better represent vitamin D storage converted into $1,25(OH)_2D$. Method 3 is considered more suitable for the determination of the active status of 25(OH)D in circulation after removal of C3-epi from 25(OH)D3. Recommendations of Endocrine Society aver that a 25(OH)D concentration of <20 ng/ml is indicative of vitamin D deficiency, whereas a concentration in the range of 21–29 ng/ml indicates insufficiency. In addition, a concentration of >30 ng/ml is considered sufficient (11).

Statistical Analyses

Statistical analyses were performed using SPSS 25.0 software (International Business Machines Corp., USA). Data were expressed as mean ± standard deviation (SD) for normally distributed continuous data and analyzed using Student's t-test between two study groups. The M=median and interquartile range (IQR) were selected for non-normally distributed variables and analyzed by Mann–Whitney U tests. One-way ANOVA was used to analyze differences between means of more than two

groups for equal variances. Welch's approximate analysis was used followed by Dunnett's T3 test if the variances are uneven. The strength of the relationship between selected metabolite parameters and commonly used fasting lipid profiles was determined using Pearson or Spearman bivariate correlation analysis for normal or skew distribution. $p < 0.05$ was considered statistically significant.

RESULTS

General Characteristics of Participants

A total of 99 eligible short-stature children aged between 1 and 18 years, including 45 males and 54 females, were recruited in the current study. In addition, 186 healthy participants were assigned to the control group, among whom were 86 males and 100 females. Influence of age on outcomes was minimized by grouping children into three subgroups: Subgroup 1 (preschool age) aged between 1 and 6 years; Subgroup 2 (school age) aged between 7 and 12 years; and Subgroup 3 (adolescence) aged between 13 and 18 years. **Table 1** shows the basic clinical characteristics of participants. The mean height SDS of the short-stature group was -2.87 ± 0.34. Patients with short stature had significantly lower height and weight compared with healthy controls (both $p < 0.001$). No significant differences in age ($t = 1.17$, $p = 0.367$), sex ($\chi 2 = 0.25$, $p = 0.426$), and BMI ($t = 1.173$, $p = 0.242$) were observed between short-stature children and healthy controls. Furthermore, biochemical indices including calcium (Ca), phosphate (PHOS), free triiodothyronine (FT3), free thyroxine (FT4), thyroid-stimulating hormone (HTSH), parathyroid hormone (PTH), and alkaline phosphatase (ALP), which are associated with children growth and development, were also not statistically significant between the two study groups.

Levels of Serum Vitamin D Components

Findings of the current study showed that both serum levels of 25(OH)D3 ($t = 3.825$, $p < 0.001$; $t = 3.121$, $p = 0.003$) and f-25(OH)D ($t = 3.848$, $p = 0.002$; $t = 2.282$, $p = 0.017$) in subgroups 2 and 3 of short-stature patients were significantly lower compared with those of healthy controls, whereas C3-epi levels ($z = 2.548$, $p = 0.023$; $z = 3.282$, $z = 0.007$; $z = 4.848$, $p < 0.001$) and C3-epi/25(OH)D3 ratios ($z = 2.845$, $p = 0.022$; $z = 2.285$, $z = 0.027$; $z = 3.788$, $p = 0.002$) were all markedly higher in subgroups 1, 2, and 3 than those of healthy participants for the corresponding control subgroups (**Figure 1**). We further compared levels of serum vitamin D components among different subgroups. Findings showed significant statistical differences in circulating 25(OH)D3 ($F = 35.63$, $p < 0.001$), C3-epi ($H = 28.62$, $p < 0.001$), and f-25(OH)D ($F = 31.25$, $p < 0.001$) levels as well as C3-epi/25(OH)D3 ratios ($H = 19.65$, $p < 0.001$) among various subgroups. Generally, increase in age correlated with decrease in all the studied indicators.

Evaluation of Vitamin D Nutritional Status

Percentages of vitamin D status among subgroups in short-stature and healthy participants are presented in **Table 2**. Specifically, sufficient storage capacities of vitamin D in short-stature subgroups were only 42.8%, 23.8%, and 9.0% as determined by Method 3, which were lower compared with those of 57.1%, 28.6%, and 18.2% as determined by Method 1 and 45.7%, 28.5%, and 13.6% as determined by Method 2. Notably, the current universally accepted clinical cutoffs (developed by Endocrine Society Recommendation) for vitamin D status were established using immunoassays, which are incapable of isolating C3-epi. Therefore, they are not suitable to evaluate the vitamin D status using Methods 2 and 3, which are likely to overestimate proportions of vitamin D deficiency and insufficiency. The current study just provided two alternative methods here to represent accurate or sufficient vitamin D storage converted into active vitamin D [1,25$(OH)_2$D]. Appropriate cutoff values for these forms of vitamin D need to be defined further, given the lack of consensus on adequate levels of vitamin D.

Vitamin D storage in all subgroups was determined, and results are presented in **Table 3**. Short-stature children in

TABLE 1 | General characteristics of the study cohort.

Items	Healthy control (n = 186)	Short stature (n = 99)	p values
Sex (male/female)	86/100	45/54	*0.426*
Age (years)	8.5 ± 2.5	8.3 ± 1.9	*0.367*
Height (cm)	139.32 ± 17.03	125.84 ± 20.04	*<0.001*
Height SDS	0.5 (-0.2, 1.0)	-2.87 (-2.7, -3.1)	*<0.001*
Weight (kg)	34.54 ± 11.17	29.03 ± 11.19	*<0.001*
Weight SDS	0.65 ± 0.21	-0.81 ± 0.24	*<0.001*
BMI (kg/m^2)	17.20 ± 1.87	17.48 ± 1.69	*0.242*
BMI SDS	0.20 (-0.19, 0.73)	0.31 (-0.12, 0.68)	*0.196*
Ca (mmol/L)	2.51 ± 0.13	2.52 ± 0.10	*0.662*
PHOS (mmol/L)	1.66 ± 0.21	1.68 ± 0.16	*0.659*
FT3 (pg/mL)	3.69 ± 0.48	3.95 ± 0.46	*0.331*
FT4 (ng/dL)	1.10 ± 0.11	0.99 ± 0.22	*0.439*
HTSH (μIU/mL)	2.30 ± 1.19	2.57 ± 1.51	*0.062*
PTH (pg/mL)	32.56 ± 17.92	36.87 ± 18.72	*0.693*
ALP (U/L)	254.52 ± 78.15	249.81 ± 76.29	*0.591*

Ca, calcium; PHOS, phosphate; FT3, free triiodothyronine; FT4, free thyroxine; HTSH, thyroid-stimulating hormone; PTH, parathyroid hormone; ALP, alkaline phosphatase.
Data were expressed by mean ± SD, or median (P25, P75).
The p value determines statistical significance between the two compared groups. P<0.001 was considered statistically significant.

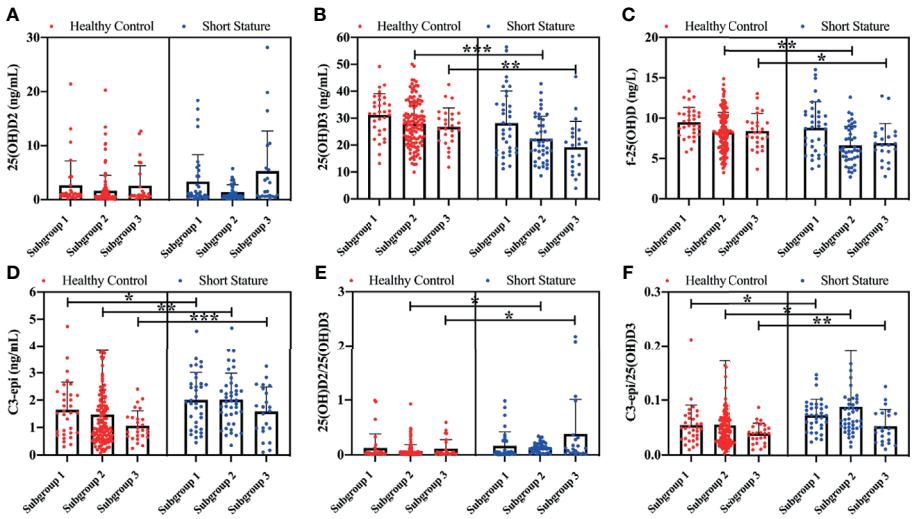

FIGURE 1 | Comparison of various vitamin D components between short stature and healthy control subgroups. Levels of 25(OH)D2 **(A)**, 25(OH)D3 **(B)**, f-25(OH)D **(C)**, and C3-epi **(D)**, and ratios of 25(OH)D2/25(OH)D3 **(E)** and C3-epi/25(OH)D3 **(F)** for all subgroups. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant. 1) Subgroup 1 (preschool age): 1~6 years old; 2) Subgroup 2 (school age): 7~12 years old; and 3) Subgroup 3 (adolescence): 13~18 years old.

subgroup 2 had significantly lower vitamin D levels (t = 4.575, p < 0.001; t = 4.212, p < 0.001; t = 2.861, p = 0.005) compared with healthy children as determined by all the three methods, whereas vitamin D levels in short-stature children in subgroup 3 only significantly decreased in comparison to healthy participants as determined by Methods 1 and 3 (t = 2.568, p = 0.007; t = 6.115, p < 0.001). Moreover, children in subgroup 1 had markedly higher vitamin D levels compared with those in subgroups 2 and 3 as determined by the three methods irrespective of short-stature subgroups (Subgroups 1 *vs.* 2: t = 6.313, p < 0.001; t = 6.103, p < 0.001; t = 5.564, p < 0.001. Subgroups 1 *vs.* 3: t = 5.896, p < 0.001; t = 6.589, p < 0.001; t = 6.352, p < 0.001) or groups of healthy children (Subgroups 1 *vs.* 2: t = 2.561, p = 0.012; t = 2.458, p = 0.020; t = 2.313, p = 0.020. Subgroups 1 *vs.* 3: t = 3.131, p = 0.004; t = 3.125, p = 0.005; t = 3.025, p = 0.004.). However, there were no statistically significant differences in vitamin D levels between subgroups 2 and 3.

Associations of Serum 25(OH)D2, 25(OH)D3, and t-25(OH)D Levels

Analysis of the results shown in **Figure 2** revealed a weak negative correlation between 25(OH)D2 and 25(OH)D3 levels in short-stature and healthy control cohorts. Higher serum 25(OH)D3 concentrations were positively associated with higher values of C3-epi. However, there was no association between serum 25(OH)D2 levels and C3-epi concentrations. In addition, f-25(OH)D levels were positively correlated with 25(OH)D3 and C3-epi levels in both cohorts but were only positively connected with 25(OH)D2 in the short-stature group.

DISCUSSION

Several methods including radioimmunoassay, ELISA, and chemiluminescence have been utilized for determination of

TABLE 2 | Evaluation of the subject's vitamin D nutritional status [%(case/total)].

Subjects		Healthy Control			Short Stature		
		Subgroup 1	Subgroup 2	Subgroup 3	Subgroup 1	Subgroup 2	Subgroup 3
Methods 1	Deficiency	0 (0/32)	10.9 (14/128)	3.8 (1/26)	14.3 (5/35)	35.7 (15/42)	22.7 (5/22)
	Insufficiency	25.0 (8/32)	34.4 (44/128)	46.2 (12/26)	28.6 (10/35)	35.7 (15/42)	59.1 (13/22)
	Sufficiency	75.0 (24/32)	54.7 (70/128)	50.0 (13/26)	57.1 (20/35)	28.6 (12/42)	18.2 (4/22)
Methods 2	Deficiency	3.1 (1/32)	13.3 (17/128)	15.4 (4/26)	22.9 (8/35)	40.5 (17/42)	45.5 (10/22)
	Insufficiency	25.0 (8/32)	38.3 (49/128)	46.2 (12/26)	31.4 (11/35)	31.0 (13/42)	40.9 (9/22)
	Sufficiency	71.9 (23/32)	48.4 (62/128)	38.4 (10/26)	45.7 (16/35)	28.5 (12/42)	13.6 (3/22)
Methods 3	Deficiency	3.1 (1/32)	14.9 (19/128)	15.4 (4/26)	28.6 (10/35)	42.9 (18/42)	45.5 (10/22)
	Insufficiency	34.4 (11/32)	45.3 (58/128)	50.0 (13/26)	28.6 (10/35)	33.3 (14/42)	45.5 (10/22)
	Sufficiency	62.5 (20/32)	39.8 (51/128)	34.6 (9/26)	42.8 (15/35)	23.8 (10/42)	9.0 (2/22)

Method 1 = 25(OH)D2+25(OH)D3+C3-epi. Method 2 = 25(OH)D2/3 + 25(OH)D3+C3-epi. Method 3 = 25(OH)D2/3 + 25(OH)D3.

TABLE 3 | Evaluation of vitamin D storage in subjects.

Subjects	Healthy control			Short stature			F, p value
	Subgroup 1	Subgroup 2	Subgroup 3	Subgroup 1	Subgroup 2	Subgroup 3	
VitD storage by Method 1 (ng/mL)	35.45 ± 6.91	31.14 ± 9.15[1]	30.39 ± 7.45[2]	33.08 ± 12.27	24.76 ± 8.89[3,a]	25.06 ± 9.24[4,b]	7.316, *p<0.001*
VitD storage by Method 2 (ng/mL)	33.70 ± 7.58	30.05 ± 8.87[1]	28.69 ± 7.11[2]	30.85 ± 12.34	23.84 ± 8.83[3,a]	21.55 ± 9.10[4]	7.964, *p<0.001*
VitD storage by Method 3 (ng/mL)	32.05 ± 7.15	28.58 ± 8.34[1]	27.63 ± 6.89[2]	29.30 ± 11.60	22.81 ± 8.39[3,a]	20.98 ± 8.74[4,b]	7.517, *p<0.001*

[a]*Compared with healthy children in subgroup 2, p < 0.05.*
[b]*Compared with healthy children in subgroup 3, p < 0.05.*
[1,2]*Compared with children in subgroup 1 in the healthy cohort, p < 0.05.*
[3,4]*Compared with children in subgroup 1 in the short stature group, p<0.05.*

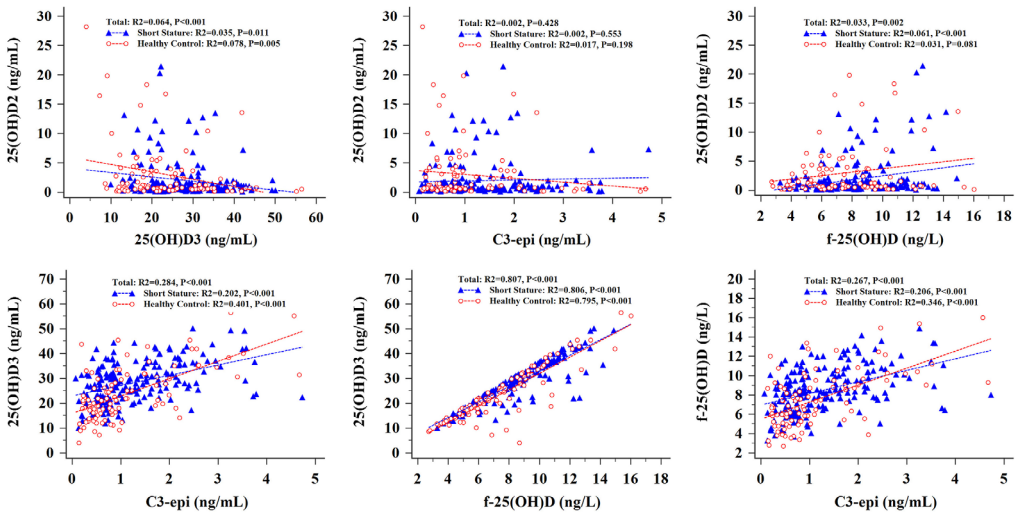

FIGURE 2 | Correlation analysis of various vitamin D components in short stature and healthy control groups.

t-25(OH)D. However, they are incapable of separating 25(OH)D2, 25(OH)D3, and C3-epi from t-25(OH)D effectively (25). It has been reported that the potential of 25(OH)D3 in maintaining 25(OH)D levels is 2–3-fold better than 25(OH)D2, and the physiological importance of the C3-epi is not as yet very well known. Therefore, even the same values of t-25(OH)D may play differing physiological roles because of the different compositions of vitamin D components. LC-MS/MS can detect 25(OH)D2 and 25(OH)D3 levels simultaneously and distinguish C3-epi from 25(OH)D3. Thus, it is considered the "gold standard" method for measurement of vitamin D status. In the present study, LC-MS/MS was used to determine the serum levels of 25(OH)D2 and 25(OH)D3 and C3-epi levels in children with short stature, thereby accurately providing more information about the nutritional status of this disease.

Several previous studies have reported inverse associations between 25(OH)D concentrations and PTH levels in humans, but these findings were commonly observed in adults and old people (11). PTH levels in children included in the current study did not show dramatic elevations when serum 25(OH)D decreased significantly. This observation was consistent with findings of a previous report (26). This may be explained by the possibility that different mechanisms regulate the secretion of PTH during childhood and adolescence unlike in adults (27). Moreover, it is likely that 1,25(OH)2D, but not 25(OH)D, can directly influence PTH secretion (27, 28) and modulate the balance in calcium/phosphate and bone health by binding to the VDR (18). While the proportion of 25(OH)D that was converted into 1,25(OH)2D was uncertain in the current study, the clear causal association between PTH and 25(OH)D could not be explored. Meanwhile, the parathyroid cells also express 1-α hydroxylase (CYP27B1) so that they can produce their own active vitamin D in an autocrine fashion to regulate PTH production (28). The interaction of circulating and locally produced active vitamin D in the regulation of PTH synthesis is not entirely clear. In light of this, large-scale multicenter studies are needed to determine the association of vitamin D and PTH with short stature in children and provide ideas for developing accurate diagnostic tools and treatments for short stature.

Our results indicated that serum 25(OH)D3 levels in short-stature patients aged 7–12 and 13–18 years were lower compared with healthy participants during the same periods. Conversely,

C3-epi levels and ratios of C3-epi/25(OH)D3 in all age ranges of short-stature children were higher than those in healthy subjects. C3-epi is the isomeric form of vitamin D3. Its active form, 3-epi-1α,25(OH)2D3, appears to have reduced calcemic effects than non-epimeric forms and can activate bone gamma-carboxy glutamic acid-containing protein (BGLAP, also called osteocalcin) at a much lower rate compared with 1α,25(OH)2D3 (29–31). However, there is still no clear causal association between C3-epi and short stature. Thus, the potential influences of C3-epi levels on height demand further elucidation. Accumulation evidence shows that the ratio of C3-epi/25(OH)D3 may be a promising tool to predict the status of various diseases such as Alzheimer's disease, rheumatoid arthritis, and type 1 diabetes (32). The ratio of C3-epi/25(OH)D3 in the current study performed statistically different in short-stature and healthy children, indicating that it might also be a novel biomarker for vitamin D catabolism in children with short stature. It is important to estimate the percentage contribution of C3-epi to 25(OH)D3 across 25(OH)D3 concentration ranges, age ranges, and varying healthy statuses, which would enable the evaluation of the physiological processes of C3-epi.

The current study showed a weak positive correlation between C3-epi and 25(OH)D3 values in both short-stature and healthy children, which was consistent with previous studies on adults. However, this relationship could not hold in infant populations because the relative C3-epimer concentration is high in neonates and declines rapidly across infancy (29). Some studies postulated that increasing serum 25(OH)D3 concentrations switch on or activate putative epimerization enzyme (15). This may be a protective mechanism against excessive levels (48–56 ng/ml) and possibly unwanted influences of vitamin because the epimeric form may be less active compared with the non-epimeric form (15, 29). However, this process is likely to become saturated when 25(OH)D3 levels reach maximum (15). Therefore, the relationship between C3-epi and 25(OH)D3 may not always be linear. The current study indicated a more linear relationship between C3-epi and 25(OH)D3 values, probably due to the limited number of study subjects with too high serum 25(OH)D3 levels. Moreover, the correlation between 25(OH)D2 and C3-epi was explored in this study. No association between the two indicators was observed, revealing that vitamin D2 may not be a source of C3-epi. However, their relationships need further confirmation.

Approximately 0.03% of total 25(OH)D and 0.4% of total 1,25 $(OH)_2D$ are free in circulation in healthy non-pregnant subjects. Its capacity depends on its physiological effects and body demands for vitamin D, rather than complex individual influencing factors (33–35). f-25(OH)D can freely move across membranes of kidney proximal tubule epithelial cells and be hydroxylated, indicating that it can be utilized by the body whenever needed (36, 37). Sufficient data from previous studies support speculation that free hormones (including free vitamin D) are more physiologically related compared with their total concentrations (38). Several scholars have argued that better skeletal conditions of African Americans despite their lower vitamin D levels (the African paradox) are likely due to the use of a "wrong" serum marker [t-25(OH)D], when f-25(OH)D should be the preferred indicator (39). Lower values of f-25(OH)D in short-stature children in age ranges of 7–12 and 13–18 years were observed in the current study, suggesting that available vitamin D levels decreased in short-stature young patients and f-25(OH)D is an alternative useful indicator for assessing vitamin D status in short-stature children. However, since several medical laboratories are incapable of determining f-25(OH)D values, assessment of the nutritional status of vitamin D in clinical practice is still challenging.

Total 25(OH)D comprising 25(OH)D2 and 25(OH)D3 is recommended by guidelines as the best indicator of vitamin D storage (40). Vitamin D3 levels are much higher compared with vitamin D2 levels, and vitamin D2/vitamin D3 ratios are extremely low in normal physiological conditions. Excessive 25(OH)D2 levels accompanied by significantly reduced 25(OH)D3 levels due to some unknown reasons may erroneously be interpreted as sufficient storage of vitamin D. Here, we found that after conversion of 25(OH)D2 to 25(OH)D3 activity (when vitamin D nutritional status is evaluated at the level of 25(OH)D3 activity equivalents), median vitamin D levels are decreased regardless of whether they are healthy or not. This may explain why serum vitamin D components and 25(OH)D2/25(OH)D3 ratios need to be determined. Findings of the current study showed that the 25(OH)D2/25(OH)D3 ratio in short stature was higher compared with that in control groups during age ranges of 7–12 and 13–18 years, indicating poor storage proportion of vitamin D3 in short-stature children.

C3-epi currently accounts for a significant proportion in neonates (21), infants, and even adults (41). The presence of C3-epi complicates the interpretation of serum 25(OH)D levels (42, 43). Otherwise, the capacity of vitamin D3 to store vitamin D is two to three times higher compared with that of vitamin D2. Therefore, three different methods were applied in the current study to determine vitamin D storage in short-stature children. Findings were totally varied, although it was clear that detection of t-25(OH)D [25(OH)D2+25(OH)D3] alone may overestimate vitamin D storage in short-stature children. Some previous studies had shown that epimeric interference does not significantly influence routine vitamin D determination for healthy adults using LC-MS/MS methods (44). This may be due to relatively low concentrations of C3-epimer in adults. However, due to lack of a more comprehensive understanding of the role of C3-epimer, determination of both 25(OH)D3 and C3-epimer in patients (especially infant and pediatric subjects) should be considered so that more accurate conclusions regarding the function of C3-epimer will be drawn with continued biological and molecular investigation.

Nevertheless, the current study had some limitations. First, it was limited by its retrospective nature with single academic center and relatively small sample size. Second, data on use of vitamin D supplements by participants were not collected. Similarly, information on sensitivity to sunlight, latitude, season, time of day, and how much direct sunlight that skin is exposed to was not included, all of which could be related to vitamin D status. In addition, methods for determining vitamin

D metabolites were not standardized. High sensitivity of LC-MS/MS and poor reproducibility of ELISA may have led to certain variations in the obtained findings. Although reliability of the current study was not entirely satisfactory, it provides important reference for design and implementation of related studies.

CONCLUSIONS

The current study revealed essential differences between various vitamin D contents in short-stature children compared with healthy ones. The findings indicated that short-stature patients had lower levels of vitamin D storage compared with healthy subjects. To accurately assess vitamin D nutritional status, kinds of vitamin D components in circulation including 25(OH)D2, 25(OH)D3, f-25(OH)D, t-25(OH)D, and C3-epi and ratios of C3-epi/25(OH)D3 and 25(OH)D2/25(OH)D3 should be determined extensively, in order to provide a scientific evidence-based basis for the diagnosis and treatment evaluation of short-stature individuals.

DATA AVAILABILITY STATEMENT

Datasets analyzed during the current study are available from corresponding author on reasonable request.

ETHICS STATEMENT

The current study was approved by Medical Ethics Committee of Mianyang Central Hospital (approval no. P2020040). Written informed consent to participate in this study was provided by legal guardian/next of kin of participants.

AUTHOR CONTRIBUTIONS

All authors contributed to the current study conception and design and take responsibility for the integrity of data and accuracy of data analyses. Data collection was undertaken by BX, LG, and YZ, and analysis was undertaken by YF. Material preparation was done by and the first draft of the manuscript written by WJ, JF, and LY, and all authors commented on the previous versions of manuscript. All authors contributed to the article and approved the submitted version.

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The Effect of BMI, Age, Gender, and Pubertal Stage on Bone Turnover Markers in Chinese Children and Adolescents

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Objectives: To ascertain the associations of serum bone turnover markers (BTMs) levels with body mass index (BMI) in Chinese children and adolescents, and whether the influence of BMI, age, pubertal stage on BTMs varied by gender.

Methods: A total of 500 students (180 controls and 320 children and adolescents with overweight/obesity) aged 9–14 years were randomly selected from the Chinese National Survey on Students Constitution and Health Cohort. Serum levels of BTMs, including bone formation marker bone alkaline phosphatase (BAP), collagen type 1 C-terminal propeptide (CICP), and bone resorption markers C-terminal telopeptide of type-I collagen (CTX) were determined by commercial enzyme-linked immunosorbent assay kits. The associations among BMI, age, gender, pubertal stage, and BTMs were analyzed.

Results: Serum levels of CICP and CTX in overweight/obese children and adolescents were lower than those in controls ($p<0.05$). Moreover, after subgroup analysis stratified by gender, the decreased serum CICP and CTX levels in overweight/obese children and adolescents were observed only in boys ($p<0.05$). After adjustment of age and pubertal stage, there was a negative correlation between serum BAP and BMI in both boys and girls ($p<0.05$). However, the correlations between serum CICP, CTX levels, and BMI were significant in boys but not in girls. Serum BAP and CICP levels were independently correlated with BMI, age, gender, and pubertal stage, while CTX levels were independently correlated with BMI, age, and gender ($p<0.05$). BAP, CICP, and CTX levels showed a clear age, gender, and pubertal stage dependence with significantly higher values in boys ($p<0.05$).

Conclusions: Our findings support the associations between serum BTMs levels and BMI in Chinese children and adolescents, and suggest age, gender, and pubertal stage differences in this relationship that warrant future studies.

Keywords: bone turnover markers (BTMs), body mass index (BMI), age, gender, pubertal stage, children

INTRODUCTION

Childhood obesity [body mass index (BMI) ≥ 95 th percentile for age and gender] has become a global epidemic (1). Its worldwide prevalence has increased by 325% since the early 1960s (2). This issue poses a heavy economic burden on health systems and becomes a major health risk factor for individuals. Obesity-related metabolic diseases, such as hypertension (3), hyperlipidemia (4), coronary artery disease (5), insulin resistance (6), diabetes mellitus (7), and fatty liver disease (8) are the research hotspots in many published literature. Bone health has only recently drawn the attention of researchers.

Osteoporosis is a systemic skeletal disease, which is characterized by a deterioration of bone tissue microarchitecture that leads to low bone mineral density (BMD) and an increased risk of bone fractures (9). Osteoporosis affects more than 200 million people globally, with significant clinical and socio-economic implications (10). Osteoporosis used to occur mainly in adults but now occurs in children too (11). Peak bone mass, i.e. the highest BMD, is a key determinant of osteoporosis and fracture risk later in life (12). Childhood and adolescence are the most critical periods for bone mineral accrual, with peak bone mass being achieved around the age of 20 years. Epidemiologic data indicate that a 10% increase in peak bone mass in childhood is estimated to decrease the risk of osteoporotic fracture in the elderly by 50%, and the relative risk of fracture increases 2.6-fold for each 1 standard deviation (SD) decrease in bone mass (13). Therefore, understanding the characteristics of bone development in children and adolescents to achieve the ideal peak bone mass is pivotal for preventing osteoporosis and osteoporosis-related fractures.

Bone metabolism is a continuous remodeling or turnover process, which relies on the tightly coupled balance between bone formation by osteoblasts and bone resorption by osteoclasts. Children, with rapid bone growth and reshaping, have larger remodeling spaces and a shorter remodeling period than adults (14). Bon| turnover markers (BTMs) are a group of metabolites or enzymes which are released by osteoblasts or osteoclasts during the bone remodeling process. BTMs could provide a dynamic picture of changes in bone remodeling, and thus identify changes in the bone remodeling within a relatively short time interval (15). BTMs are independent predictors of fracture risk and future bone loss (16). The most studied BTMs are bone alkaline phosphatase (BAP), collagen type 1 C-terminal propeptide (CICP), reflecting bone formation by osteoblasts, and C-terminal telopeptide of type-I collagen (CTX), reflecting bone resorption by osteoclasts. Moreover, recent studies identified the bone as an endocrine organ, by producing and secreting "hormone-like factors" such as fibroblast growth factor 23 (FGF23) (17), which can affect bone remodeling and even the complete metabolism of the whole organism. BTMs and bone-derived factors levels could be affected by many factors, such as age, gender, pubertal stage, hormones, and nutritional status. Accurate measurement of BTMs and bone-derived factors are important for better understanding the connection between obesity and osteoporosis.

However, previous studies on serum BTMs and bone-derived factors levels in overweight/obese children provided conflicting results. For example, in an observational cross-sectional study involving 81 Italian children, Radetti et al. reported that serum CTX levels were significantly increased in obese children compared to controls (18). Furthermore, Dimitri et al. studied 103 United Kingdom (UK) children and demonstrated that serum CTX levels were significantly higher in obese children, even after correction for pubertal stage and sex (19). Otherwise, in a case-control study performed in 68 European children, Viljakainen et al. showed that obese subjects with early-onset severe obesity had significantly lower CTX levels than the sex- and age-matched controls (20).

As the data concerning serum BTMs and bone-derived factors levels in obese children were controversial, and few reported studies were performed in Chinese children, the objectives of the present study were (1) to evaluate serum BTMs and bone-derived factors levels in overweight/obese Chinese children and adolescents (2); to assess the associations between serum BTMs, bone-derived factors, and BMI (3); to explore the effects of age, sex, and pubertal stage on serum BTMs and bone-derived factors.

MATERIALS AND METHODS

Participants and Study Design

This was a school-based cross-sectional study that was carried out in Beijing, the capital of China. Our study was a part of the Chinese National Survey on Students Constitution and Health. We screened the database and randomly selected 500 students aged 9–14 years, which were divided into two groups, the control group (n=180) and the overweight/obesity group (n=320). Children and adolescents were excluded if they had any of the following: chronic illness, metabolic bone disease, endocrine or known chromosomal abnormalities, or if they did not complete the questions in the questionnaires, or did not sign the informed consent. The ethics committee of Beijing Children's Hospital, Capital Medical University approved this study, and all children's parents signed the written informed consent.

Anthropometric Measurements and Definitions

Anthropometric measurements including body height, body weight, and waist circumference (WC) were accomplished by the well-trained study assistants using uniform instruments. Body height (to the nearest 0.1 cm) was measured with subjects in the erect position without shoes (Seca 213 stadiometer, Hamburg, Germany), and weight (to the nearest 0.1 kg) was measured with subjects wearing light clothing (Tanita HA 503, Tanita Corporation, Tokyo, Japan). WC (to the nearest 0.1 cm) was obtained at the midpoint between the inferior costal margin and the superior border of the iliac crest on the midaxillary line (WT-21, Wintape, Guangdong, China). Two measurements (measurement error ≤ 0.1 cm) were recorded and the average was used for the analysis. BMI (kg/m^2) was calculated as weight (kg) divided by height squared (m^2). According to age- and sex-specific BMI cut-off points

recommended by the Working Group for Obesity in China, overweight was defined as BMI between the 85 th and 95 th percentile, while obesity was defined as BMI ≥95 th percentile (21). Puberty was assessed by the well-trained study assistants using the standardized method of Tanner stages (22).

Questionnaire

Information about each subject regarding demographic and lifestyle factors was collected by the trained investigators through a structured parent questionnaire (23, 24). Children's lifestyle habits were ascertained on the questionnaire by asking "On average, how many times a week does your child exercise? (≤3 times/week or >3 times/week) ", "How many minutes per week does your child exercise? (≤120 minutes/week or >120 minutes/week) ", "How many hours per week does your child spend watching television (TV)? (≤2 hours/day or >2 hours/day)", "On most nights, how many hours does your child sleep each night? (<9 hours/day or ≥9 hours/day)".

Blood Sample Collection and BTMs, FGF23 Assays

Venous blood samples were taken from all subjects after an overnight fast. According to the manufacturer's instructions, serum BAP was determined using an immune-enzymatic assay (Octeia Ostase BAP, IDS ltd, Baldon, UK). The lower limit of detection was 0.7 ug/L. Serum CICP was analyzed using an enzyme-linked immunoadsorbent assay (ELISA) with a Metra CICP EIA kit (Quidel Corporation, San Diego, CA, USA). The lower limit of detection was 0.02 ng/mL. Serum CTX was measured using the IDS-iSYS CTX (CrossLaps®) assay (Immunodiagnostic Systems, plc, Tyne and Wear, UK). The lower limit of detection was 0.2 ng/mL. Serum FGF23 was tested by a commercially available ELISA kit (Wuhan Cusabio Biotech Co., Ltd., Wuhan, China). The minimum detectable concentration was 0.78 pg/mL.

Statistical Analysis

Values were expressed as mean ± SD for continuous variables, and as numbers (percentages) for categorical variables. Comparisons for continuous and categorical variables were made by the independent t-test and chi-square test, respectively. Spearman partial correlation coefficients were used to describe the associations between BTMs, FGF23, and BMI. Stepwise multiple regression analysis was executed to explore the variables independently related to serum BTMs and FGF23 levels. The statistical analyses were achieved using SPSS version 20.0 for Windows (SPSS Inc, Chicago, IL, USA) and a p-value <0.05 was considered statistically significant.

RESULTS

Serum BTMs, FGF23 Levels, and Other Characteristics in the Study Population

Table 1 presented the baseline characteristics, serum BTMs and FGF23 levels of all subjects. The lifestyle characteristics of all participants were shown in **Supplementary Table 1**. Overweight/obese children had significantly higher body weight, BMI, and WC than controls (p<0.05), regardless of their gender (p<0.05). The proportion of girls presenting in the advanced Tanner stages (stage III-V) was greater than that of boys, both in the control group and the overweight/obesity group (p<0.05). Serum levels of CICP and CTX in overweight/obese children were lower than that in controls (p<0.05). Moreover, after subgroup analysis stratified by gender, the decreased serum CICP and CTX levels in overweight/obese children were observed only in boys (p<0.05). Serum BAP and FGF23 levels were not different between the control group and the overweight/obesity group in both genders (p>0.05). Boys had higher levels of BAP, CICP as well as CTX than girls, both in the control group and the overweight/obesity group (p<0.05). However, no difference was detected between boys and girls in FGF23 levels (p>0.05).

Association of BTMs, FGF23 with BMI

The associations of serum BTMs and FGF23 levels with BMI were studied separately for boys and girls. As illustrated in **Figure 1A**, when age and pubertal stage were taken into account, serum BAP levels were negatively correlated with BMI in both genders (r=−0.130 for BAP in boys, r=−0.164 for BAP in girls, p all<0.05). After adjusting for age and pubertal stage, serum CICP and CTX levels were negatively associated with BMI only amomg boys (r=−0.162 for CICP in boys, r=−0.200 for CTX in boys, p all<0.05; r=−0.127 for CICP in girls, r=−0.069 for CTX in girls, p all>0.05) (**Figures 1B, C**). There was no significant relationship between serum FGF23 levels and BMI neither in boys nor in girls (p>0.05).

Next, stepwise multivariate linear regression was performed. As displayed in **Table 2**, BMI, age, sex, pubertal stage, and frequency of exercise were independently associated with serum BAP levels after adjusting for WC, exercise time, watching TV, and sleep duration. Among them, BMI was independently and negatively related to serum BAP levels (β=-0.130, p<0.05), which agreed with the results shown in **Figure 1** by partial correlation analysis. After adjusting for WC, exercise time, watching TV, and sleep duration, BMI, age, sex, and pubertal stage were independent factors associated with serum CICP levels, while BMI, age, and sex were independent factors associated with serum CTX levels. Moreover, serum CICP and CTX were also independently and negatively correlated with BMI (β=-0.151 for CICP, β=-0.152 for CTX, p all<0.05), which was consistent with the results demonstrated in **Table 1**. In addition, age was independently and negatively related to FGF23 levels after controlling for other confounders.

Association of BTMs, FGF23 with Age

Since age was found to be an independent influence factor for serum BTMs levels, serum levels of BTMs and FGF23 were further examined in different age groups stratified by gender. In boys, serum BAP levels fluctuated with age, with a nadir value at 14 years (93.64 ug/L) and a peak value at 12 years (126.68 ug/L) (**Figure 2A**). In girls, BAP started with high values at 9 years of age (125.66 ug/L) and subsequently decreased until reaching a nadir at 14 years of age (42.61 ug/L) (**Figure 2B**). In boys, CICP

TABLE 1 | Comparison of subjects with and without overweight/obesity according to age, anthropometric measures, Tanner stage, and bone turnover markers of both sexes.

Parameters	Overall cohorts (n=500)			Control group (n=180)			Overweight/obesity group (n=320)		
	All (n=500)	Boys (n=297)	Girls (n=203)	All (n=180)	Boys (n=109)	Girls (n=71)	All (n=320)	Boys (n=188)	Girls (n=132)
Age (years)	12.18 ± 1.79	12.22 ± 1.72	12.13 ± 1.89	12.33 ± 1.74	12.33 ± 1.68	12.33 ± 1.84	12.10 ± 1.82	12.15 ± 1.75	12.02 ± 1.92
Body weight (kg)	61.00 ± 20.82	63.16 ± 22.18	57.85 ± 18.24[b]	44.73 ± 10.75	45.47 ± 11.10	43.60 ± 10.17	70.16 ± 19.48[a]	73.42 ± 20.48[e]	65.51 ± 16.98[df]
Body height (cm)	157.79 ± 12.81	159.93 ± 13.53	154.65 ± 10.97[b]	156.33 ± 12.44	157.59 ± 13.35	154.39 ± 10.70	158.61 ± 12.96	161.28 ± 13.48[e]	154.80 ± 11.15[d]
BMI (kg/m^2)	23.99 ± 5.98	24.11 ± 6.03	23.81 ± 5.90	17.99 ± 2.16	17.97 ± 2.02	18.00 ± 2.38	27.36 ± 4.64[a]	27.66 ± 4.55[e]	26.94 ± 4.74[f]
WC (cm)	76.67 ± 14.52	79.31 ± 15.15	72.80 ± 12.62[b]	63.69 ± 6.41	64.58 ± 6.39	62.32 ± 6.25[c]	83.96 ± 12.57[a]	87.85 ± 11.82[e]	78.44 ± 11.54[df]
Tanner stage									
I	108 (21.6%)	92 (31.0%)	16 (7.9%)[b]	38 (21.1%)	30 (27.5%)	8 (11.3%)[c]	70 (21.9%)	62 (33.0%)	8 (6.1%)[d]
II	38 (7.6%)	27 (9.1%)	11 (5.4%)	16 (8.9%)	9 (8.3%)	7 (9.9%)	22 (6.9%)	18 (9.6%)	4 (3.0%)
III	121 (24.2%)	67 (22.6%)	54 (26.6%)	42 (23.3%)	27 (24.8%)	15 (21.1%)	79 (24.7%)	40 (21.3%)	39 (29.5%)
IV	119 (23.8%)	48 (16.2%)	71 (35.0%)	47 (26.1%)	21 (19.3%)	26 (36.6%)	72 (22.5%)	27 (14.4%)	45 (34.1%)
V	114 (22.8%)	63 (21.2%)	51 (25.1%)	37 (20.6%)	22 (20.2%)	15 (21.1%)	77 (24.1%)	41 (21.8%)	36 (27.3%)
BAP (ug/L)	97.90 ± 45.50	110.38 ± 39.48	79.64 ± 47.61[b]	100.66 ± 46.71	113.45 ± 41.81	81.02 ± 47.31[c]	96.35 ± 44.80	108.60 ± 38.07	78.90 ± 47.93[d]
CICP (ng/mL)	256.23 ± 132.96	293.19 ± 127.15	202.15 ± 122.63[b]	274.42 ± 145.85	315.11 ± 137.60	211.95 ± 136.57[c]	246.00 ± 124.20[a]	280.48 ± 119.21[e]	196.88 ± 114.63[d]
CTX (ng/mL)	1.62 ± 0.74	1.85 ± 0.70	1.29 ± 0.66[b]	1.73 ± 0.81	2.00 ± 0.80	1.30 ± 0.64[c]	1.56 ± 0.69[a]	1.76 ± 0.63[e]	1.28 ± 0.67[d]
FGF23 (pg/mL)	51.60 ± 69.75	54.29 ± 70.95	47.68 ± 67.94	50.22 ± 64.68	51.80 ± 64.67	47.79 ± 65.38	52.39 ± 72.53	55.73 ± 74.57	47.62 ± 69.52

BMI, body mass index; WC, waist circumstance; TV, television; BAP, bone-specific alkaline phosphatase; CICP, C-propeptide of type I procollagen; CTX, collagen type 1 C-terminal propeptide; FGF23, fibroblast growth factor 23. Values were presented as N (%) or mean ± SD as appropriate. P values were obtained by Student's t-test or chi-square test. [a]P < 0.05 between control group and overweight/obesity group in the overall cohorts; [b]P < 0.05 between boys and girls in the overall cohorts; [c]P < 0.05 between boys and girls in the control group; [d]P < 0.05 between boys and girls in the overweight/obesity group; [e]P < 0.05 between control group and overweight/obesity group in boys; [f]P < 0.05 between control group and overweight/obesity group in girls.

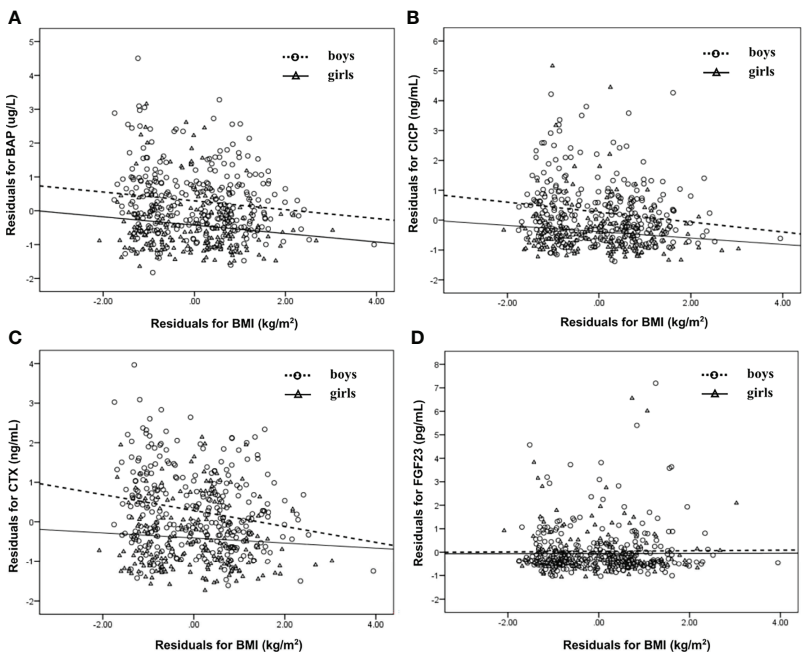

FIGURE 1 | Scatter plot that showing the relationship between BAP **(A)**, CICP **(B)**, CTX **(C)**, FGF23 **(D)** and BMI in boys and girls, respectively. Partial correlation analysis was performed after adjustment for age and pubertal stages. BAP, bone-specific alkaline phosphatase; CICP, C-propeptide of type I procollagen; CTX, collagen type 1 C-terminal propeptide; FGF23, fibroblast growth factor 23; BMI, body mass index.

TABLE 2 | Multiple regression analysis for the variables independently related to serum BTMs and FGF23 levels in all subjects.

	B	se of B	β	*p*
Serum BAP (R^2 = 0.301)				
BMI	-0.993	0.300	-0.130	<0.05
Age	-12.023	1.595	-0.474	<0.05
Sex	-36.538	3.842	-0.395	<0.05
Pubertal stage	5.982	1.987	0.189	<0.05
Frequency of exercise	-8.084	3.789	-0.089	<0.05
(Constant)	312.404	17.157		<0.05
Serum CICP (R^2 = 0.174)				
BMI	-3.349	0.951	-0.151	<0.05
Age	-20.093	4.900	-0.271	<0.05
Sex	-104.680	12.162	-0.387	<0.05
Pubertal stage	14.314	6.305	0.154	<0.05
(Constant)	682.889	54.467	12.538	
Serum CTX (R^2 = 0.181)				
BMI	-0.019	0.005	-0.152	<0.05
Age	-0.046	0.018	-0.112	<0.05
Sex	-0.572	0.061	-0.380	<0.05
(Constant)	3.441	0.234	14.720	<0.05
Serum FGF23 (R^2 = 0.042)				
Age	-8.108	1.703	-0.209	<0.05
(Constant)	150.369	20.972		<0.05

Variables also entered multiple regression analysis but not included in the equation: waist circumstance, exercise time, watching TV, sleep duration. R^2, multiple determination coefficient; B, unstandardized regression coefficient; se of B, standard error of the unstandardized regression coefficient; β, standardized regression coefficient.

levels were low at 9 years (248.08 ng/mL), then revealed a clear tendency to increase until 12 years (344.13 ng/mL) and followed by decreasing values after 12 years (**Figure 2C**). In girls, CICP started with high values at 9 years (274.61 ng/mL) and reached a peak value at 10 years (343.85 ng/mL), and subsequently decreased until reaching a nadir at 14 years (128.98 ng/mL) (**Figure 2D**). Serum CTX levels also showed similar trends, with a peak value at age 12 in boys (2.14 ng/mL) and at age 10 (1.95 ng/mL) in girls, a nadir at age 9 in boys (1.60 ng/mL) and age 14 in girls (0.78 ng/mL) (**Figures 2E, F**). Serum FGF23 levels exhibited similar trends in both sexes across all age groups, with a peak value at 10 years (115.25 pg/mL for boys, 79.01 pg/mL for girls) and a nadir at 14 years (33.81 pg/mL for boys, 26.96 pg/mL for girls) (**Figures 2G, H**).

Association of BTMs, FGF23 with Pubertal Stage

Additionally, serum BTMs and FGF23 concentrations were further explored in different pubertal stages stratified by gender. In boys, serum BTMs levels presented similar trends across Tanner stages, with peak values at Tanner stages II and III and nadir levels at Tanner stages IV and V (**Figures 3A, C, E**). In girls, serum BTMs levels have also shown similar trends with Tanner stages. Serum BTMs levels started with high values at Tanner stage I, remained relatively constant in Tanner stages II-III, and then decreased until reaching a nadir at Tanner stage V (**Figures 3B, D, F**). For FGF23 levels, the trends among boys and girls at different Tanner stages were quite different. In boys, FGF23 peaked at Tanner stage I, then decreased until reaching a nadir at Tanner stage III, followed by increasing values after Tanner stage III (**Figure 3G**). In girls, FGF23 started with high values at Tanner stage I, and subsequently decreased until reaching a nadir at Tanner stage V (**Figure 3H**).

DISCUSSION

Obesity is closely associated with impaired bone homeostasis. BTMs, which reflect the dynamic changes in bone remodeling, are sensitive indicators of early bone metabolism disturbances. Although many studies have examined the associations between serum BTMs and BMI in children and adolescents, the results were inconsistent and were largely based on foreign studies (18–20, 25–31). To date, this is the first study to investigate serum BTMs levels in Chinese overweight/obese children and adolescents.

In the current study, we showed that serum levels of CICP (a marker of bone formation) and CTX (a marker of bone resorption) were lower in overweight/obese children than in controls. This is in agreement with a recent study among children in Europe, which demonstrated that all BTMs, except BAP, were lower in obese children compared with the age-and sex-matched controls (20). Similarly, in the USA, Reinehr et al. observed significantly lower circulating osteocalcin (OC, a marker of bone formation) levels in obese children than in normal-weight children (25). Moreover, after one-year lifestyle intervention, serum OC levels in obese children with substantial weight loss were significantly increased (25). However, there are also several studies presenting inconsistent results. In both the studies conducted in Italy (18) and the UK (19), serum CTX levels were significantly increased in obese children compared to controls. There are several reasons for inconsistent results in the different studies. Firstly, Radetti et al. recruited 81 children, which were further divided into the overweight/obese group (n=44) and lean group (n=37), the small sample size of fewer than 50 subjects in each group (18) may cause false positives and low power. Secondly, Dimitri et al. explored serum BTMs levels in obese children, particularly in those who have fractured, the high proportion of children with previous fractures (26/103) (19)

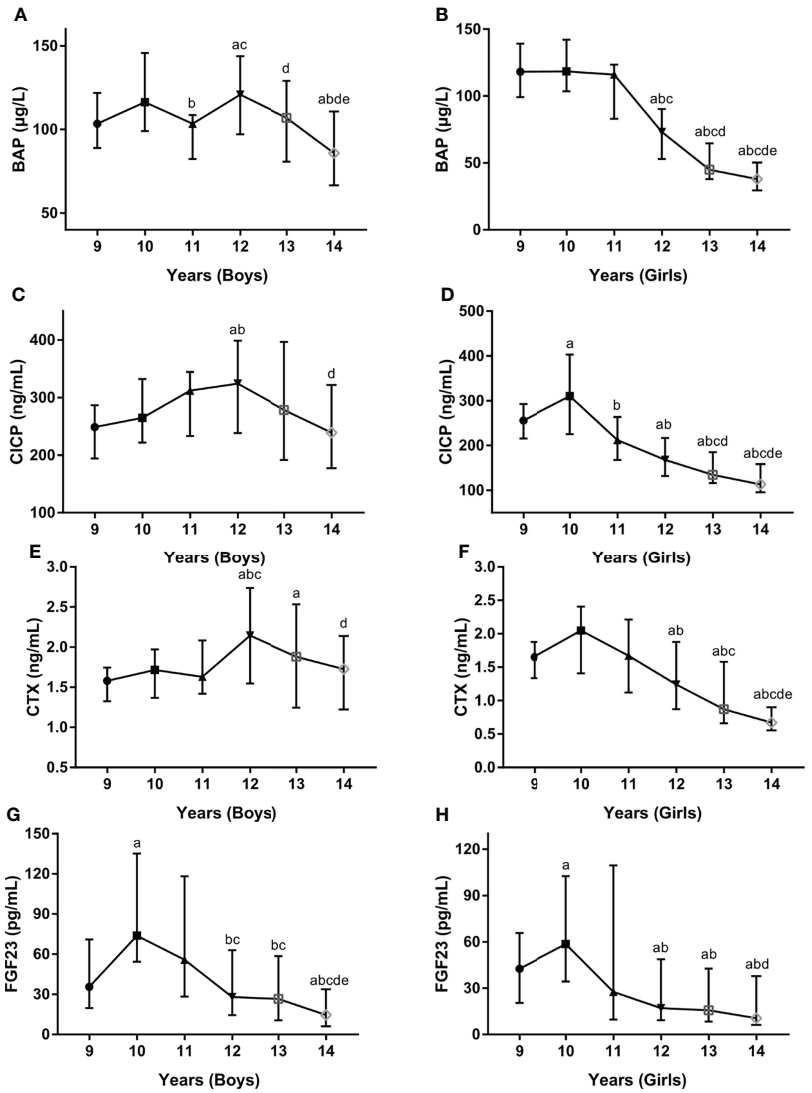

FIGURE 2 | Serum levels for BAP **(A, B)**, CICP **(C, D)**, CTX **(E, F)**, FGF23 **(G, H)** by age and gender. BAP, bone-specific alkaline phosphatase; CICP, C-propeptide of type I procollagen; CTX, collagen type 1 C-terminal propeptide; FGF23, fibroblast growth factor 23. $^{a}P < 0.05$ compared with children aged 9 years; $^{b}P < 0.05$ compared with children aged 10 years; $^{c}P < 0.05$ compared with children aged 11 years; $^{d}P < 0.05$ compared with children aged 12 years; $^{e}P < 0.05$ compared with children aged 13 years.

may also affect the results of serum BTMs. Thirdly, race differences may be also an important factor. In brief, previous studies on serum BTMs in overweight/obese children were mainly carried out in Caucasian populations, our study showed decreased serum BTMs levels in Chinese overweight/obese children.

As we all know, obese children often manifested an earlier onset of puberty in comparison with children with normal weight (32), which was also observed in our study. We then explored the associations of serum BTMs and BMI after adjusting the age and pubertal Tanner stage, we found that all BTMs, including BAP, CICP, and CTX had a negative correlation with BMI. Moreover, multivariate linear regression showed that all serum BTMs were independently and negatively associated with BMI after controlling for other confounders. Similarly, Geserick et al. showed that age- and gender-adjusted BTMs were significantly lower in obese children than in controls independent of their pubertal development (27). Thus, all these findings together with our results suggest the negative associations between serum BTMs and BMI. According to previous studies, the decreased process of bone turnover in overweight/obese children could influence BMD, bone quality, and bone strength, and consequently increased the risk of fracture (33). However, given the cross-sectional design of this study, the directionality of the association between serum BTMs and BMI cannot be conclusively established.

In addition, our study observed a clear gender difference in serum BTMs. Previously, Mayer et al. studied 397 German

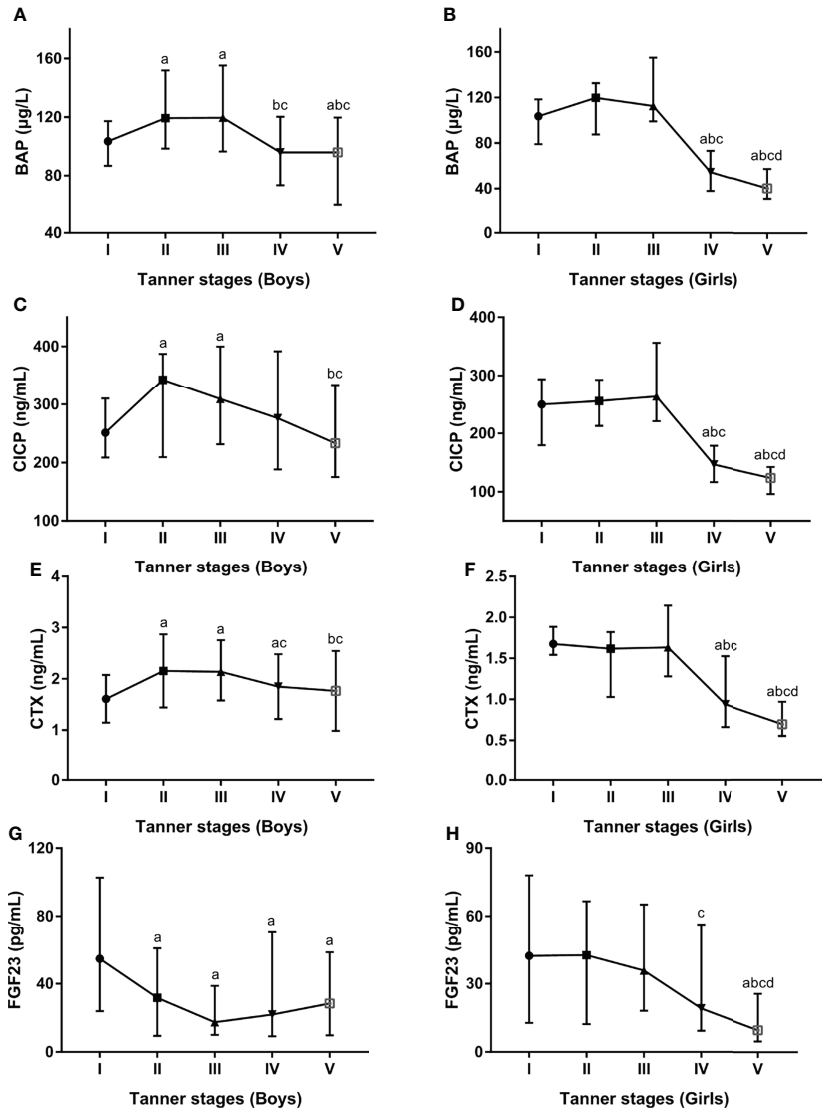

FIGURE 3 | Serum levels for BAP **(A**, **B)**, CICP **(C**, **D)**, CTX **(E**, **F)**, FGF23 **(G**, **H)** by developmental staging and gender. BAP, bone-specific alkaline phosphatase; CICP, C-propeptide of type I procollagen; CTX, collagen type 1 C-terminal propeptide; FGF23, fibroblast growth factor 23. [a]P < 0.05 compared with Tanner stage I; [b]P < 0.05 compared with Tanner stage II; [c]P < 0.05 compared with Tanner stage III; [d]P < 0.05 compared with Tanner stage IV.

children with a wide range of BMI (217 subjects without obesity, 180 subjects with obesity) and reported significantly higher bone formation as well as resorption markers in boys than in girls (34). In our present study, higher levels of serum BTMs in boys were observed both in the control and overweight/obese groups. The higher serum BTMs in boys may be attributed to physical factors (higher physical activity and muscle strength in boys) and biological factors (gender hormones) (34). As a result of testosterone acting on periosteal apposition, boys have a greater width and size of bones than girls of the same age (35).

Interestingly, the correlations between serum BTMs and BMI were more pronounced in boys. On the one hand, the decreased serum CICP and CTX levels in overweight/obese children were observed only in boys. On the other hand, the correlations between serum CICP, CTX levels, and BMI were significant in boys but not in girls. These findings suggest that gender differences should be considered by local policy-makers when designing initiatives to address issues around bone health in children with overweight/obesity. Further studies are needed to uncover the underlying mechanism. Although we are unclear about what may drive the gender differences, the interconnections of adipokines, BTMs, and sex hormones warrant further investigation.

Apart from gender, age and puberty also were the significant and independent determinants of serum BTMs levels, which was expected given the growth and development present during

childhood and adolescence. Interestingly, when both age and gender were considered simultaneously, gender demonstrated different effects in different age bands. In girls, serum BTMs showed a peak at 10 years of age and then dropped rapidly thereafter, while in boys peak value of those biomarkers was observed at 12 years of age. These results were similar to previous studies conducted in Finish (girls, peak levels at 11 years; boys, peak levels at 14 years) (36), German (girls, peak levels at 10 to 11 years; boys, peak levels at 13 years) (27), and Polish (girls, peak levels at 8 to 13 years; boys, peak levels at 10 to 15 years) (37) children and adolescents. When both puberty and gender were considered simultaneously, serum BTMs levels presented similar trends across Tanner stages in boys and girls, with peak values at Tanner stages II and III and nadir levels at Tanner stages IV and V. Consistent with our findings, a cross-sectional study conducted by Bayer et al. among 439 Caucasian children reported the peak values of serum OC and procollagen type I N-terminal propeptide with the pubertal growth spurt at second-third Tanner stages (38). As age and pubertal stage are closely linked, older age of onset of puberty was associated with later peak values of serum BTMs observed in boys. However, given our present study was conducted in children and adolescents aged 9–14 years, further research is required on the influence of age, gender, and pubertal stages on serum BTMs, in particular in different age groups.

In recent research, the bone has been identified as an important endocrine organ. FGF23, one of the bone-derived factors, plays an important role not only in bone homeostasis but also in regulating whole-body energy metabolism. However, the data from existing research on serum FGF23 levels in children with obesity are inconsistent. Some studies reported an increase in serum FGF-23 concentration in overweight/obese children (39, 40) and a positive association between serum FGF23 and BMI (39). In contrast, some studies detected decreased serum FGF-23 levels in obese subjects (41) and inverse correlations with fasting insulin levels (41) as well as fasting glucose levels (42). In our present study, we did not detect a significant difference in serum FGF-23 levels between the overweight/obese group and the control group. Noteworthy, we found that age was independently and negatively related to FGF23 levels after controlling for other confounders. Our findings were in line with a previous study in German children and adolescents that showed serum FGF-23 was significantly correlated with age (43). However, contrary to that study, we also found that when both puberty and gender were considered at the same time, the trends for serum FGF23 levels across Tanner stages were quite different between boys and girls. Thus, future studies are needed to have a better understanding of the association between serum FGF23 and BMI in age-gender-puberty populations.

This study has some limitations that should be taken into consideration. Firstly, since it was a cross-sectional study, the causality cannot be determined. Secondly, there was a large difference in the number of respondents between the two groups, which probably affected the results obtained in our study. Thirdly, BMD, bone mineral content, and bone areas were not assessed in our study. Fourthly, in terms of external validity, this was a single-center study with a relatively small sample size, our results cannot be extrapolated to the whole country.

In conclusion, the present study revealed that serum BTMs levels were significantly decreased in overweight/obese Chinese children and adolescents, and were independently negatively associated with BMI. Furthermore, serum BTMs levels showed a clear age, gender, and pubertal stage dependence with significantly higher values in boys. Future studies should be performed to establish age- gender- and puberty-dependent references of serum BTMs levels in Chinese children.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The ethics committee of Beijing Children's Hospital, Capital Medical University approved this study, and all children's parents signed the written informed consent. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors helped to perform the research; BC contributed to the project management; MJL wrote the manuscript; QL participated in the interpretation of data; QW, ML, XL, DW, WL, CS, and JC took part in the collection of clinical samples; CG conceived and designed the project as well as revised the manuscript. All listed authors revised the paper critically and approved the final version of the submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.880418/full#supplementary-material

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Blood Lead Level Is Negatively Associated With Bone Mineral Density in U.S. Children and Adolescents Aged 8-19 Years

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Context: The relationship of lead (Pb) exposure with bone health in children and adolescents remains controversial.

Objection: We aimed to investigate the association of blood lead levels (BLL) with bone mineral density (BMD) in American children and adolescents using data from the National Health and Nutrition Examination Survey (NHANES), 2005-2010.

Methods: We analyzed 5,583 subjects aged 8-19 years (mean age, 13.49 ± 3.35 years) from the NHANES 2005-2010. BLL was tested using inductively coupled plasma mass spectrometry. BMD was measured by dual-energy X-ray absorptiometry (DXA) at the lumbar spine, total femur, and femur neck. Multivariate linear regression models were used to explore the association between BLL and BMD, adjusting for age, gender, race/ethnicity, poverty income ratio (PIR), body mass index (BMI), serum calcium, and serum phosphorus.

Results: BLL was negatively correlated with BMD at different sites of interest in children and adolescents. For every 1mg/dl increase in BLL, the BMD of the total spine, total hip, and femoral neck decreased by 0.011 g/cm^2, 0.008 g/cm^2, and 0.006 g/cm^2. In addition, Pb affected the lumbar spine more than the femur. The effect estimates were stronger in girls than boys at the lumbar spine (*P* for interaction= 0.006). This negative association remained significant in American children and adolescents after excluding individuals with BLL more than 3.5 ug/dl.

Conclusion: Our study indicates that BLL is negatively correlated with BMD at different sites of interest in children and adolescents aged 8-19 years, even in the reference range. More research is needed to elucidate the relationships between Pb and bone health in children and adolescents, including specific mechanisms and confounding factors like race/ethnicity, gender, and age.

Keywords: lead, bone mineral density, children, adolescent, NHANES

INTRODUCTION

Osteoporosis is a multifactorial skeletal disease characterized by low bone mineral density (BMD) and increased fragility fracture risks. In 2010, more than 24 million people aged 50 and older in the United States were estimated to have osteoporosis (1). The peak bone mass (PBM) in adolescence and bone loss with age are two major factors in the process of osteoporosis. PBM in adolescence has been shown to be a significant predictor of osteoporosis in old age (2). Population-based studies have proved that a 10% increase in PBM could reduce fracture risk by 50% in old age (3). Several factors influencing PBM formation have been explored, including race, gender, genetics, and lifestyle (4, 5).

Lead (Pb), listed as one of the ten most harmful metals by the World Health Organization (WHO), has been reported to be associated with osteoporosis (6–10). Despite a significant decline in blood lead (BLL) in the U.S. population, many sources of Pb exposure still exist including drinking water and contaminated soil particles (11, 12). Studies have explored the molecular mechanism of Pb damage to bone health. Bone is the main site of lead storage in the body (13). Some studies suggest that calcium in hydroxyapatite crystals can be exchanged by Pb, which leads to a decrease in bone mass (14). Moreover, Pb has a substantial regulatory effect on growth plate chondrocytes and inhibits the process of endochondral bone formation (14). Previous studies have found the adverse effects of Pb on bone health in adults (10, 15, 16). However, evidence for the association between Pb exposure and BMD in children and adolescents was scarce and inconsistent. An early study in America stated that children with higher BLL were associated with an increased BMD. However, it recruited smaller samples, which may result in an unreliable conclusion (17). A recent study conducted by Li et al. (18) showed an N-shaped curve association between BLL and BMD in children and adolescents.

Pb exposure is especially hazardous for children since they are more likely to absorb Pb than adults (19). In order to prevent future osteoporosis, it is crucial to explore the association of Pb exposure with BMD in children and adolescents, as they acquire most of their PBM at the end of puberty (20). Therefore, the focus of our study is to evaluate the correlation of BLL with BMD in American children and adolescents. We hypothesize that BLL is negatively associated with BMD in children and adolescents.

MATERIALS AND METHODS

Study Population

National Health and Nutrition Examination Survey (NHANES), using a stratified, multi-stage random sampling design, is a nationally representative nutrition survey of general populations in the United States. Three consecutive cycles of NHANES (2005–2006, 2007–2008, 2009–2010) were selected since the femur BMD of American children and adolescents are only available in these cycles. We initially included 7,313 subjects aged 8–19 years from NHANES 2005-2010. After excluding 1,729 subjects with missing BMD (n = 1,157) and blood lead (n = 573) data, this study includes 5,583 eligible subjects for analysis.

Variables

The venous blood of the subjects was collected during the interview. Whole blood specimens were processed and shipped to the National Center for Environmental Health for testing and analysis. BLL was tested using inductively coupled plasma mass spectrometry. A detailed manual of laboratory procedures is available on the NHANES website (21). The dependent variables were the total femur, femur neck, and total spine BMD measured by DXA (Hologic, Inc., Bedford, Massachusetts). Total spine BMD was the average BMD of the L1 to L4. The left hip was routinely scanned to estimate the total femur, and femoral neck BMD. The right hip was selected for scanning if participants had left hip arthroplasty or metal object implantation. BMD values were collected and standardized by professionals.

Based on previous studies (18, 22–24), confounders that may influence BMD were chosen to eliminate potential effects on the results. We also performed a multicollinearity analysis of these covariates and did not find the presence of multicollinearity. Finally, the following covariates were collected and adjusted, including gender, age, race/ethnicity, PIR (poverty income ratio), body mass index (BMI), serum calcium, and serum phosphorus. Details on the covariates can be found on the NHANES website (http://www.cdc.gov/nchs/nhanes/).

Statistical Analysis

All statistical analyses were performed using Package R and EmpowerStats (http://www.empowerstats.com), with a complex weighted sampling design from NHANES. The characteristics of participants were described according to quartile of BLL (Categories 1: 0.18-0.59 ug/dl; Categories 2: 0.59-0.82 ug/dl; Categories 3: 0.82-1.20 ug/dl; Categories 4: >1.20 ug/dl). We used percentages for categorical variables and means ± standard deviations for continuous variables. To compare the difference among the groups, we employed the weighted $\chi 2$ test and linear regression model to analyze categorical and continuous variables, respectively. Weighted multivariate linear regression models were used to assess the association between BLL and the total femur, femur neck, and total spine BMD. An unadjusted model was created first (Model 1), and then a minimally adjusted model (Model 2) was built after adjusting age, gender, and race/ethnicity. Finally, a fully adjusted model (Model 3) was calculated, adjusting for age, gender, race/ethnicity, PIR, BMI, serum calcium, and serum phosphorus. Then stratified analyses were performed by age, gender, and race/ethnicity, and their interactions were tested. As the current normal reference value range of BLL in the U.S. is 3.5 ug/dl (25), we performed another weighted multivariate linear regression analysis after excluding individuals whose BLL was more than 3.5 ug/dl to exclude the influence of the children and adolescents with very high BLL. *P* values less than 0.05 were considered statistically significant in the analyses.

RESULTS

Study Participants and Baseline Characteristics

A total of 5,583 participants with a mean age of 13.49 ± 3.35 years were enrolled. BLL values were detected in all participants, with a mean of 1.04 ± 0.87 ug/dL. In this study, 59.28% of the participants were non-Hispanic white, 13.94% were non-Hispanic black, 13.77% were Mexican American, and 7.16% were other races (including multiracial population), and 5.85% were other Hispanic. The weighted characteristics of participants were described according to quartile of BLL (Categories 1: 0.18-0.59 ug/dl; Categories 2: 0.59-0.82 ug/dl; Categories 3: 0.82-1.20 ug/dl; Categories 4: >1.2 ug/dl), as listed in **Table 1**. According to the BLL quartiles, the participants' characteristics were significantly different except for cholesterol and total protein. Participants in the highest quartile of BLL were more likely to be younger, men, and non-Hispanic Blacks. They had a higher value of serum uric acid and a lower value of PIR, BMI, total femur, femur neck, and total spine BMD.

Correlation Between Blood Lead and Bone Mineral Density

Overall

The results of weighted multivariate regression analyses for the association between BLL and BMD in American children and adolescents were shown in **Table 2**. BLL was negatively correlated to BMD in the three models at all sites of interest. After adjusting all covariates (model 3), BLL was negatively correlated to total spine BMD (β= -0.011 95% CI: -0.015, -0.007, *P*<0.001), total femur BMD (β= -0.008 95% CI: -0.012, -0.003, *P*=0.001), and femur neck (β= -0.006 95% CI: -0.010, -0.002, *P*=0.005). Smooth curve fittings of the association between BLL and BMD at lumbar spine and femur were shown in **Figure 1** and **Appendices 1**, **2**. We converted BLL from a continuous variable to a categorical variable (quartiles). Individuals in the highest BLL quartile had a lower mean BMD than those in the lowest quartile, with -0.018 g/cm^2 lower BMD at total spine (β= -0.018 95% CI: -0.027, -0.010, *P*<0.001), -0.013 g/cm^2 at the total femur (β= -0.013 95% CI: -0.022, -0.004, *P*<0.001), and -0.010 g/cm^2 at femur neck (β= -0.010 95% CI: -0.018, -0.009, *P*=0.029) (**Table 2**). This association remained significant in American children and adolescents after excluding participants with BLL more than 3.5 ug/dl (**Table 3**).

Stratified Analyses by Age, Gender, and Race/Ethnicity

Stratified analyses were performed by age (8-13 and 14-19) (**Table 2**). In the fully adjusted models, the negative correlation was also significant in ages between 8-13 and 14-19 years at all sites of interest, with no interactive effect. In aged between 8-13, the BLL was negatively correlated to total spine BMD (β= -0.014 95% CI: -0.020, -0.008, *P*<0.001), total femur BMD (β= -0.010 95% CI: -0.015, -0.004, *P*<0.001), and femur neck (β= -0.007 95% CI: -0.012, -0.002, *P*<0.001). In aged between 14-19, the BLL was negatively correlated to total spine BMD (β= -0.013 95% CI: -0.020, -0.006, *P*<0.001), total femur BMD (β= -0.011 95% CI: -0.019, -0.004, *P*=0.004), and femur neck (β= -0.009 95% CI: -0.017, -0.002, *P*=0.015). For gender, the negative correlation was also significant in males and females at all sites of interest in the fully

TABLE 1 | Characteristics of the study population based on BLL quartiles.

		Blood lead (μg/dL)				
	total	**Q1 (0.18-0.59)**	**Q2 (0.59-0.82)**	**Q3 (0.82-1.20)**	**Q4 (>1.20)**	***P* value**
Number of subjects (n)	5583	1386	1386	1391	1420	
Age (years)	13.49 ± 3.35	14.09 ± 3.06	13.65 ± 3.36	13.28 ± 3.45	12.70 ± 3.43	<0.001
Gender (%)						<0.001
Men	53.27%	36.74%	53.72%	60.84%	66.86%	
Women	46.73%	63.26%	46.28%	39.16%	33.14%	
Race/ethnicity (%)						<0.001
Mexican American	13.77%	14.41%	11.98%	12.77%	16.39%	
Other Hispanic	5.85%	5.11%	5.36%	6.20%	7.11%	
Non-Hispanic White	59.28%	67.64%	62.35%	56.40%	47.00%	
Non-Hispanic Black	13.94%	8.39%	12.89%	15.81%	20.87%	
Other Race (Including Multi-Racial)	7.16%	4.45%	7.42%	8.83%	8.63%	
PIR	2.61 ± 1.60	3.00 ± 1.60	2.82 ± 1.55	2.41 ± 1.58	2.01 ± 1.49	<0.001
Blood urea nitrogen (mmol/L)	3.67 ± 0.96	3.62 ± 0.90	3.64 ± 0.93	3.73 ± 1.09	3.71 ± 0.93	0.007
Serum total calcium (mmol/L)	2.41 ± 0.06	2.41 ± 0.07	2.41 ± 0.06	2.42 ± 0.07	2.42 ± 0.06	<0.001
Serum phosphorus (mmol/L)	1.42 ± 0.18	1.40 ± 0.17	1.42 ± 0.18	1.44 ± 0.19	1.44 ± 0.18	<0.001
Cholesterol (mmol/L)	4.24 ± 0.64	4.21 ± 0.69	4.24 ± 0.64	4.24 ± 0.61	4.27 ± 0.60	0.188
Total protein (g/L)	7.20 ± 0.33	7.19 ± 0.34	7.20 ± 0.35	7.19 ± 0.33	7.21 ± 0.30	0.508
Serum uric acid (mmol/L)	301.80 ± 58.97	291.10 ± 60.81	303.55 ± 59.76	304.96 ± 55.87	310.74 ± 56.69	<0.001
BMI (kg/m^2)	22.49 ± 5.68	23.61 ± 5.91	22.95 ± 5.81	22.15 ± 5.53	21.27 ± 5.19	<0.001
Total femur BMD (g/cm^2)	0.90 ± 0.19	0.92 ± 0.18	0.92 ± 0.19	0.89 ± 0.19	0.87 ± 0.19	<0.001
Femur neck (g/cm^2)	0.84 ± 0.17	0.86 ± 0.16	0.85 ± 0.18	0.82 ± 0.17	0.81 ± 0.17	<0.001
Total spine BMD (g/cm^2)	0.85 ± 0.20	0.90 ± 0.19	0.86 ± 0.20	0.83 ± 0.20	0.79 ± 0.20	<0.001
Blood lead (μg/dL)	1.04 ± 0.87	–	–	–	–	–

Mean ± SD for continuous variables: the P value was calculated by the weighted linear regression model. (%) for categorical variables. The P value was calculated by the weighted chi-square test. BLL Blood lead levels. BMD bone mineral density. PIR poverty income ratio. BMI body mass index.

TABLE 2 | Results of weighted linear regression modeling for associations of the BLL with BMD at different sites.

	Total spine			Total femur			Femur neck		
	Model 1 β (95% CI) *P* value	**Model 2 β (95% CI) *P* value**	**Model 3 β (95% CI) *P* value**	**Model 1 β (95% CI) *P* value**	**Model 2 β (95% CI) *P* value**	**Model 3 β (95% CI) *P* value**	**Model 1 β (95% CI) *P* value**	**Model 2 β (95% CI) *P* value**	**Model 3 β (95% CI) *P* value**
Per 1 ug/dL increase BLL (Quartile)	0.046 (-0.053, -0.038) <0.001	-0.016 (-0.021, -0.012) <0.001	-0.011 (-0.015, -0.007) <0.001	-0.026 (-0.033, -0.019) <0.001	-0.015 (-0.020, -0.010) <0.001	-0.008 (-0.012, -0.003) 0.001	-0.022 (-0.028, -0.016) <0.001	-0.014 (-0.018, -0.009) <0.001	-0.006 (-0.010, -0.002) 0.005
Q1 (0.18-0.59)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Q2 (0.59-0.82)	-0.036 (-0.050, -0.022) <0.001	-0.008 (-0.017, 0.000) 0.052	-0.004 (-0.012, 0.003) 0.266	-0.007 (-0.020, 0.006) 0.283	-0.002 (-0.011, 0.007) 0.592	0.003 (-0.010, 0.008) 0.812	-0.006 (-0.018, 0.006) 0.304	-0.002 (-0.011, 0.006) 0.597	0.003 (-0.005, 0.011) 0.437
Q3 (0.82-1.20)	-0.071 (-0.085, -0.057) <0.001	-0.024 (-0.032, -0.015) <0.001	-0.014 (-0.022, -0.006) <0.001	-0.034 (-0.047, -0.020) <0.001	-0.021 (-0.030, -0.012) 0.001	-0.007 (-0.026, 0.008) 0.114	-0.033 (-0.045, -0.020) <0.001	-0.022 (-0.031, -0.013) <0.001	-0.008 (-0.016, -0.001) 0.048
Q4 (>1.20)	-0.106 (-0.121, -0.091) <0.001	-0.031 (-0.040, -0.021) <0.001	-0.018 (-0.027, -0.010) <0.001	-0.058 (-0.072, -0.044) <0.001	-0.031 (-0.041, -0.021) <0.001	-0.013 (-0.022, -0.004) <0.001	-0.050 (-0.063, -0.037) <0.001	-0.028 (-0.037, -0.018) <0.001	-0.010 (-0.018, -0.001) 0.029
P for trend	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	<0.001	<0.001	0.005
Stratified by age									
8–13 years	-0.025 (-0.032, -0.018) <0.001	-0.025 (-0.032, -0.018) <0.001	-0.014 (-0.020, -0.008) <0.001	-0.014 (-0.021, -0.008) <0.001	-0.023 (-0.029, -0.016) <0.001	-0.010 (-0.015, -0.004) <0.001	-0.011 (-0.017, -0.005) <0.001	-0.020 (-0.026, -0.014) <0.001	-0.007 (-0.012, -0.002) <0.001
14-19 years	-0.017 (-0.025, -0.009) <0.001	-0.013 (-0.021, -0.005) 0.001	-0.013 (-0.020, -0.006) <0.001	0.005 (-0.004, 0.014) 0.320	-0.015 (-0.023, -0.006) 0.001	-0.011 (-0.019, -0.004) 0.004	0.003 (-0.006, 0.012) 0.539	-0.012 (-0.021, -0.003) 0.006	-0.009 (-0.017, -0.002) 0.015
Test for interaction	0.158	0.025	0.833	<0.001	0.142	0.705	<0.001	0.142	0.579
Stratified by sex									
Men	-0.021 (-0.031, -0.012) <0.001	-0.011 (-0.016, -0.005) <0.001	-0.008 (-0.012, -0.003) <0.001	-0.020 (-0.029, -0.011) <0.001	-0.013 (-0.019, -0.007) <0.001	-0.008 (-0.014, -0.002) 0.006	-0.017 (-0.025, -0.009) <0.001	-0.012 (-0.018, -0.006) <0.001	-0.007 (-0.012, -0.002) 0.002
Women	-0.079 (-0.091, -0.067) <0.001	-0.029 (-0.037, -0.021) <0.001	-0.019 (-0.026, -0.012) <0.001	-0.060 (-0.070, -0.049) <0.001	-0.024 (-0.032, -0.016) <0.001	-0.011 (-0.018, -0.004) <0.001	-0.050 (-0.060, -0.040) <0.001	-0.020 (-0.028, -0.012) <0.001	-0.007 (-0.014, 0.001) 0.054
Test for interaction	<0.001	<0.001	0.006	<0.001	<0.030	0.523	<0.001	0.117	0.974
Stratified by race/ethnicity									
Mexican American	-0.028 (-0.038, -0.017) <0.001	0.019 (-0.026, -0.013) <0.001	-0.013 (-0.019, -0.007) <0.001	-0.014 (-0.024, -0.004) 0.005	-0.015 (-0.022, -0.008) <0.001	-0.007 (-0.013, -0.001) 0.017	-0.014 (-0.023, -0.005) 0.001	-0.015 (-0.022, -0.009) <0.001	-0.007 (-0.013, -0.002) 0.012
Other Hispanic	-0.035 (-0.059, -0.012) 0.003	-0.012 (-0.026, 0.002) 0.103	-0.011 (-0.024, 0.002) 0.103	-0.016 (-0.037, 0.005) 0.129	-0.011 (-0.026, 0.003) 0.134	-0.011 (-0.024, 0.003) 0.136	-0.007 (-0.026, 0.012) 0.473	-0.002 (-0.017, 0.013) 0.792	-0.002 (-0.015, 0.012) 0.823
Non-Hispanic White	-0.051 (-0.067, -0.034) <0.001	-0.015 (-0.025, -0.005) 0.003	-0.011 (-0.020, -0.001) 0.024	-0.031 (-0.046, -0.015) <0.001	-0.018 (-0.029, -0.007) <0.001	-0.010 (-0.020, -0.001) 0.042	-0.027 (-0.041, -0.014) <0.001	-0.017 (-0.027, -0.006) 0.001	-0.009 (-0.019, -0.001) 0.048
Non-Hispanic Black	-0.068 (-0.080, -0.057) <0.001	-0.019 (-0.026, -0.011) <0.001	-0.012 (-0.019, -0.005) <0.001	-0.047 (-0.057, -0.036) <0.001	-0.013 (-0.021, -0.005) 0.001	-0.005 (-0.012, 0.002) 0.165	-0.042 (-0.051, -0.032) <0.001	-0.013 (-0.020, -0.005) 0.001	-0.005 (-0.012, -0.002) 0.183
Other Race	-0.046 (-0.085, -0.007) 0.022	-0.002 (-0.025, 0.021) 0.880	0.004 (-0.018, 0.025) 0.733	-0.024 (-0.060, 0.012) 0.186	-0.004 (-0.029, 0.021) 0.755	-0.001 (-0.022, 0.023) 0.976	-0.013 (-0.045, 0.020) 0.441	0.006 (-0.018, 0.029) 0.646	0.012 (-0.009, 0.032) 0.272
Test for interaction	<0.001	0.556	0.637	0.027	0.748	0.834	0.017	0.231	0.334

Model 1 unadjusted. Model 2 adjusted for age, gender, and race/ethnicity. Model3 adjusted for age, gender, and race/ethnicity, PIR, body mass index, serum calcium, serum phosphorus. BLL Blood lead levels. BMD bone mineral density. PIR poverty income ratio.

adjusted models. Effect estimates were stronger in girls than boys at the lumbar spine (*P* for interaction= 0.006). For race/ethnicity, the negative association between BLL and BMD was pronounced in Mexican American, Non-Hispanic White, and Non-Hispanic Black in the fully adjusted models, but not in Other Hispanic and Other Races (Including Multi-Racial). However, no interactive effects were observed.

DISCUSSION

The present study found a negative association between BLL and BMD at the spine and femur in children and adolescents. For every 1mg/dl increase in BLL, the BMD of the total spine, total hip, and femoral neck decreased by 0.011 g/cm^2, 0.008 g/cm^2, and 0.006 g/cm^2, respectively. The results showed that Pb affected the lumbar spine more than the femur. The effect estimates were stronger in girls than boys at the lumbar spine. This association remained significant in American children and adolescents after excluding participants with BLL more than 3.5 ug/dl. It means that even at the reference concentration (<3.5 ug/dl), BLL still has a negative correlation with BMD in children and adolescents.

Pb has been proved to be a hazardous metal toxic to many organs and systems, including the kidneys, bones, blood system, digestive system, nervous system, and so on (26). BLL in Americans

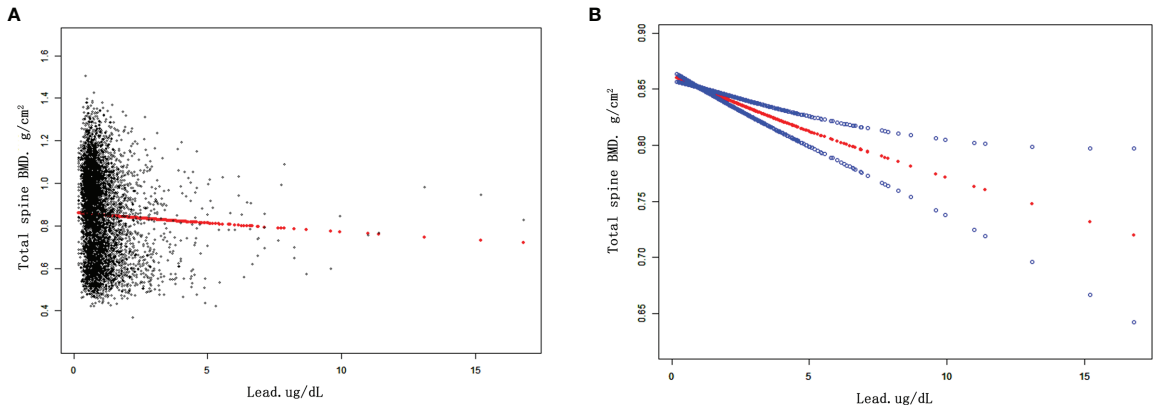

FIGURE 1 | The associations between BLL and total spine BMD. **(A)** Each black point represents a sample. **(B)** Red line represents the smooth curve fit between variables. Blue lines represent the 95% of confidence interval from the fit. Age, gender, race/ethnicity, PIR, BMI, serum calcium, serum phosphorus were adjusted. Abbreviations: BLL, blood lead levels. PIR, poverty income ratio. BMI, body mass index. BMD, bone mineral density.

has been reported to decline in recent years (27). In 2012, the Centers for Disease Control (CDC) identified blood lead reference value (BLRV) as 5.0 ug/dl using the BLL distribution in American children aged 1–5 years from NHANES 2007-2010. Then, CDC updated BLRV in children to 3.5 μg/dL using NHANES 2015-2018 (25). The mean concentration of BLL in our study was 1.04 ± 0.87 ug/dl, which was lower than in previous studies (17, 28). Among the 5,583 subjects, only 103 (1.84%) participants were above 3.5μg/dL. Nevertheless, our study demonstrated that Pb exposure had an adverse effect on BMD in children and adolescents, even at a low level (<3.5 μg/dL). Cumulative evidence has suggested that there was no safe BLL for children since even very low levels cause harm (29). Further actions are needed to be taken by individuals, healthcare providers, and policymakers to eliminate Pb exposure among children and adolescents, especially in some areas with potential threats (30).

Many studies have explored the molecular mechanism between Pb and bone metabolism. *In vitro*, experiments showed that Pb could inhibit the normal physiological metabolism of chondrocytes and osteoblasts (31, 32). In addition, Pb could inhibit the function of active vitamin D and the absorption of calcium from the diet (33). Moreover, Pb could interfere with normal bone metabolism through competitive inhibition of osteocalcin (34). Studies investigating the association of Pb exposure and BMD have drawn different conclusions for adults (10, 15, 35). Lu et al. (15) found that BLL was negatively associated with BMD in American female adults. However, this negative correlation was not found in men. Another study using the NHANES database showed a negative association of blood lead and urine lead concentrations with BMD in American women older than 40 years (10). Wei et al. (35) found that Pb exposure was negatively associated with BMD in American adults aged ≥20 years. Studies that reported the relationship between BLL and BMD in children and adolescents were scarce and inconsistent. An early study measuring BMD using DXA at one-third of the radius, investigated the association between BLL and BMD in American children (28). They included 59 black children who attended the Medical and Lead Poisoning Clinic. However, this small-sample study did not find any association between BLL and BMD. In another early cross-sectional study by James et al. (17), they recruited African American children aged 8-10 years for analysis. The children were divided into two cohorts by BLL. They found that the cohort with high BLL had higher BMD, which was contrary to the finding of our study. They made some explanations for the results. Pb can inhibit the parathyroid hormone-related peptide (PTHrP) and transform growth factor-β1, leading to the chondrocyte precocity (36). However, the higher BMD associated with the PTHrP suppression was transient. Although PTHrP-deficient mice were born with higher BMD, their BMD got lower in later years (37). Different study populations, designs, and statistical methods could explain these inconsistent results. First, the two studies included a small sample size, which can affect the reliability of the results. Moreover, they only included African American children, which was far from representative because the

TABLE 3 | Results of weighted linear regression modeling for associations of the BLL with BMD at different sites after excluding individual blood lead more than 3.5 ug/dl.

	Model 1 β (95% CI) *P* value	Model 2 β (95% CI) *P* value	Model 3 β (95% CI) *P* value
Total spine	-0.069 (-0.080, -0.059) <0.001	-0.022 (-0.029, -0.016) <0.001	-0.014 (-0.020, -0.008) <0.001
Total femur	0.039 (-0.049, -0.030) <0.001	-0.024 (-0.031, -0.017) <0.001	-0.012 (-0.018, -0.005) <0.001
Femur neck	-0.033 (-0.042, -0.024) <0.001	-0.021 (-0.027, -0.014) <0.001	-0.009 (-0.015, -0.003) 0.004

Model 1 unadjusted. Model 2 adjusted for age, gender, and race/ethnicity. Model3 adjusted for age, gender, and race/ethnicity, PIR, body mass index, serum calcium, serum phosphorus. BLL Blood lead levels. BMD bone mineral density. PIR poverty income ratio.

United States was a multiracial country. Furthermore, these were comparative studies without adjusting relevant variables and performing regression analyses. A recent study conducted by Li et al. (18) found an N-shaped curve relationship between BLL and BMD in children and adolescents. However, there are also some limitations to this study. The outcome variables were lumbar spine BMD, limb BMD, subtotal BMD, and total BMD, but not femur BMD. As we all know, the lumbar spine and femur are usually the sites of most concern to individuals and clinicians regarding osteoporosis (38, 39). The strength of our study was that we included all cycles of NHANES (2005-2010) that included both lumbar spine and femoral bone density data of children and adolescents. In addition, the study did not perform multiple regression analysis, and we could not quantify the relationship between BLL and BMD. Furthermore, we noticed that the study adjusted height and weight, variables with potential multicollinearity in children and adolescents when multivariable generalized additive models (GAMS) were performed. It may lead to inaccurate results.

A study by Campbell JR et al. using data of people older than 50 years from the NHANES database confirmed that Pb exposure was significantly negatively associated with BMD in white subjects, but not in Blacks (16) after adjusting numerous variables. However, we could not determine whether the effect of Pb on BMD was race-specific because no interaction effect was found. Although our results showed that the effects were pronounced in Mexican American, Non-Hispanic White, and Non-Hispanic Black, the effects were not statistically significant for Other Hispanic and Other Races. Further studies with stronger evidence are needed to elucidate the effect of race on Pb exposure and bone mineral density in children and adolescents.

Another remarkable result was that females were more sensitive to Pb exposure than males at the lumbar spine, as proven by the interaction test (P= 0.006 for interaction). Several previous studies have come up with the same conclusion for adults (10, 15). Since adolescent women seldom experience menopause, pregnancy, lactation, and so on, further research is needed to explore the underlying mechanisms. Our study also showed that BLL had more effect on the lumbar spine than the femur in American children and adolescents, which was consistent with previous research (40). One explanation is that the trabecular bone (spine) has a larger surface area than cortical bone (femur), causing the spine to absorb more Pb than the femur.

To our knowledge, this is the first large-sample cross-sectional study that found an adverse association between Pb exposure and BMD in children and adolescents. The sample of our study adopts multi-layer random sampling with high reliability and standardization of data, which represents the general population of the United States. However, some limitations should be acknowledged. First, since this is a cross-sectional study, no causal relationship can be inferred. Second, we did not adjust variables such as calcium intake, diet, and exercise, which could bias the results. Third, since DXA is only available to children over 8 years, we were not able to investigate the association between BLL and BMD in children younger than 8 years old.

CONCLUSIONS

Our study indicates that BLL is negatively correlated with BMD at different sites of interest in children and adolescents aged 8-19 years, even in the reference range. In addition, the results show that Pb affects the lumbar spine more than the femur. The effect estimates are stronger in girls than boys at the lumbar spine. Considering the possible adverse effects of Pb exposure on BMD in children and adolescents, individuals, healthcare providers, and policymakers should make efforts to eliminate Pb exposure among children and adolescents. More research is needed to elucidate the relationships between Pb and bone health in children and adolescents, including specific mechanisms and confounding factors like race/ethnicity, gender, and age.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics review board of the National Center for Health Statistics. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Conceptualization, AC, PX, FZ and YZ; Data curation, AC, PX, and BH; Formal analysis, AC, PX, BH, YZM, ZF, HW, FZ, and YZ; Investigation, AC, PX, BH, YZM, ZF, HW, FZ, and YZ; Methodology, AC, PX, and BH; Project administration, AC, PX, FZ and YZ; Software, AC, PX, and BH; Visualization, AC, PX, BH, YZM, ZF, HW, FZ, and YZ; Writing – review & editing, AC, PX, BH, YZM, ZF, HW, FZ, and YZ. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.928752/full#supplementary-material

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APPENDIX 1

The associations between BLL and total femur BMD. **(A)** Each black point represents a sample. **(B)** Red line represents the smooth curve fit between variables. Blue lines represent the 95% of confidence interval from the fit. Age, gender, race/ethnicity, PIR, BMI, serum calcium, serum phosphorus were adjusted. Abbreviations: BLL, blood lead levels. PIR, poverty income ratio. BMI, body mass index. BMD, bone mineral density.

APPENDIX 2

The associations between BLL and femur neck BMD. **(A)** Each black point represents a sample. **(B)** Red line represents the smooth curve fit between variables. Blue lines represent the 95% of confidence interval from the fit. Age, gender, race/ethnicity, PIR, BMI, serum calcium, serum phosphorus were adjusted. Abbreviations: BLL, blood lead levels. PIR, poverty income ratio. BMI, body mass index. BMD, bone mineral density.

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Saturation Effect of Body Mass Index on Bone Mineral Density in Adolescents of Different Ages: A Population-Based Study

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Background: Adolescence is a critical period for bone development, and peak bone mass may be reached in late adolescence. Boosting bone accumulation at this time can help preserve adult bone health and avoid osteoporosis later in life. Body mass index (BMI) has been found to have a favorable impact on bone mineral density (BMD) in previous research. However, excessive obesity is harmful to health and may lead to various systemic diseases. Therefore, finding an appropriate BMI to maintain a balance between obesity and BMD is critical for adolescents.

Methods: The datasets from the National Health and Nutrition Examination Survey (NHANES) 2011–2020 were used in a cross-sectional investigation. Multivariate linear regression models were used to examine the linear connection between BMI and BMD. Fitted smoothing curves and threshold effect analysis were used to describe the nonlinear relationship. Subgroup analyses were then conducted based on gender and age.

Results: This population-based study included a total of 6,143 adolescents aged 8–19 years. In a multivariate linear regression analysis, a good association between BMI and total BMD was shown [0.014 (0.013, 0.014)]. This positive association was maintained in all subgroup analyses grouped by sex and age. Furthermore, the association between BMI and BMD was nonlinear with a saturation point present, as evidenced by smoothed curve fitting. According to the threshold effect study, with an age group of two years, adolescents of different ages had different BMI saturation values with respect to BMD.

Conclusions: Our study showed a significant positive and saturated association between BMI and BMD in adolescents aged 8–19 years. Maintaining BMI at saturation values may reduce other adverse effects while achieving optimal BMD.

Keywords: bone mineral density, osteoporosis, NHANES, obese, body mass index, adolescent

BACKGROUND

Osteoporosis is a long-term disorder marked by reduced bone mineral density (BMD) that affects a huge number of people (1). Adolescence is a critical period for bone development, and peak bone mass (PBM) may be reached in late adolescence (2, 3). There is evidence that increasing PBM by 5% throughout childhood and adolescence reduces the risk of osteoporotic fractures by 40%, whereas increasing PBM by 10% reduces the risk by half (4, 5). As a result, boosting bone accumulation at this time can help preserve adult bone health and avoid osteoporosis later in life (6, 7). In addition to metabolic disorders such as lipids (8, 9), serum calcium (10), and non-alcoholic fatty liver disease (11), obesity has been shown to have an impact on adolescent BMD (12). Meanwhile, scientists are working to discover novel ways to prevent and treat osteoporosis.

Obesity is a major health issue that affects individuals all over the globe (13). The prevalence of overweight and obesity among children and adolescents aged 5–19 years rose sharply from 4% in 1975 to more than 18% in 2016 (14). Previous research has shown that body mass index (BMI) and BMD have a significant positive relationship (15, 16). However, excessive obesity not only has very serious consequences for various organs and systems (17, 18) but may also increase the risk of fractures in children (19). We hypothesized that BMI had a saturation point, and that keeping BMI at this level would provide the greatest balance between obesity and BMD. Therefore, finding an appropriate BMI to maintain a balance between obesity and BMD is critical for adolescents. As a result, we examined the relationship between BMI and BMD in adolescents in this study, utilizing a large sample of people aged 8–19 from the National Health and Nutrition Examination Survey (NHANES).

METHODS

Study Population

The National Health and Nutrition Examination Survey is a representative survey of the US national population that uses a complicated, multistage, and probabilistic sampling methodology to provide a wealth of information about the general US population's nutrition and health (20). The 2011–2020 continuous cycle of the US NHANES dataset was used for this investigation. In this round, there were 68,394 participants. After eliminating patients who lacked information on laboratory and demographic characteristics, a total of 6,143 subjects were included in the analysis. **Figure 1** illustrates the sample selection flow chart.

Study Variables

The dependent variable in this study is BMI, with total BMD as the intended independent variable. Weight divided by height squared is how BMI is computed according to the international guidelines. To ensure that the data are trustworthy, outliers will be subjected to appropriate scrutiny. The age, weight, height, and gender of the individual are used to verify the accuracy of the data. After verification, inaccurate data were removed. A dual-energy X-ray absorptiometry scan was used to calculate the total BMD. Covariates included age, gender, race, standing height, education level, family income-to-poverty ratio, activities status, diabetes status, alanine transaminase (ALT), weight, alkaline phosphatase (ALP), waist circumference, aspartate aminotransferase (AST), total calcium, total cholesterol, direct high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglyceride, phosphorus, blood urea nitrogen, and serum glucose. For more detailed information on BMI, total BMD, and confounders, visit http://www.cdc.gov/nchs/nhanes/.

Statistical Analysis

The statistical study was carried out using the statistical computing and graphics software R (version 4.1.3), Origin (version 2021b), and EmpowerStats (version 2.0). Baseline tables for the study population were statistically described by BMI subgroup, and continuous variables are described using means ± standard deviation and weighted linear regression models. The beta values and 95% confidence intervals (CI) were calculated using multivariate linear regression analysis between the BMI and BMD. The multivariate test was built using three models: Model 1: no variables adjusted; Model 2: gender, age, and race adjusted; Model 3: adjusted for all covariates except for height and weight, which had a large effect on exposure factors. By adjusting the variables, smoothed curve fits were done simultaneously. A threshold effects analysis model was used to examine the relationship and saturation value between BMI and BMD. Finally, the same statistical study methods described above were conducted for the gender and BMI subgroups. It was determined that $P < 0.05$ was statistically significant. We used a weighting approach to reduce the significant volatility of our dataset.

RESULTS

Baseline Characteristics

A total of 6,143 adolescents were included in this study based on the inclusion and exclusion criteria, and the average age of the participants was 13.10 ± 3.46 years. Among these participants, 51.77% were boys, 48.23% were girls, 27.53% were non-Hispanic white, 23.98% were non-Hispanic black, 20.56% were Mexican-American, and 27.93% were other races. The mean (SD) concentrations of BMI and total BMD were 22.28 (5.99) kg/m^2 and 0.95 (0.16) g/cm^2, respectively. **Table 1** lists the clinical features of the study participants, and column stratified grouping was based on BMI dividing all participants equally into four groups by number. **Figure 2** shows the frequency distribution of BMI for total participants and for participants of different genders. In comparison to the bottom quartile, those in the top quartile with higher BMI were more likely to be females and older, with a higher proportion of non-Hispanic blacks and

Abbreviations: BMD, bone mineral density; BMI, Body Mass Index; NHANES, National Health and Nutrition Examination Survey; PBM, peak bone mass.

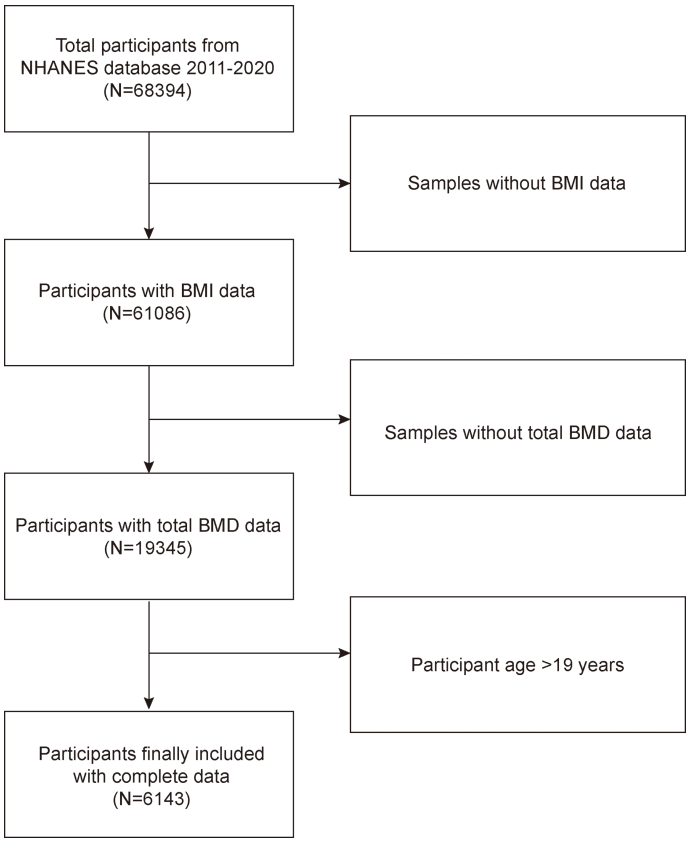

FIGURE 1 | Flow chart of participants selection. NHANES, National Health and Nutrition Examination Survey; BMD, bone mineral density; Body Mass Index, BMI.

Mexican-Americans, with higher prevalence of diabetes, and with higher levels of weight, standing height, waist circumference, AST, ALT, Total cholesterol, LDL cholesterol, serum glucose, total BMD, triglyceride, and total BMD but with lower levels of ratio of family income to poverty, ALP, total calcium, direct HDL cholesterol, phosphorus, and blood urea nitrogen ($P < 0.05$).

Association Between BMI and Total BMD

Table 2 shows the results of the multivariate regression analysis. In the unadjusted model [0.014 (0.013, 0.014)], BMI was highly associated with total BMD. In addition, this relationship remained significant after adjusting corresponding variables in Model 2 [0.006 (0.005, 0.006)] and Model 3 [0.005 (0.004, 0.005)]. In the unadjusted model, the beta value was 0.014, meaning that, for every unit increase in BMI, the total BMD increased by 0.014 g/cm^2.

In all subgroups, BMI showed a significant positive association with total BMD. In the subgroup analysis stratified by sex, the effect values were closer for boys and girls, 0.015 and 0. 013, respectively. Whereas in the subgroup analysis stratified by age, the effect values for adolescents aged 8–15 years were significantly larger than those for adolescents aged 16–19 years, implying that, for each unit increase in BMI for adolescents aged 8–15 years, BMD increased by 0.07 g/cm^2 and, for each unit increase in BMI for adolescents aged 16–19 years, BMD increased by only 0.04 g/cm^2. In addition, the results of the BMI quartile subgroup analysis showed that there was a dose-response relationship between BMI and total BMD.

Non-Linearity and Saturation Effect Analysis Between BMI and Total BMD

Smooth curve fittings were performed to characterize the non-linear relationship and saturation effect between BMI and total BMD (**Figures 3**, **4**). We discovered that the saturation effect value between BMI and total BMD was 21.5 kg/m^2 in total participants (**Table 3**). When the BMI was under 21.5 kg/m^2, the effect value was 0.036. However, when BMI exceeded 21.5 kg/m^2, the effect value became 0.005. All participants were divided into six groups according to an age group of 2 years and the saturation values of BMI for total BMD of adolescents at different ages were determined using smoothed fitted curves and saturation effects analysis (**Table 3**).

TABLE 1 | Characteristics of the participants.

Outcome	BMI (kg/m^2) Quartiles				*P*-value
	Q1, 18.1< (N = 1,524)	Q2, 18.1-21.0 (N = 1,532)	Q3, 21.1-23.4 (N = 1,549)	Q4, >23.4 (N = 1,538)	
Age (years)	10.406 ± 2.425	13.053 ± 3.233	14.206 ± 3.286	14.699 ± 3.128	<0.001
Gender (%)					<0.001
Male	55.381	48.825	53.777	49.090	
Female	44.619	51.175	46.223	50.910	
Race (%)					<0.001
Non-Hispanic White	31.234	27.350	28.018	23.537	
Non-Hispanic Black	23.031	24.086	21.821	26.983	
Mexican-American	16.929	18.277	21.562	25.423	
Other race	28.806	30.287	28.599	24.057	
Weight (kg)	33.496 ± 7.889	47.850 ± 9.186	59.129 ± 10.108	81.402 ± 19.604	<0.001
Standing height (cm)	142.756 ± 13.682	155.671 ± 14.374	160.363 ± 12.984	162.745 ± 11.633	<0.001
Waist circumference(cm)	60.464 ± 4.989	70.018 ± 4.380	78.462 ± 5.098	96.614 ± 13.295	
Ratio of family income to poverty	2.270 ± 1.664	2.147 ± 1.577	2.110 ± 1.577	1.838 ± 1.394	<0.001
Moderate activities (%)					0.133
Yes	52.742	51.949	50.442	50.485	
No	47.258	48.051	49.558	49.515	
Diabetes status (%)					<0.001
Yes	0.000	0.196	0.646	0.845	
No	100.000	99.804	99.364	99.155	
ALT (U/L)	16.027 ± 5.400	16.173 ± 7.555	18.196 ± 9.688	23.701 ± 17.523	<0.001
AST (U/L)	24.545 ± 6.062	23.028 ± 6.887	23.608 ± 9.358	24.823 ± 15.572	0.002
ALP (U/L)	201.910 ± 105.025	150.348 ± 106.815	118.515 ± 82.951	109.776 ± 69.877	<0.001
Total calcium (mmol/L)	2.415 ± 0.074	2.407 ± 0.073	2.400 ± 0.073	2.386 ± 0.079	<0.001
Total cholesterol (mmol/L)	4.075 ± 0.733	3.990 ± 0.672	4.043 ± 0.743	4.164 ± 0.804	<0.001
Direct HDL cholesterol (mmol/L)	1.548 ± 0.337	1.450 ± 0.308	1.353 ± 0.300	1.207 ± 0.270	<0.001
LDL cholesterol (mmol/L)	2.059 ± 0.575	2.121 ± 0.619	2.282 ± 0.689	2.422 ± 0.723	<0.001
Triglyceride(mmol/L)	0.677 ± 0.366	0.716 ± 0.389	0.806 ± 0.517	1.088 ± 0.702	<0.001
phosphorus(mmol/L)	1.536 ± 0.214	1.445 ± 0.223	1.383 ± 0.206	1.356 ± 0.203	<0.001
Blood urea nitrogen(mmol/L)	3.857 ± 1.289	3.883 ± 1.204	4.014 ± 1.232	3.826 ± 1.129	0.003
Serum glucose(mmol/L)	4.913 ± 0.537	4.891 ± 0.770	4.853 ± 0.586	5.016 ± 0.746	<0.001
Body Mass Index(kg/m^2)	16.199 ± 1.210	19.558 ± 0.849	22.825 ± 1.135	30.467 ± 5.324	<0.001
Lumbar bone mineral density(g/cm^2)	0.708 ± 0.116	0.855 ± 0.166	0.922 ± 0.190	0.968 ± 0.184	<0.001
Total bone mineral density(g/cm^2)	0.968 ± 0.184	0.932 ± 0.130	1.000 ± 0.143	1.042 ± 0.140	<0.001

Mean ± SD for continuous variables: P-value was calculated by weighted linear regression model. % for categorical variables: P-value was calculated by weighted chi-square test.

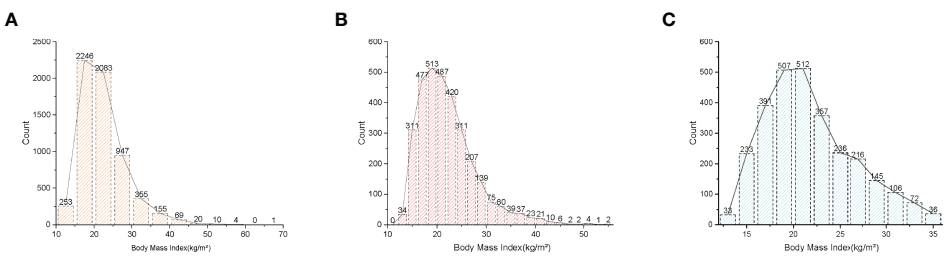

FIGURE 2 | Distribution histogram of BMI. **(A)** Among all participants. **(B)** Among all males. **(C)** Among all females. Body Mass Index, BMI.

DISCUSSION

Higher BMI was linked to higher total BMD in a weighted analysis involving US adolescents aged 8–19. We also performed a threshold effect analysis based on multiple regression analysis for different age groups of adolescents, and the results supported our hypothesis that the presence of a saturation value of BMI on total BMD among different age groups of adolescents could maintain a relatively healthy BMI while maintaining a higher BMD.

Several epidemiological studies in the past have demonstrated that BMD in adolescents is closely related to BMI (21–23). A cross-sectional study from Korea that included 1,063 adolescents found that BMI, lean body mass, and fat mass were all positively associated with BMD (24). Similarly, a study from Lebanon showed that obese and overweight boys had significantly higher

TABLE 2 | Association between BMI (kg/m²) and total bone mineral density (g/cm²).

	Model 1β (95% CI) *P*-value	Model 2β (95% CI) *P*-value	Model 3β (95% CI) *P*-value
Body mass index(kg/m²)	0.014 (0.013, 0.014) <0.001	0.006 (0.005, 0.006) <0.001	0.005 (0.004, 0.005) <0.001
Subgroup analysis stratified by gender			
Males	0.015 (0.014, 0.015) <0.001	0.005 (0.005, 0.006) <0.001	0.005 (0.003, 0.006) <0.001
Females	0.013 (0.012, 0.014) <0.001	0.006 (0.005, 0.006) <0.001	0.004 (0.003, 0.006) <0.001
Subgroup analysis stratified by age			
8–9 years (n = 1,223)	0.007 (0.006, 0.008) <0.001	0.007 (0.006, 0.008) <0.001	0.007 (0.006, 0.008) <0.001
10–11 years (n = 1,180)	0.007 (0.006, 0.008) <0.001	0.007 (0.006, 0.008) <0.001	0.007 (0.006, 0.008) <0.001
12–13 years (n = 944)	0.007 (0.006, 0.008) <0.001	0.006 (0.005, 0.007) <0.001	0.006 (0.004, 0.008) <0.001
14–15 years (n = 959)	0.007 (0.006, 0.008) <0.001	0.007 (0.006, 0.008) <0.001	0.006 (0.004, 0.007) <0.001
16–17 years (n = 961)	0.004 (0.003, 0.005) <0.001	0.004 (0.004, 0.005) <0.001	0.004 (0.003, 0.005) <0.001
18–19 years (n = 876)	0.004 (0.002, 0.005) <0.001	0.003 (0.002, 0.004) <0.001	0.004 (0.002, 0.006) <0.001
Subgroup analysis stratified by BMI			
Q1, 18.1<	0.040 (0.036, 0.044) <0.001	0.022 (0.019, 0.025) <0.001	0.020 (0.016, 0.024) <0.001
Q2, 18.1-21.0	0.032 (0.024, 0.039) <0.001	0.014 (0.010, 0.019) <0.001	0.014 (0.009, 0.018) <0.001
Q3, 21.1–23.4	0.014 (0.008, 0.020) <0.001	0.007 (0.004, 0.011) <0.001	0.012 (0.004, 0.020) 0.002
Q4, >23.4	0.005 (0.004, 0.006) <0.001	0.001 (0.000, 0.002) <0.001	0.001 (0.001, 0.002) 0.042
P for trend	<0.001	<0.001	<0.001

*Model 1: No covariates were adjusted. Model 2: Age, gender, and race were adjusted. Model 3: Age, gender, race, education level, ratio of family income to poverty, activities status, diabetes status, ALT, ALP, AST, total calcium, total cholesterol, direct HDL cholesterol, LDL cholesterol, triglyceride, phosphorus, blood urea nitrogen, and serum glucose were adjusted. *In the subgroup analysis stratified by gender or race, the model is not adjusted for the stratification variable itself.*

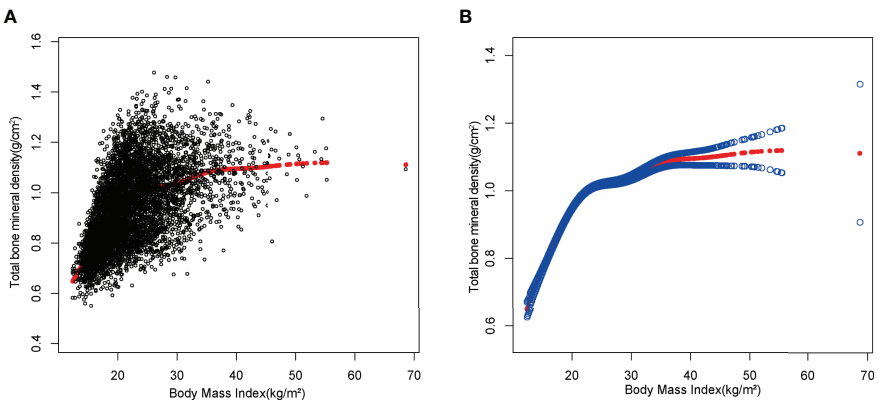

FIGURE 3 | The association between BMI and total bone mineral density. **(A)** Each black point represents a sample. **(B)** The solid red line represents the smooth curve fit between variables. Blue bands represent the 95% confidence interval from the fit.

total hip BMD and femoral neck BMD compared to boys with normal BMI (25). Our findings also demonstrate that higher BMI is associated with higher BMD in both boys and girls.

The mechanisms behind the obesity-BMD connection are unclear. One explanation is that people with obesity have a greater BMD because of the mechanical impact of their weight on their bones (26–28). Animal studies have revealed that osteocytes are particularly vulnerable to biomechanical stressors (29). They die from apoptosis in the absence of load (30), whereas when osteoblasts receive shear stress signals (31), they do not undergo apoptosis and their sclerostin secretion is inhibited (32). At the same time, osteoclast activity is slowed and osteoblast differentiation is boosted (33–35). In the population with obesity, Garnero et al. discovered a decrease in biochemical bone indicators, with a higher fall in bone resorption markers than bone production markers (36). This finding supports the theory that increased body weight causes orthostatic equilibrium. In addition to mechanical considerations, the increased BMD associated with obesity appears to be linked to estrogen activity. Estrogen has been shown to play a significant function in bone metabolism, promoting bone growth and inhibiting bone resorption (37, 38). The metabolism of estrogen and fat tissue are inextricably linked. In reality, adipose tissue is a major source of aromatase enzymes, which are needed for estrogen synthesis. Obese postmenopausal women had greater serum estrogen concentrations than non-obese women, and 17β-estradiol levels were higher in obese patients (39).

Although it is well proven that a higher BMI leads to a higher BMD, this does not mean that the risk of fracture is reduced (19). The "obesity paradox" is the name given to this phenomena (40).

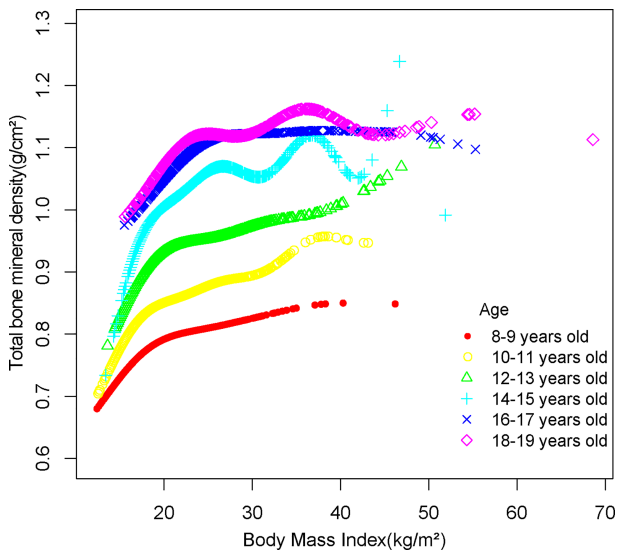

FIGURE 4 | The association between BMI and total bone mineral density stratified by age.

In children and adolescents, even being overweight has a positive effect on BMD, but the incidence of fractures is higher than in non-obese individuals (41). Preschool obesity was linked to an increased incidence of fracture in adolescents, according to a comprehensive study conducted by Lane et al. in Catalonia (42). This could be owing to excessive mechanical loading generated by extra adipose tissue (43). Whether from the perspective of reducing other systemic diseases caused by obesity or reducing the incidence of fractures in adolescents, we should find an appropriate BMI while striving for a higher BMD. Ma et al. found that, for Americans over 50 years of age, keeping the BMI at a slightly overweight value (about 26 kg/m^2) may reduce other adverse effects while obtaining an optimal BMD (44). While in adolescents, BMI saturation values for BMD may change substantially with age, and our findings are the first to investigate BMI saturation values for BMD in US adolescents aged 8–19 years at different ages.

The mechanism of maintaining a BMI of saturation value and hence achieving optimal BMD is still unknown. Bone development trajectories and PBM are established early in life, which could explain why adult BMD does not rise after a time of restricted growth (45, 46). Another reason for BMI saturation effects is the presence of a separate bone–fat axis *in vivo* between adipose and bone tissues (47); supporting bone homeostasis and linked by numerous bioactive substances, bone and adipocytes are descended from the same stem cell parent and are competitive, according to existing research, with an increase in extra fat leading to bone loss (48). According to investigations in animal models caused by increased fat intake, BMD decreases as obesity increases in obese animals (49, 50). As a result, we hypothesized that maintaining a saturated BMI would retain enough BMD while reducing the risk of obesity-related diseases and comorbidities.

Our study has some limitations. First, this is a cross-sectional analysis; thus, temporality cannot be ascertained. Second, due to database limitations, we were unable to obtain data on participants taking calcium supplements, dietary intake of calcium, vitamin D, and lipid-lowering medications that may

TABLE 3 | Saturation effect analysis of BMI (kg/m^2) on total BMD (g/cm^2).

Total bone mineral density	Model: saturation effect analysis
BMI turning point (K),kg/m^2	21.5
<K, effect 1	0.036 (0.034, 0.037) <0.001
>K, effect 2	0.005 (0.004, 0.006) <0.001
Subgroup analysis stratified by age	
BMI turning point for 8–9 years old(K),kg/m^2	16.9
<K, effect 1	0.023 (0.019, 0.027) <0.001
>K, effect 2	0.004 (0.003, 0.005) <0.001
BMI turning point for 10–11 years old(K),kg/m^2	16.4
<K, effect 1	0.035 (0.027, 0.042) <0.001
>K, effect 2	0.006 (0.005, 0.007) <0.001
BMI turning point for 12–13 years old(K),kg/m^2	17.2
<K, effect 1	0.050 (0.038, 0.062) <0.001
>K, effect 2	0.005 (0.004, 0.006) <0.001
BMI turning point for 14–15 years old(K),kg/m^2	20.9
<K, effect 1	0.026 (0.021, 0.030) <0.001
>K, effect 2	0.004 (0.002, 0.005) <0.001
BMI turning point for 16–17 years old(K),kg/m^2	24.2
<K, effect 1	0.017 (0.014, 0.020) <0.001
>K, effect 2	0.000 (-0.001, 0.002) 0.621
BMI turning point for 18-19 years old(K),kg/m^2	22
<K, effect 1	0.021 (0.015, 0.027) <0.001
>K, effect 2	0.001 (0.000, 0.003) 0.028

Age, gender, race, education level, ratio of family income to poverty, activities status, ciabetes status, ALT, ALP, AST, total calcium, total cholesterol, direct HDL cholesterol, LDL cholesterol, triglyceride, phosphorus, blood urea nitrogen, and serum glucose were adjusted.

have an effect on BMD; therefore, our findings should be interpreted with caution. Finally, given the database limitations, we were unable to obtain a history of fractures in adolescent participants; therefore, we were unable to assess whether fracture rates were higher in adolescents with high BMI than in the general population. Despite these limitations, our study has several advantages. Because we used a nationally representative sample, our study is representative of a multi-ethnic and gender-diverse population of adolescents in the United States. In addition to this, because of the large sample size included in our study, this allowed us to divide adolescents aged 8–19 years into multiple age groups for subgroup analysis. To our knowledge, past studies have demonstrated the saturating effect of adult BMI on BMD, and the present study is the first to investigate the saturating effect of BMI on BMD in adolescents of different ages.

CONCLUSION

In this study, we used multiple linear regression models, smoothed curve fitting, and saturation effect analysis models to examine the relationship between BMI and BMD in US adolescents aged 8–19 years. We found not only a simple linear positive correlation between BMI and BMD but also a saturation value that persisted across gender and age subgroups in the analysis. This work suggests that keeping BMI at saturation values may provide benefits for adolescents to maintain optimal BMD and reduce other obesity-related diseases.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: www.cdc.gov/nchs/nhanes/.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by NCHS Ethics Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YO and RX designed the research. YO, YQ, and XJH collected and analyzed the data. YO, CG, SX, CL, and NM drafted the manuscript. YC, XX, and RX revised the manuscript. All authors contributed to the article and approved the submitted version.

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Skeletal outcomes of patients with osteogenesis imperfecta during drug holiday of bisphosphonates: a real-world study

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Purpose: This study aimed to investigate the skeletal outcomes of patients with osteogenesis imperfecta (OI) who received bisphosphonate (BP) treatment and entered drug holiday after achieving an age- and sex-specific bone mineral density (BMD) reference.

Methods: Patients with OI receiving BP treatment were enrolled when they entered drug holidays of BPs. The skeletal outcomes were evaluated in detail during the drug holiday, including BMD, X-ray of the bone, bone fracture incidence, and bone turnover biomarkers. The pathogenic mutations of OI were identified by next-generation sequencing and confirmed by Sanger sequencing.

Results: A total of 149 OI patients (127 juveniles and 22 adults) who entered drug holidays after nearly 4 years of BP treatment were included. Areal BMD at the lumbar spine increased from 0.934 ± 0.151 to 0.990 ± 0.142 g/cm^2 and was stable in the second (1.029 ± 0.176 g/cm^2) and third years (1.023 ± 0.174 g/cm^2) of BP drug holidays, and BMD at the femoral neck, trochanter, and total hip had no significant change, but it was gradually inferior to that of the same-gender juveniles in the second and third years of the drug holiday. BMD at the lumbar spine and proximal hip did not change and was inferior to that of the same-gender adults. The average time of fractures fluctuated from 0.18 to 0.08 per year in juveniles, while only one adult suffered from a fracture during BP drug holidays. Bone turnover markers were in the normal range, except for a mildly high level of β-carboxy-terminal cross-linked telopeptide of type 1 collagen in the juvenile group. A total of 17 (11.4%) patients received BP retreatment because of bone loss during the drug holiday. OI type III and type IV and *COL1A2* mutation were correlated to a longer duration of BP treatment to enter

drug holidays (all $p < 0.05$). Old age at initial treatment (OR, 1.056) and OI type III (OR, 10.880) were correlated to a higher risk of BP retreatment.

Conclusions: OI patients will undergo nearly 4 years of BP treatment to achieve drug holidays. During the 3 years of the drug holiday, the patients' BMD is stable, and fracture incidence does not increase significantly. Patients are more inclined to need retreatment during drug holidays owing to the late start of BP treatment and more severe OI phenotypes.

KEYWORDS

skeletal outcomes, bisphosphonate, drug holiday, osteogenesis imperfecta, long-term therapy

Introduction

Osteogenesis imperfecta (OI) is an inherited skeletal dysplasia characterized by bone fragility and skeletal deformities, with an estimated incidence of 1 in 10,000–20,000 neonates (1, 2). OI also leads to dental and craniofacial abnormalities, muscle weakness, hearing loss, and respiratory and cardiovascular complications (1). The majority of OI patients are associated with pathogenic variants in *COL1A1* and *COL1A2*, the encoding genes of type I collagen, and the minority of OI patients are related to mutations in other genes that are involved in type I collagen biosynthesis or osteoblast differentiation or bone mineralization (3). The clinical classification of OI includes type I to type V, of which the severity broadly ranges from nearly asymptomatic cases with a normal life span to severe bone deformities, mobility impairment, and even perinatal mortality (4).

Treatment for OI is a great challenge, which is primarily supportive and symptomatic, including management with medications, physical therapy, and even orthopedic interventions to improve bone strength, reduce fracture risk, and improve mobility (5, 6). Bisphosphonates (BPs) are the most commonly used medications for OI, which can increase bone mineral density (BMD), reduce bone fracture risk, and lead to the reshaping of the vertebra (7–10), but the optimal duration of BP therapy in OI patients is unknown. Studies have shown that the therapeutic benefits for OI patients from BP treatment are more apparent in the first 2 to 4 years (11). Otherwise, iatrogenic osteopetrosis has been described with excessive treatment, and long-term BP therapy is associated with an increased risk of atypical femoral fracture in patients with OI (12, 13). Thus, many clinical concerns about BP treatment are worthy of investigation in OI patients, including when to enter the drug holiday of BPs, how long the drug holiday should hold, and how the skeletal outcomes during BP discontinuation.

Therefore, we aimed to investigate the skeletal outcomes during the drug holiday of BPs and their associated factors for OI patients when they entered the drug holiday.

Materials and methods

Study participants

Patients were diagnosed with OI if they had either a history of at least one non-traumatic or low-impact fracture and an age-adjusted and sex-adjusted areal BMD Z-score of –1.0 or less for either total body or lumbar spine sites, or an adjusted areal BMD Z-score of –2.0 or less irrespective of a history of fractures (14, 15). For patients without a family history of non-traumatic fracture, a diagnosis of OI was made if they had more than one non-traumatic fracture and at least a kind of extra-skeletal manifestations or with a genetic diagnosis of OI (14, 15).

The study comprised patients with OI who received BP treatment (alendronate or ibandronate or zoledronic acid) between the years 2003 and 2019 in the Endocrinology Department of Peking Union Medical College Hospital (PUMCH) and who discontinued BP treatment after achieving the age- and sex-specific normal BMD of juveniles (16, 17) and adults (18), termed drug holiday (19).

The study was approved by the ethics committee of PUMCH. Written informed consent was obtained from the patient or legal guardian of the patients before they participated in this study.

Data collection

The medical history was collected in detail, including the age of onset; the clinical information of bone pain, bone fracture, and bone deformity; and a family history of OI. The bone, joint,

sclera, ears, and teeth were examined carefully. Detailed information about fractures, including time of the initiation, site, degree of trauma, frequency, and radiological evidence of fracture, was collected. The frequency of clinical fracture was calculated as the number of clinical fractures/disease courses. Bone deformities were evaluated, including limb bending, thoracic deformity, scoliosis, and pelvic deformity (20). The height of the juvenile was measured using a Harpenden stadiometer (Seritex Inc., Farmingdale, NJ, USA) and adjusted to age- and sex-specific Z-scores on the basis of reference data from the Chinese National Centers for Disease Control and Prevention (21). For patients who were unable to stand, their height was replaced by a body length in a supine position. Serious events were observed, including new bone fractures, osteonecrosis of the jaw, and atypical femoral fracture. Delayed fracture healing was also recorded during the follow-up.

Biochemical measurements

Blood samples were obtained after overnight fasting for at least 8 h. The serum concentrations of calcium (Ca), phosphate (P), total alkaline phosphatase (ALP), alanine aminotransferase (ALT), and creatinine (Cr) were measured using an automatic biochemical analyzer (ADVIA 1800, Siemens Inc., Munich, Germany). The serum levels of β-cross-linked C-telopeptide of type I collagen (β-CTX), N-terminal propeptide of type I precollagen (P1NP), 25-hydroxyvitamin D (25OHD), and intact parathyroid hormone (PTH) were measured with an automated electrochemiluminescence system (Roche Diagnostics, Basel, Switzerland). The biochemical measurements were completed in the central laboratory of PUMCH.

Bone mineral density and radiographic assessments

The BMD at the lumbar spine, femoral neck, trochanter, and total hip was measured by dual-energy X-ray absorptiometry (Lunar Prodigy Advance, GE Healthcare, Chicago, IL, USA). The BMD phantom scan was performed daily and detected no significant machine drifts during the 5-year study. The areal BMD values were converted into age- and sex-specific Z-scores using reference data from previous studies (16–18). The radiologic views of the skull, spine, hip, and limb were performed by the radiologists of PUMCH.

Detection of pathogenic mutation of osteogenesis imperfecta patients

The pathogenic mutations of OI were detected using a panel for next-generation sequencing (NGS) (Illumina HiSeq2000 platform, Illumina, Inc., San Diego, CA, USA), which covered 20 known candidate genes of OI (*COL1A1*, *COL1A2*, *IFITM5*, *SERPINF1*, *FKBP10*, *CRTAP*, *P3H1*, *PPIB*, *SERPINH1*, *BMP1*, *PLOD2*, *SP7*, *TMEM38B*, *WNT1*, *CREB3H1*, *SPARC*, *PLS3*, *P4HB*, *SEC24D*, and *MBTPS2*). The experimental procedures followed a previously described protocol (15). The mutations identified by NGS were further confirmed by Sanger sequencing.

Classification of osteogenesis imperfecta

Patients with OI were classified into subtypes based on Sillence classification and molecular diagnosis (22): type I, mild phenotype; type II, perinatally lethal; type III, a severe form with progressive deformity; type IV, with moderate severity; and type V, characterized by calcification of the forearm interosseous membrane, radial head dislocation, and hyperplastic callus formation (23). No patients with OI type II were included in this study because of perinatal death. According to molecular diagnosis, genetic mutations leading to an early stop codon or frameshift in *COL1A1* were regarded as the quantitative reduction group (haploinsufficiency). Mutations causing amino acid substitutions in the triple-helical domain of *COL1A1* or *COL1A2* were classified into the qualitative defect group. As the effects of splice site mutation were difficult to predict, we did not include splice site mutations in the analyses (15).

Statistical analysis

Normally distributed data, such as BMD and height, were presented as mean ± standard deviation, while those with abnormal distribution were expressed as medians (interquartile ranges (IQRs)), or proportions. The differences among each time of BMD, height, and so on, during BP discontinuation were analyzed using a generalized linear mixed model. Multiple linear and binary logistic regression analyses were used to analyze related factors of the BP treatment course and restart BP treatment. A p-value of less than 0.05 indicated a statistically significant difference. The statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Graphs were drawn using GraphPad Prism software version 6.0.

Results

Patients' characteristics at bisphosphonate discontinuation

A total of 149 patients with OI received BP treatment and entered drug holiday, 127 of whom received BP treatment while they were still juveniles, designated as the juvenile group, while 22 patients received initial treatment during adulthood,

designated as the adult group. These patients either received alendronate (12 in the juvenile group and 6 in the adult group) or zoledronate (95 in the juvenile group and 9 in the adult group) or were sequentially treated with alendronate or ibandronate and then with zoledronate (20 in the juvenile group and 7 in the adult group). The detailed follow-up records are shown in Figure 1. The median age of OI onset, age at initial treatment, and duration of BP treatment were 1.5 years (IQR 0.77–4.75 years), 7.0 years (IQR 3.1–11.8 years), and 3.95 years (IQR 2.2–5.0 years) in the juvenile group and were 2.0 years (IQR 1–11 years), 32.7 years (IQR 23.0–43.0 years), and 4.2 years (IQR 3.0–5.0 years) in the adult group, respectively, as shown in Table 1. Up to now, the time when OI patients entered drug holidays was as follows: 21 patients within 1 year, 23 patients within 2 years, 33 patients within 3 years, and 72 patients over 3 years (Figure 1). One patient had the longest follow-up of 7 years after BP discontinuation.

The pathogenic variants of OI were identified in the majority of the patients. Sixty patients carried *COL1A1* mutation, 30 with *COL1A2* mutation, 9 with *IFITM5* mutation, 3 with *SERPINF1* mutation, 3 with *FKBP10* mutation, 2 with *PLOD2* mutation, and 2 with *WNT1* mutation, and mutations in *TMEM38B*, *CRTAP*, *PLS3*, and *P4HB* were found in one patient. Non-mutation was detected in 36 OI patients (Table 1).

Skeletal outcomes after bisphosphonate discontinuation

After BP discontinuation, the height of juveniles with OI increased from 139.72 ± 24.28 to 145.51 ± 25.43, 147.05 ± 20.60, and 148.77 ± 19.38 cm in the first, second, and third years of drug holiday, respectively, but the juveniles had lower height Z-scores, which suggested that the OI patients were shorter than their peers. The heights of adults had no obvious change during the drug holidays (Figures 2A, B). Meanwhile, no significant change was observed in the serum levels of β-CTX, Ca, P, 25OHD, and PTH during the 3 years of the drug holidays in juveniles and adults, except for mildly increased β-CTX levels in juveniles (Figures 3A–F). Liver and kidney functions were normal during the 3 years of BP discontinuation (Supplementary Figures 1A, B). No patients suffered from osteonecrosis of the jaw and atypical femoral fracture during the whole drug holiday.

In juveniles, the areal BMD at the lumbar spine increased from 0.934 ± 0.151 to 0.990 ± 0.142 g/cm^2 from the baseline (0′) to the first year of BP discontinuation and was stable in the second (1.029 ± 0.176 g/cm^2) and third years (1.023 ± 0.174 g/cm^2) in the juveniles, with no significant change in BMD Z-score (Figures 2C, D). The femoral neck BMD of juveniles was stable, but the Z-score gradually declined during the drug holidays (Figures 2E, F). No significant change was observed in the trochanter and the total hip BMD during the 3 years of BP discontinuation. However, the BMD of the juvenile was inferior to that of the peers in trochanter during the first and second years of the drug holidays (Figures 2G–J). The bone pain incidence fluctuated from 3.1% to 4.0%, and the average times of fractures of the juveniles fluctuated from 0.18 to 0.08 per year (Figures 2K, L).

In the adults, the BMD at the lumbar spine did not change in the 3 years of the drug holidays with a lower Z-score in the first 2 years until the third year (Figures 2C, D). The femoral neck BMD and Z-score of adults remained stable during the whole drug holiday (Figures 2E, F). Similarly, no significant change was observed in the trochanter and the total hip BMD during the 3 years of BP discontinuation. However, the BMD of adults was inferior to that of peers in trochanter during the 3 years of the drug holidays (Figures 2G–J). The bone pain incidence fluctuated from 31.8% to 12.5%, while only one adult suffered from a fracture in the second year of BP discontinuation.

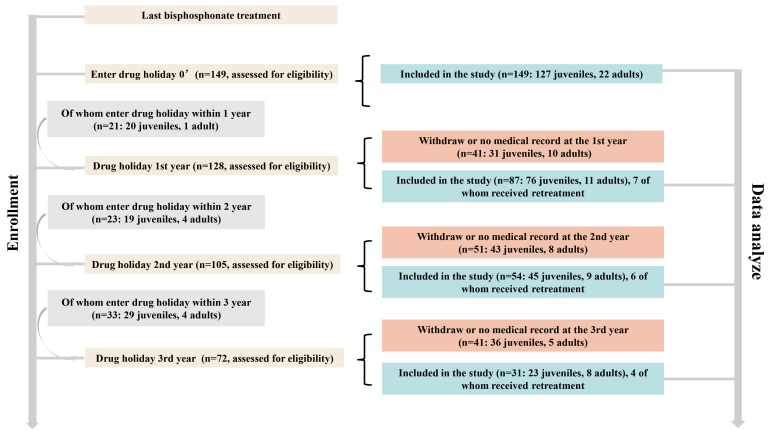

FIGURE 1
Details of the patients' enrollment and the study design.

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TABLE 1 Baseline characteristics of OI patients.

Characteristic	Juvenile group (n = 127)	Adult group (n = 22)
Male, n (%)	83 (65.4)	7 (31.8)
Age of onset, years, median (IQR)	1.5 (0.77–4.75)	2 (1–11)
Age at initial treatment, years, median (IQR)	7.0 (3.1–11.8)	32.6 (23.0–43.0)
Age of stopping BPs, years, median (IQR)	11.3 (7.7–15.8)	35.3 (27.6–46.8)
Duration of BP treatment, years, median (IQR)	3.95 (2.2–5.0)	4.2 (3.0–5.0)
Family history of fractures, n (%)	53 (41.7)	16 (72.7)
Family history of blue sclera, n (%)		
Father, n (%)	10 (7.8)	1 (4.5)
Mother, n (%)	9 (7.1)	3 (13.6)
Other consanguinity, n (%)	0 (0)	1 (4.5)
Fracture times, median (IQR)	3 (3)	5 (19)
Multiple fractures, n (%)	84 (66.1)	16 (72.7)
Difficulty walking, n (%)	32 (25.2)	4 (18.2)
Bone deformities, n (%)	35 (27.6)	6 (27.3)
Bone bending, n (%)	31 (24.4)	5 (22.7)
Loose joint, n (%)	62 (48.8)	11 (50)
Blue sclera, n (%)	103 (81.1)	17 (77.3)
Dentin deficiency, n (%)	23 (18.1)	5 (22.7)
Hearing impairment, n (%)	4 (3.1)	3 (13.6)
Medical Imaging		
Thin long bone cortex, n (%)	101 (79.5)	10 (45.5)
Interstitial bone, n (%)	75 (59.1)	9 (40.9)
Hypertrophic callus, n (%)	4 (3.1)	1 (4.5)
Sillence classification		
I, n (%)	80 (63)	17 (77.3)
III, n (%)	10 (7.9)	2 (9.1)
IV, n (%)	24 (18.9)	2 (9.1)
V, n (%)	13 (10.2)	1 (4.5)
Inheritance pattern		
Autosomal recessive inheritance, n (%)	38 (29.9)	8 (36.4)
Autosomal and X-linked dominant inheritance, n (%)	89 (70.1)[note]	14 (63.6)
Genetic mutations		
COL1A1, n (%)	54 (42.5)	6 (27.3)
COL1A2, n (%)	25 (19.7)	5 (22.7)
IFITM5, n (%)	8 (6.3)	1 (4.5)
SERPINH1, n (%)	3 (2.4)	0 (0)
FKBP10, n (%)	3 (2.4)	0 (0)
Others, n (%)	34 (26.8)	10 (45.5)
Collagen defects		
Quantitative reduction	22 (17.3)	6 (27.3)
Qualitative defect	47 (37.0)	4 (18.2)
Unclassified	58 (45.7)	12 (54.5)

One child with OI inherited in an X-linked dominant way. BPs, bisphosphonates; OI, osteogenesis imperfecta; IQR, interquartile range.

Related factors of bisphosphonate treatment course and restart treatment

We evaluated the related factors regarding the duration of BP treatment to enter the drug holiday. As shown in Figure 4, OI type III (β = 1.542, $p = 0.018$), OI type IV (β = 1.155, $p = 0.014$), and *COL1A2* mutation (β = 1.091, $p = 0.020$) were positively correlated with a longer duration of BP treatment before patients entered drug holiday.

There were 17 patients (11.4%, a median drug holiday of 2 years) who started the retreatment of BPs because of a decrease in BMD, including 12 juveniles (9.4%) and 5 adults (22.7%). The

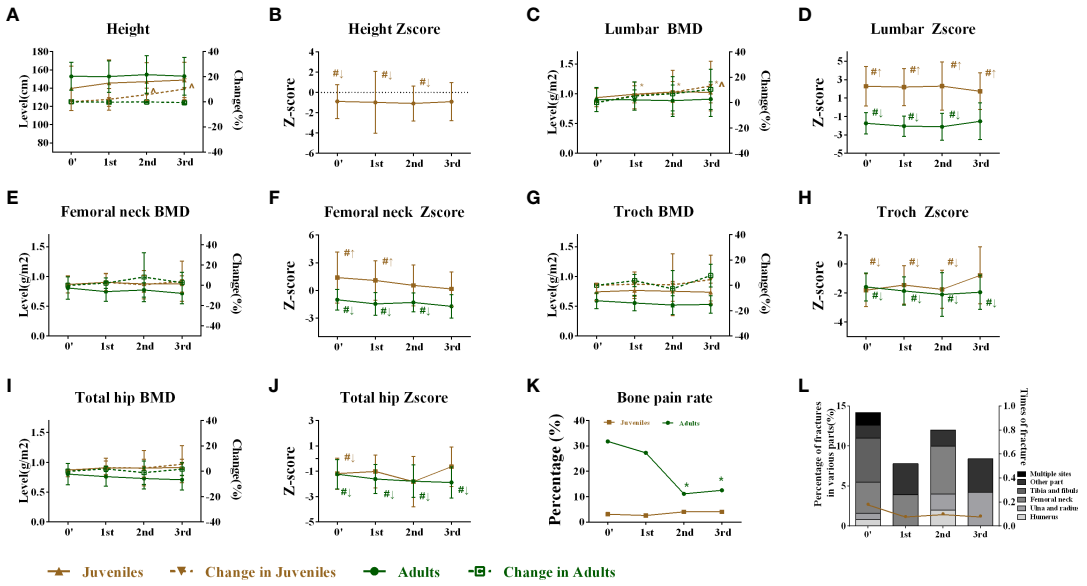

FIGURE 2
Changes in height and skeletal outcomes after bisphosphonate discontinuation. **(A)** Changes in height during the follow-up. **(B)** Changes in the height Z-score during the follow-up. **(C)** Changes in the lumbar BMD during the follow-up. **(D)** Changes in the lumbar Z-score during the follow-up. **(E)** Changes in the femoral neck BMD during the follow-up. **(F)** Changes in the femoral neck Z-score during the follow-up. **(G)** Changes in the trochanter BMD during the follow-up. **(H)** Changes in the trochanter Z-score during the follow-up. **(I)** Changes in the total hip BMD during the follow-up. **(J)** Changes in the total hip Z-score during the follow-up. **(K)** Changes in the bone pain rate during the follow-up. **(L)** Changes in average times per year of fractures and fracture rates during the follow-up. *Level compared with baseline after bisphosphonate discontinuation, $p < 0.05$. †Level compared with the first year after bisphosphonate discontinuation, $p < 0.05$. &Level compared with the second year after bisphosphonate discontinuation, $p < 0.05$. ^Change rate compared with 0′ after bisphosphonate discontinuation, $p < 0.05$. #Level compared with average Z-score of their peers (value = 0). ↑Superior to their same-sex peers. ↓Inferior to the same-sex peers. BMD, bone mineral density.

old age at initial treatment (OR, 1.056; 95% CI, 1.003–1.111) and type III OI (OR, 10.880; 95% CI, 1.429–82.816) were significantly correlated with the retreatment of BPs. However, no significant association was found between gender, age of OI onset, genotype, patterns of inheritance, and retreatment of BPs (Figure 4). The longest drug holiday was observed in an OI patient with a mutation in *COL1A2*, who received 2 years of zoledronic acid treatment and then entered a drug holiday. During the 7 years of the drug holiday, his lumbar and proximal hip BMD continued to increase (Supplementary Table 1). As he still had age- and sex-specific normal BMD, he did not receive retreatment.

Discussion

This was a novel clinical study to evaluate the skeletal outcome during drug holidays of BPs in a large cohort of juveniles and adults with moderate-to-severe OI. During the 3-year observation in drug holidays, the serum levels of biochemical indexes were normal, except for a mildly high level of β-CTX in juveniles. The lumbar spine BMD increased in the first year and was stable in the second and third years of drug holiday in the juveniles. The proximal hip BMD had no significant change during the drug holiday, but its Z-score tended to decline gradually during the drug holidays in juveniles. Both lumbar spine and proximal hip BMD remained stable in the adult group. New fracture incidences remained at a lower level, which did not increase during the drug holiday. Meanwhile, OI type III and type IV and *COL1A2* mutation were positively correlated with a longer duration of BP treatment to achieve the drug holiday, while patients with a later onset of BP treatment and severe clinical phenotypes were associated with a higher risk of BP retreatment.

BPs are the most commonly used medications for osteoporosis, which play important roles by effectively inhibiting osteoclast activities, increasing BMD, and reducing bone fracture incidence (24). Nitrogenous BPs could disrupt osteoclasts formation, survival, and cytoskeletal dynamics, and alendronate, pamidronate, and zoledronate were commonly used BPs for patients with OI. However, long-term BP treatment could increase the microcracks in the bone, thereby increasing bone fragility and the risk of atypical fracture (25, 26). The persistent effects of BPs on the bone led to the concept of a drug holiday, which was designed to minimize side effects and maximize benefits. As the mechanical properties and structure of the bone in OI patients were markedly different from

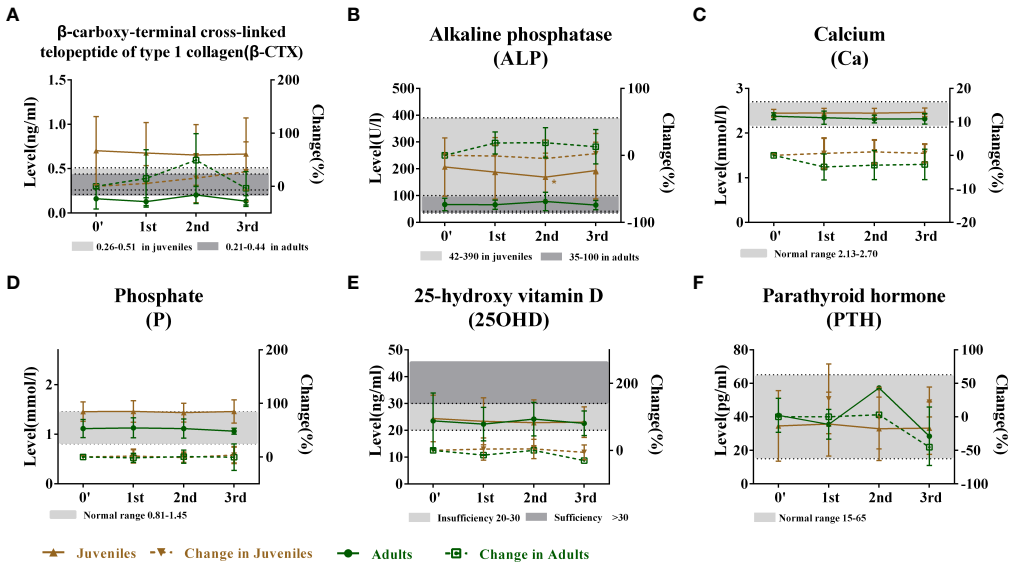

FIGURE 3
Changes in bone metabolic markers after bisphosphonate discontinuation. **(A)** Changes in the serum ALP level during the follow-up. Normal range of ALP (42–390 U/L in juvenile group and 35–100 U/L in adult group) marked by gray. **(B)** Changes in the serum β-CTX level during the follow-up. Normal range of β-CTX (0.26–0.51 ng/ml in juvenile group and 0.21–0.44 ng/ml in adult group) marked by gray. **(C)** Changes in serum Ca level during the follow-up. Normal range of Ca (2.13–2.7 mmol/L) marked by gray. **(D)** Changes in the serum P level during the follow-up. Normal range of P (0.81–1.45 mmol/L) marked by gray. **(E)** Changes in the serum 25OHD level during the follow-up. Level of 25OHD with more than 20 ng/ml marked by gray. **(F)** Changes in the serum PTH level during the follow-up. Normal range of PTH (15–65 pg/ml) marked by gray. *Level compared with baseline after bisphosphonate discontinuation, $p < 0.05$. †Level compared with the first year after bisphosphonate discontinuation, $p < 0.05$. &Level compared with the second year after bisphosphonate discontinuation, $p < 0.05$. ^Change rate compared with 0′ after bisphosphonate discontinuation, $p < 0.05$. ALP, alkaline phosphatase; PTH, parathyroid hormone.

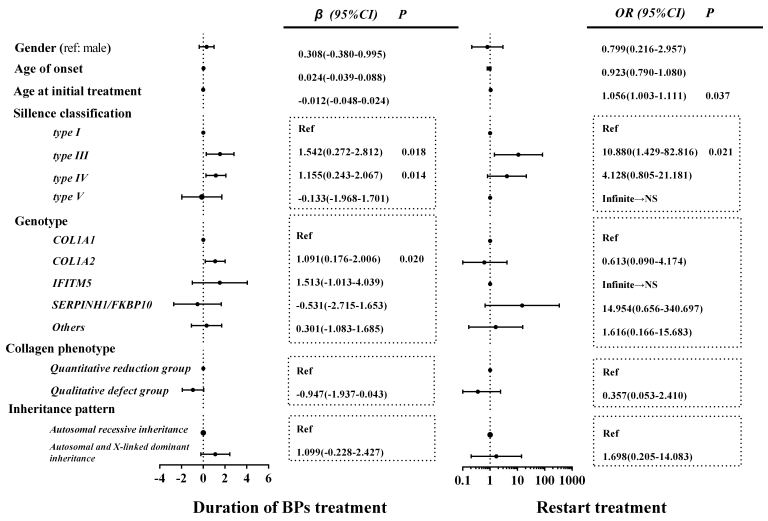

FIGURE 4
Related factors of BP treatment course and restart treatment of BPs. In the collagen phenotype group, those other than the quantitative and qualitative defect groups were classified as other groups to ensure that all data were fully analyzed. Data of other groups are not shown. NS, non-significant; BP, bisphosphonate.

those of the normal bone, bone fragility was significantly high in OI patients. Therefore, we should consider the appropriate course of BP treatment in OI patients.

Recently, the optimal duration and long-term safety of BP therapy were worthy of further investigation. A meta-analysis showed that oral or intravenous BPs increased the BMD of children and adults with OI (27). However, whether oral or intravenous BP treatment could consistently reduce fracture occurrence was controversial (27). Although specific data were not extracted, BP therapy for 1 to 3 years appeared to be beneficial, with the maximum benefits in the first year of treatment in adults and children with OI patients (27, 28). The increase in load to fracture after BP treatment came at the cost of a trend toward a decline in bone material properties, decreased strength and elastic modulus, and decreased matrix production by osteoblasts, which could be avoided by a shorter treatment duration (29). In humans, the volume of the bone increased after BP treatment, while the intrinsic material properties, stiffness, and hardness of bone tissue remained unaffected (30). However, the average length of BP treatment preceding fractures was 6.5 years without standard drug holidays in the study by Nicolaou et al. (31). Thus, in another study, drug holidays were achieved after the average treatment duration of 4.1 years (7). In our study, a median of nearly 4.0 years of BP treatment was safe according to the skeletal outcomes. However, patients with OI are often not treated in a timely manner. OI is an extremely rare disease, and both doctors and patients might have relatively insufficient knowledge of disease treatment. As a result, the patients were diagnosed with OI but did not receive timely treatment until repeated fractures and even bone deformities occurred (32).

Additionally, when to terminate BP drug holiday and restart treatment were unclear in patients with OI. Rauch et al. suggested that treatment was restarted after 15 and 16 months of cessation of pamidronate when some patients began to feel unwell and lacked stamina (33). Another study indicated that BP treatment had to be restarted owing to the decreased BMD, increased fracture rate, and recurrence of bone pain (34). In our study, most patients with OI remained stable during the first 2 years of BP discontinuation. However, 17 (11.4%) patients received the retreatment after a median 2 years of drug holiday due to the obvious decrease in BMD.

Very few studies have comprehensively evaluated the skeletal outcome of OI patients during BP discontinuation. A study reported that the lumbar spine BMD and its *Z*-scores decreased, while the fracture rate increased in OI patients after 1.5 years of BP discontinuation (34). Another study indicated that the effects of pamidronate discontinuation were more obvious at the radial metaphysis than at the diaphysis (35). Recently, increased lumbar spine areal BMD and a 19% decrease in the trabecular volumetric BMD at the distal metaphysis were observed after 4 years of BP discontinuation in OI patients (36). Moreover, we evaluated the changes in bone turnover markers in juveniles and adults after BP discontinuation. BP discontinuation leads to an obvious decrease in bone turnover biomarkers. As OI children were in the stage of growth and development, juveniles with OI had increased BMD and slightly increased β-CTX levels during the BP drug holiday. Interestingly, the lumbar spine BMD continued to increase, and the proximal hip BMD had no significant change during the drug holidays in juveniles. The curative effects seemed to be weaker in OI adults than in OI juveniles, which may be related to high bone remodeling and bone growth speed in juveniles. The possible mechanism of site-specific changes in juveniles' BMD was as follows: as the spine is rich in cancellous bone, the effects of BPs on the spine BMD were more obvious than in other sites (27, 37). BPs could not alter the genetic defects of OI, and the BMD would decrease again after the long-term discontinuation of BP therapy (33). The demineralization was predominant on sites rich in cortical bone (38), and a fall in BMD was significant in the proximal hip after BP discontinuation (39). Moreover, most studies focused on the safety profile of OI patients during treatment (40). No patients suffered from osteonecrosis of the jaw and atypical bone fracture in this study. Our findings might broaden the long-term safety spectrum of BPs in OI patients.

Several factors associated with the duration to enter a drug holiday and termination of the drug holidays were investigated. We found that OI type III and type IV and *COL1A2* mutation were positively correlated with a longer BP treatment duration to enter the drug holiday. Meanwhile, OI patients with later initiation of BP treatment or with the severe OI phenotype tended to require retreatment. As patients with OI type III or IV, or with a qualitative defect in type I collagen, usually had a severe phenotype, they might need a longer duration of BP treatment and need to be treated again (41, 42). As BPs were less effective in OI adults than in OI children (43, 44), the early initiation of BP treatment might have more benefits (40).

In this study, we comprehensively assessed the skeletal outcomes after BP discontinuation for the first time in a large cohort of Asian OI patients. We investigated the factors correlated with the BP treatment course to enter the drug holidays and retreatment during the drug holiday. Meanwhile, all the measurements were performed in a single center, avoiding measurement bias. However, this study had many limitations. It was a retrospective study. Patients were administered various BPs, and the duration of BP treatment and lengths of drug holidays were diverse.

Conclusions

Patients with osteogenesis imperfecta will undergo nearly 4 years of BP treatment to achieve drug holidays. During the 3 years of the drug holiday, the patients' BMD is stable, and fracture incidence does not increase significantly. Patients are more inclined to need retreatment during the drug holidays owing to the late start of BP treatment and more severe phenotypes This study provides valuable information for the long-term rational treatment of BPs in juveniles and adults with osteogenesis imperfecta.

Data availability statement

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding authors. The datasets presented in this article are not readily available since no access to raw dataset of NGS is allowed other than the Beijing Genomics institution in charge of NGS. Requests to access the datasets should be directed to https://www.genomics.cn/contact.html.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of PUMCH. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

YZ collected the clinical data from the patients, analyzed the data, and wrote the manuscript. JH, XL, and LS contributed to the data collection and blood sample collection. QZ, SY, YJ, OW, WX, and XX contributed to the review of the manuscript. ML contributed to the conception and design of the research, and acquisition and interpretation of the data, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.901925/full#supplementary-material

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Association between perfluoroalkyl substances concentration and bone mineral density in the US adolescents aged 12-19 years in NHANES 2005-2010

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Background: Reports on the association of perfluoroalkyl substances (PFASs) exposure with adolescent bone health are scarce, and studies have primarily targeted maternal serum.

Objective: We evaluated the relationship between autologous serum perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), perfluorohexane sulfonic acid (PFHxS) and perfluorononanoic acid (PFNA) levels and bone mineral density (BMD) in adolescents.

Methods: We analyzed data from 1228 adolescents aged 12-19 years in the National Health and Nutrition Examination Survey (NHANES) 2005-2010 and used multiple regression analysis to identify the relationship between serum PFOA, PFOS, PFHxS, and PFNA concentrations and total femur, femoral neck, and lumbar spine BMD, in addition to multiple stratified subgroup analyses.

Results: The mean age of participants was 15 years, males had higher serum PFAS concentrations than females. The results of multiple regression analysis showed that the natural log(ln)-transformed serum PFOA, PFOS, and PFNA concentrations were negatively correlated with total femur, femoral neck, and lumbar spine BMD (all $p < 0.05$), and ln-PFHxS was positively correlated with total femur and femoral neck BMD (all $p< 0.05$). In males, ln-PFOA was negatively associated with total femur and lumbar spine BMD (all $p< 0.05$), ln-PFOS was associated with the reduced total femur, femoral neck, and lumbar spine BMD (all $p< 0.05$), while ln-PFHxS and ln-PFNA were not observed to correlate with BMD at these three sites. In females, both ln-PFOA and ln-PFOS were negatively correlated with total femur and lumbar spine BMD (all $p< 0.05$), ln-PFHxS is associated with the increased total femur and femoral neck BMD (all $p< 0.05$), and ln-PFNA was negatively correlated with total femur and femoral neck BMD (all $p< 0.05$), most of the associations

were confined to females. The associations of ln-PFOS with femoral neck BMD and ln-PFNA with total femur BMD were more significant in those who were overweight/obese and had anemia, respectively (all p for interaction < 0.05).

Conclusions: In this representative sample of US adolescents aged 12-19 years, certain PFAS were associated with lower bone mineral density, and most of the associations were confined to females. The negative effect of PFAS on BMD is more pronounced in those who are overweight/obese and have anemia. However, further studies are needed to confirm this finding.

KEYWORDS

perfluoroalkyl substances, perfluorooctanoic acid, perfluorooctane sulfonic acid, perfluorohexane sulfonic acid, perfluorononanoic acid, bone mineral density

Introduction

Osteoporosis is one of the most common skeletal diseases and a very important public health problem in populations worldwide, characterized mainly by low bone mineral density, which predisposes to fractures in the affected skeletal areas (1). The critical period of skeletal development during adolescence is important for lifelong bone health because bone mass increases rapidly during adolescence and peaks in late adolescence (2, 3), and peak bone mass during this period may have a significant impact on the onset and diagnosis of osteoporosis in later life (4).

Perfluoroalkyl substances (PFASs) are one of the most stable classes of chemicals in industrial history and have become widespread persistent environmental pollutants since the 1950s due to their widespread use and presence in items we use every day, as well as their long-term and stable presence in the environment (5). PFAS can be exposed to humans and accumulate in the body through a variety of pathways (6, 7), and have been reported to be able to be detected in 95% of the American population (8). Perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), perfluorohexane sulfonic acid (PFHxS), and perfluorononanoic acid (PFNA), the most commonly used PFASs, have been studied extensively. Although the use PFASs is now widely restricted (www.oecd.org/officialdocuments), a significant percentage of the global population is still exposed to them. PFAS have been classified as endocrine-disrupting chemicals (EDCs) (9), which together with other EDCs have been shown to be strongly associated with a wide range of human health issues including male and female reproductive health, obesity and metabolism, neurodevelopment, and bone health (10, 11), however, few studies have reported on the effects of such environmental pollutant exposures on adolescent bone health (12, 13), and previous studies on PFAS exposure in adolescent bone health have only primarily collected maternal prenatal PFAS exposure levels (14, 15), therefore the extent of the effect of different PFAS on BMD in adolescents is unclear. However, on the basis of the limited available data suggesting a negative association between PFAS exposure and BMD, we proceeded to test the hypothesis that higher PFAS concentrations are associated with lower BMD in the NHANES 2005-2010 cross-sectional survey of adolescents aged 12-19 years.

Abbreviations: NHANES, National Health and Nutrition Examination Survey; PFOA, Perfluorooctanoic acid; PFOS, Perfluorooctane sulfonic acid; PFHxS, Perfluorohexane sulfonic acid; PFNA, Perfluorononanoic acid; BMD, Bone mineral density; PFASs, Perfluoroalkyl substances; Ln, Natural log; EDCs, Endocrine-disrupting chemicals; CDC, Centers for Disease Control and Prevention; LOD, Limit of detection; DXA, Dual-energy X-ray absorptiometry; BMI, Body mass index; ACR, Albumin/creatinine ratio; ETS, Environmental tobacco smoke; SE, Standard error; β, Regression coefficients; OR, Odds ratio; CI, Confidence intervals; BUA, Broadband-ultrasound attenuation; SOS, Speed of sound waves; SI, Stiffness index; PFDA, Perfluorodecanoic acid; OCN, Osteocalcin; PPARγ, Peroxisome proliferator-activated receptor-γ; PFDE, Perfluorodecanoic acid.

Methods

Study methods and participants

NHANES is a nationally representative cross-sectional survey of the health and nutritional status of civilians, noninstitutional adults, and children conducted by the Centers for Disease Control and Prevention, the details of the survey design and methodology can be found on the NHANES website [Centers for Disease Control and Prevention (CDC), http://cdc.gov/nchs/nhanes)]. We selected only three cycles of NHANES 2005-2006, 2007-2008, and 2009-2010 to investigate the relationship between perfluorinated alkyl substances concentrations and bone mineral density in adolescents aged

12-19 years, since bone mineral density was measured only for those aged 40 (or 50) years or older from the NHANES 2013-2014 cycle, and bone mineral density of the femur, femoral neck, and lumbar spine was not measured in the 2011-2012 cycle. In the three cycles, a total of 31,034 people participated in the survey, including 4,865 adolescents aged 12-19 years, and only 1,361 had data on serum PFAS, among which we finally selected 1,228 people with complete BMD data on the total femur and femoral neck or lumbar spine (Figure 1).

PFAS measurements

The quantification of PFAS in CDC is derived from a combination of solid-phase extraction and high-performance liquid chromatography-turbine ionization tandem mass spectrometry as also described in other cases (16). Concentrations below the limits of detection (LOD) were replaced with LOD divided by the square root of 2 (17), We selected four PFAS biomarkers that were detected in > 98% of participants: PFOA, PFOS, PFHxS, and PFNA, and we performed a natural logarithmic transformation of the serum PFAS concentrations because they showed a significantly skewed distribution.

BMD measurements

Dual-energy X-ray absorptiometry (DXA) is the most widely accepted method of measuring bone density due to its speed, ease of use, and low radiation dose (18), and the bone mineral density of the total femur, femoral neck, and lumbar spine is also measured by experienced professional technicians using a dual x-ray absorptiometry technique (QDR 4500A fan-beam densitometers [Hologic Inc]), lumbar bone mineral density is the average of the first to fourth lumbar vertebrae, the detailed measurements for each part can be found on the NHANES website (http://cdc.gov/nchs/nhanes).

Other covariates

We identified potential confounding factors associated with strong predictors of serum PFAS levels and bone mineral density based on previous studies, which included demographic information such as age, sex, race, and family income to poverty ratio, but also body mass index (BMI), smoking (serum cotinine), exercise status (performing vigorous or moderate exercise), serum lead, albuminuria and anemia, the demographic information was collected from questionnaires

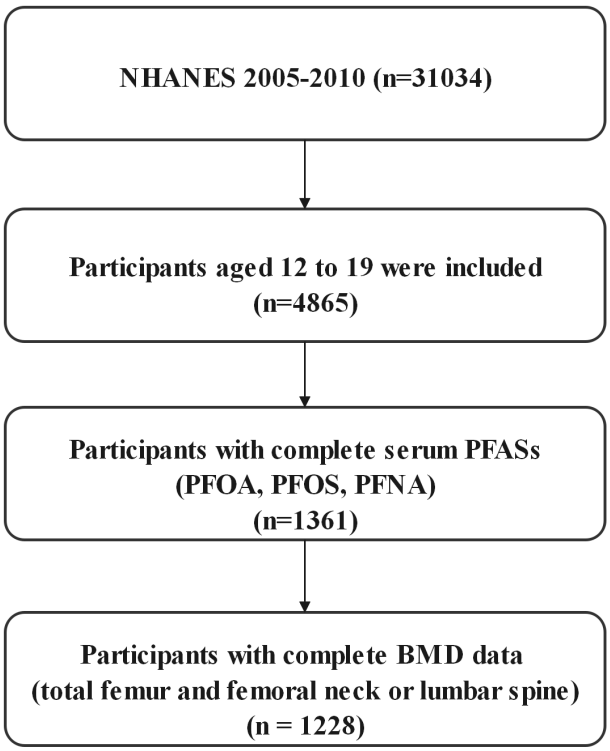

FIGURE 1
Flow chart algorithm.

administered during home visits. BMI is calculated by dividing body weight (kg) by body height (m^2), vigorous physical activity was identified from the questionnaire (did you do any vigorous activities for at least 10 minutes that caused heavy sweating, or large increases in breathing or heart rate such as running, lap swimming, aerobics classes or fast bicycling). Moderate physical activity was determined from the questionnaire (did you do moderate activities for at least 10 minutes that cause only light sweating or a slight to moderate increase in breathing or heart rate such as brisk walking, bicycling for pleasure, golf, and dancing).

Continuous variables such as age, income poverty rate, BMI, and serum cotinine were categorized using different criteria when stratifying the covariates, and continuous variable such as serum lead was mainly stratified by quartiles. Families with an income poverty ratio of <1.3 are classified as low-income families, and≥1.3 are classified as middle to high-income families (https://www.cbpp.org/research/food-assistance), for the BMI classification, we used the traditional percentile thresholds from the Centers for Disease Control and Prevention growth charts to identify subjects with a BMI below the 5th percentile as underweight, a BMI between the 5th and 85th percentile as normal weight, a BMI between the 85th and 95th percentile as overweight, and a BMI above the 95th percentile as obese (19), and we classified individuals people with serum cotinine levels <1.0 ng/mL as non-smokers, those with levels between 1.0 and 9.9 ng/mL as people exposed to environmental tobacco smoke (ETS), and those with levels ≥ 10.0 ng/mL as smokers (http://www.cdc.gov/exposurereport). We classified people with an albumin/creatinine ratio (ACR) greater than 30 mg/g as having albuminuria, females with a whole blood hemoglobin concentration <12 g/dL, and males with a whole blood hemoglobin concentration <13 g/dL as having anemia (20, 21).

Statistical analysis

In all analyses of the article, Continuous variables are represented by the mean standard error (SE), whereas categorical variables are represented by numbers and percentages, and gender differences were tested using the Student's two-tailed t-test or the Rao–Scott chi-square test. We utilized a multiple regression model to assess the relationship between individual ln-PFAS and the availability of bone mineral density in the total femur, femoral neck, and lumbar spine, results Expressed as regression coefficients and 95% confidence intervals (CI), then divided the ln-PFAS levels into quartiles for quartile-based repeated analyses, and set the lowest quartile as the reference. Since previous studies have shown that the association between PFAS and BMD has been observed mainly in females, we conducted stratified analysis by gender to assess the potential effect modification. Models were adjusted for sex, age, race, income poverty rate, BMI, serum cotinine, vigorous physical activity, moderate physical activity, serum lead, albuminuria, and anemia.

Stratified analyses, as well as a significance test of the interaction term with exposure, were conducted to explore the effect modification by BMI groups [underweight/normal weight (BMI< 85th percentile), overweight/obese (BMI ≥ 85th percentile)], albuminuria (yes/no) and anemia (yes/no), due to these three factors that can affect PFAS and BMD (22–27).

NHANES makes the data collected nationally representative through a complex sampling design and by using sample weights. We weighted the data according to the NHANES recommended sample weight calculation method, the six-year weights for the 2005-2006, 2007-2008, and 2009-2010 estimates were calculated by multiplying the two-year weights by one-third. We used Empowerstats software (www.empowerstats.com) and R (http://www.R-project.org) for all data analysis, the significance of the data is shown by the p-value < 0.05.

Results

The research population's characteristics

All participants were 15.44 ± 2.23 years old on average, non-Hispanic whites make up the majority of the study population (Table 1). The average family income poverty rate was 2.71 ± 1.68, with no significant difference in gender. Males exhibited greater rates of smoking and vigorous physical activity than females (all $p < 0.05$), but females had significantly higher rates of albuminuria and anemia than males (all $p < 0.05$), moderate physical activity and BMI had no significant differences.

In terms of bone mineral density, the bone mineral density of the total femur and femoral neck in males was 8% and 6% higher than in females respectively, while the bone mineral density of the lumbar spine was 4% lower than in females ($p<0.001$). Concerning serum PFAS levels, serum PFOA, PFOS, PFHxS, and PFNA levels were 15%, 26%, 37%, and 14% higher in males than in females respectively.

In the Supplementary Material, Table S1 summarizes the different stratified covariates and PFAS concentrations. PFOA was significantly correlated with all stratified covariates except BMI category (all $p < 0.05$), PFOS was correlated with all stratified covariates except anemia (all $p < 0.05$), PFHxS was significantly associated with sex, race, family income status, smoking status, vigorous physical activity, albuminuria (all $p < 0.05$), PFNA was only correlated with sex, race and family income status (all $p < 0.05$). Table S2 analyzes the covariates and bone mineral density for the different strata. Total femur and femoral neck BMD were significantly associated with all stratified covariates except family income status, moderate physical activity, serum lead quartiles, and albuminuria (all $p< 0.05$). In addition to family income status, vigorous physical

TABLE 1 Characteristics of the study population, overall and by sex, NHANES 2005-2010.

Characteristic variable	Overall		Male		Female		p-Value[a]
	n	Mean ± SE or percent	n	Mean ± SE or percent	n	Mean ± SE or percent	
Age (years)	1228	15.44 ± 2.23	670	15.44 ± 2.22	558	15.43 ± 2.25	0.983
Race/ethnicity	1228						0.722
Non-Hispanic white	346	60.04	199	61.74	147	57.84	
Non-Hispanic black	337	13.92	182	13.22	155	14.82	
Mexican American	374	13.33	196	12.6	178	14.28	
Other Hispanic	111	6.08	63	6.02	48	6.14	
Other multiracia	60	6.63	30	6.42	30	6.91	
Income poverty ratio	1147	2.71 ± 1.68	629	2.75 ± 1.65	518	2.67 ± 1.71	0.411
Smoking status[b]	1228		670		558		0.001
Nonsmokers	949	76.35	492	73.24	457	80.38	
ETS	129	9.81	72	9.78	57	9.84	
Smoker	150	13.84	106	16.98	44	9.78	
BMI (kg/m)	1225	23.68 ± 5.75	668	23.78 ± 5.51	557	23.55 ± 6.03	0.501
Serum lead (μg/dL)	1227	0.92 ± 0.71	670	1.07 ± 0.83	557	0.73 ± 0.44	<0.001
Vigorous physical activity	1205		660		545		<0.001
Yes	821	69.42	512	77.64	309	58.71	
No	384	30.58	148	22.36	236	41.29	
Moderate physical activity	1205		660		545		0.173
Yes	683	59.38	375	61.08	308	57.18	
No	522	40.62	285	38.92	237	42.82	
Albuminuria	1223						0.023
Yes	96	6.2	46	4.82	50	7.99	
No	1127	93.8	621	95.18	506	92.01	
Anemia	1221						<0.001
Yes	161	13.88	54	8.92	107	20.27	
No	1060	86.12	610	91.08	450	91.08	
Total femur BMD (g/cm^2)	1211	0.99 ± 0.16	656	1.02 ± 0.17	555	0.94 ± 0.13	<0.001
Femoral neck BMD (g/cm^2)	1211	0.91 ± 0.15	656	0.93 ± 0.16	555	0.87 ± 0.13	<0.001
Lumbar spine BMD (g/cm^2)	1191	0.95 ± 0.15	662	0.93 ± 0.17	529	0.97 ± 0.13	<0.001
PFOA (ng/mL) [c]	1228	3.80 ± 1.78	670	4.03 ± 1.75	558	3.50 ± 1.79	<0.001
PFOS (ng/mL) [c]	1228	12.96 ± 9.04	670	14.02 ± 9.65	558	11.60 ± 7.97	<0.001
PFHxS (ng/mL) [c]	1228	3.88 ± 4.95	670	4.41 ± 5.52	558	3.20 ± 4.01	<0.001
PFNA (ng/mL) [c]	1228	1.23 ± 0.72	670	1.30 ± 0.77	558	1.14 ± 0.65	<0.001

p-Value[a], validation of differences between males and females, t-test for continuous variables, and Rao-Scott chi-square test for categorical variables.
Smoking status[b], classification according to serum cotinine levels.
PFOA (ng/mL)[c], PFOS (ng/mL)[c], PFHxS (ng/mL)[c], PFNA (ng/mL)[c], untransformed serum perfluoroalkyl concentrations of the environment.

activity and moderate physical activity lumbar spine BMD were significantly correlated with all stratified covariates (all $p< 0.05$).

Associations of PFAS with BMD

Table 2 shows the outcomes of multivariate regression analysis of ln-transformed serum PFAS with total femur BMD, femoral neck BMD, and lumbar spine BMD separately. In the adjusted model, ln-PFOA, ln-PFOS, and ln-PFNA were negatively correlated with BMD of the three sites (all $p< 0.05$), and ln-PFHxS was positively correlated with total femur and femoral neck BMD (all $p< 0.05$). ln-PFOA with lumbar spine BMD, ln-PFOS with BMD of the three sites, and ln-PFNA with lumbar spine BMD after repeated analysis of quartiles in the trend test remained significant (all p for trend < 0.05).

Subgroup analysis

In the subgroup analysis of gender (Figures 2–4), in males, ln-PFOA was negatively associated with femoral neck and lumbar spine BMD (all $p< 0.05$), ln-PFOS was associated with reduced total femur, femoral neck, and lumbar spine BMD (all

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TABLE 2 Association between PFAS concentrations and BMD in young adults aged 12-19 years, NHANES 2005-2010.

ln-PFAS n = 1174	Total femur BMD β (95% CI)	Femur neck BMD β (95% CI)	Lumbar spine BMD β (95% CI)
ln-PFOA	**-0.017 (-0.031, -0.002)**	**-0.017 (-0.031, -0.003)**	**-0.020 (-0.033, -0.007)**
Q1	Reference	Reference	Reference
Q2	-0.003 (-0.025, 0.020)	0.006 (-0.015, 0.027)	-0.006 (-0.026, 0.014)
Q3	-0.009 (-0.031, 0.013)	-0.006 (-0.028, 0.015)	-0.010 (-0.029, 0.010)
Q4	-0.017 (-0.040, 0.006)	-0.016 (-0.038, 0.006)	-0.026 (-0.046, -0.006)
P for trend	0.095	0.054	**0.008**
ln-PFOS	**-0.021 (-0.032, -0.010)**	**-0.019 (-0.030, -0.009)**	**-0.022 (-0.032, -0.012)**
Q1	Reference	Reference	Reference
Q2	-0.025 (-0.045, -0.004)	-0.021 (-0.041, -0.001)	-0.014 (-0.032, 0.004)
Q3	-0.025 (-0.046, -0.005)	-0.022 (-0.042, -0.002)	-0.017 (-0.036, 0.001)
Q4	-0.051 (-0.072, -0.030)	-0.045 (-0.065, -0.025)	-0.040 (-0.059, -0.022)
P for trend	**<0.001**	**<0.001**	**<0.001**
ln-PFHxS	**0.007 (0.000, 0.014)**	**0.008 (0.001, 0.015)**	-0.004 (-0.010, 0.002)
Q1	Reference	Reference	Reference
Q2	-0.004 (-0.026, 0.017)	-0.011 (-0.032, 0.009)	-0.011 (-0.030, 0.008)
Q3	0.012 (-0.009, 0.034)	0.012 (-0.009, 0.032)	-0.012 (-0.031, 0.007)
Q4	0.017 (-0.005, 0.038)	0.016 (-0.005, 0.037)	-0.015 (-0.034, 0.004)
P for trend	0.058	0.067	0.171
ln-PFNA	**-0.014 (-0.027, -0.001)**	**-0.014 (-0.026, -0.002)**	**-0.016 (-0.027, -0.004)**
Q1	Reference	Reference	Reference
Q2	-0.022 (-0.044, -0.001)	-0.010 (-0.031, 0.011)	-0.021 (-0.040, -0.001)
Q3	-0.014 (-0.036, 0.007)	-0.008 (-0.029, 0.012)	-0.023 (-0.037, 0.001)
Q4	-0.017 (-0.039, 0.004)	-0.013 (-0.034, 0.007)	-0.025 (-0.044, -0.006)
P for trend	0.285	0.268	**0.024**

Adjusted for age, gender, race, income poverty ratio, serum cotinine, vigorous physical activity, moderate physical activity, BMI, serum lead, albuminuria, and anemia.
The bold values indicate significance (p<0.05).

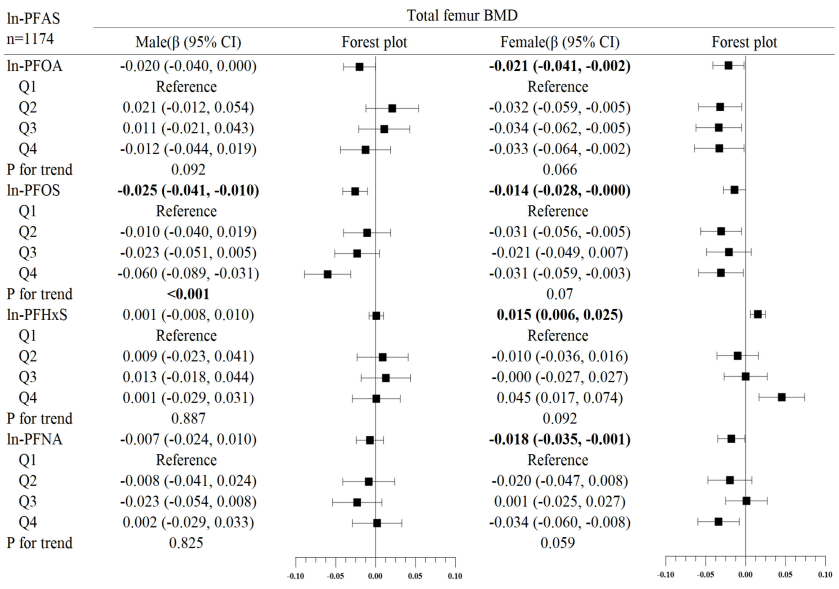

FIGURE 2
Association between ln-PFOA and bone mineral density, stratified by gender. Adjusted for age, race, income poverty ratio, serum cotinine, vigorous physical activity, moderate physical activity, BMI, serum lead, albuminuria, and anemia. The bold values indicate significance (p<0.05).

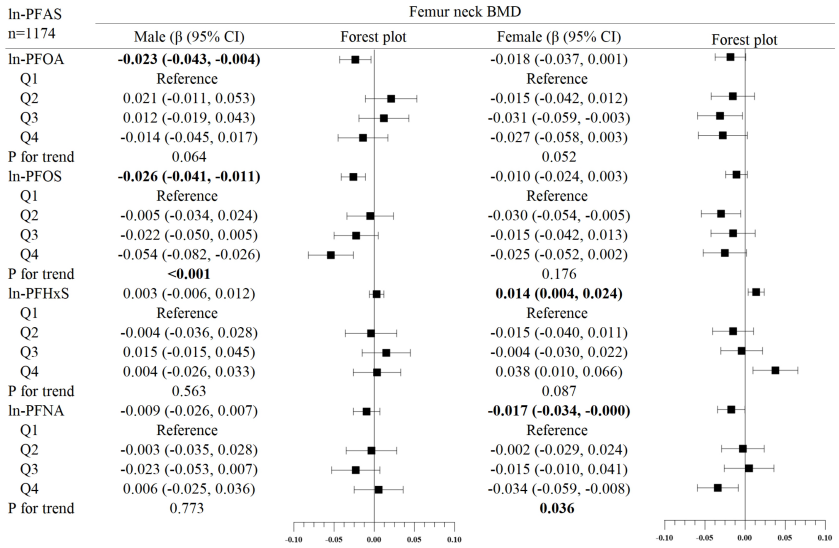

FIGURE 3
Association between ln-PFOS and bone mineral density, stratified by gender. Adjusted for age, race, income poverty ratio, serum cotinine, vigorous physical activity, moderate physical activity, BMI, serum lead, albuminuria, and anemia. The bold values indicate significance (p<0.05).

p< 0.05), while ln-PFHxS and ln-PFNA were not observed to correlate with BMD at these three sites. ln-PFOS was significantly associated with the quartile trend of BMD at all three sites in males (all p for trend < 0.05). In females, both ln-PFOA and ln-PFOS were negatively correlated with total femur and lumbar spine BMD (all p< 0.05), ln-PFHxS is associated with the increased total femur and femoral neck BMD (all p< 0.05), and ln-PFNA was negatively correlated with total femur and femoral neck BMD (all p< 0.05). ln-PFOA and ln-PFOS had significant quartile trends with lumbar spine BMD, as well as ln-PFNA with femoral neck BMD (all p for trend < 0.05). By observing the forest plots of PFAS and its quartiles with BMD, we could find that most of the associations were confined to females.

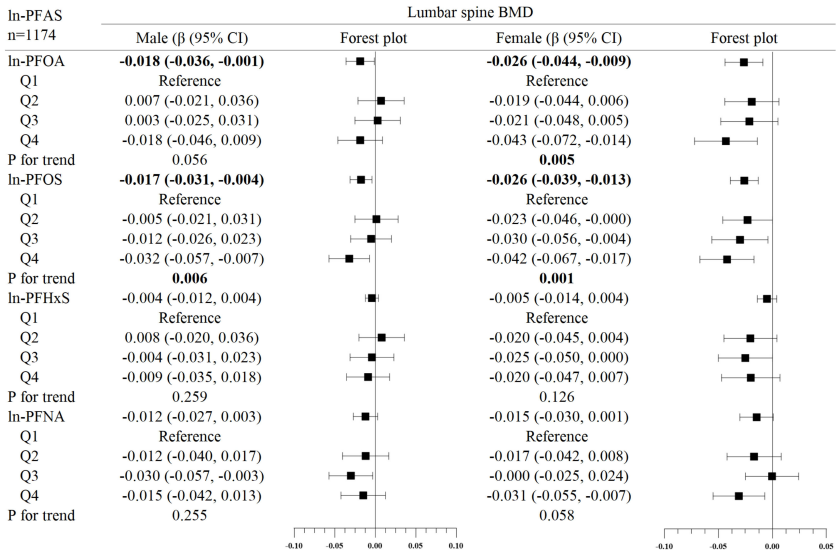

FIGURE 4
Association between ln-PFHxS and bone mineral density, stratified by gender. Adjusted for age, race, income poverty ratio, serum cotinine, vigorous physical activity, moderate physical activity, BMI, serum lead, albuminuria, and anemia. The bold values indicate significance (p<0.05).

Table 3 presents the results of the follow-up stratified (BMI, albuminuria, and anemia) analysis, which showed that the associations of ln-PFOS with femoral neck BMD and ln-PFNA with total femur BMD were more significant in those who were overweight/obese and had anemia, respectively (all p for interaction < 0.05), the results were consistent with the results of the regression analysis.

Discussion

In this study, we evaluated the correlation between exposure to specific PFASs (PFOA, PFOS, PFHxS, and PFNA) and bone mineral density in the total femur, femoral neck, and lumbar spine in adolescents aged 12-19 years at NHANES from 2005-2010. Some of the results showed that exposure to PFASs was

TABLE 3 Association between PFAS concentration and BMD, stratified by BMI groups, albuminuria (yes/no), and anemia (yes/no).

Subgroup	PFOA β (95% CI)	PFOS β (95% CI)	PFHxS β (95% CI)	PFNA β (95% CI)
Total femur BMD				
BMI category				
<85th percentile	-0.014 (-0.033, 0.005)	-0.020 (-0.034, -0.006)	0.005 (-0.004, 0.014)	-0.008 (-0.026, 0.010)
≥85th percentile	-0.029 (-0.055, -0.002)	-0.040 (-0.060, -0.020)	0.010 (-0.002, 0.022)	-0.026 (-0.046, -0.006)
P for interaction	0.378	0.111	0.509	0.178
Albuminuria				
Yes	0.007 (-0.028, 0.042)	-0.013 (-0.042, 0.015)	-0.004 (-0.024, 0.016)	0.005 (-0.028, 0.038)
NO	-0.020 (-0.037, -0.004)	-0.021 (-0.033, -0.009)	0.009 (0.002, 0.017)	-0.017 (-0.031, -0.003)
P for interaction	0.157	0.648	0.201	0.226
Anemia				
Yes	-0.035 (-0.076, 0.006)	-0.025 (-0.061, 0.011)	0.002 (-0.026, 0.030)	**-0.072 (-0.127, -0.016)**
No	-0.016 (-0.032, 0.000)	-0.023 (-0.035, -0.011)	0.007 (0.000, 0.015)	-0.011 (-0.024, 0.002)
P for interaction	0.401	0.907	0.721	**0.034**
Femoral neck BMD				
BMI category				
<85th percentile	-0.011 (-0.030, 0.007)	0.017 (-0.030, -0.003)	0.005 (-0.003, 0.014)	-0.006 (-0.023, 0.011)
≥85th percentile	-0.032 (-0.057, -0.006)	**-0.042 (-0.061, -0.022)**	0.012 (0.000, 0.023)	-0.027 (-0.047, -0.007)
P for interaction	0.195	**0.034**	0.407	0.107
Albuminuria				
Yes	0.016 (-0.018, 0.049)	-0.006 (-0.034, 0.021)	-0.005 (-0.024, 0.014)	0.009 (-0.022, 0.041)
NO	-0.024 (-0.040, -0.008)	-0.021 (-0.033, -0.010)	0.010 (0.003, 0.018)	-0.018 (-0.032, -0.005)
P for interaction	0.079	0.332	0.143	0.108
Anemia				
Yes	-0.017 (-0.056, 0.022)	-0.013 (-0.047, 0.022)	0.001 (-0.026, 0.028)	-0.052 (-0.106, 0.001)
No	-0.020 (-0.035, -0.004)	-0.022 (-0.034, -0.011)	0.009 (0.002, 0.016)	-0.012 (-0.025, 0.000)
P for interaction	0.900	0.605	0.576	0.149
Lumbar spine BMD				
BMI category				
<85th percentile	-0.020 (-0.037, -0.003)	-0.023 (-0.035, -0.011)	-0.001 (-0.009, 0.007)	-0.013 (-0.028, 0.003)
≥85th percentile	-0.023 (-0.046, 0.001)	-0.032 (-0.050, -0.014)	-0.008 (-0.018, 0.003)	-0.021 (-0.039, -0.003)
P for interaction	0.834	0.397	0.313	0.489
Albuminuria				
Yes	-0.018 (-0.049, 0.013)	-0.019 (-0.044, 0.007)	-0.021 (-0.039, 0.004)	-0.029 (-0.059, 0.000)
NO	-0.019 (-0.033, -0.004)	-0.021 (-0.031, -0.010)	-0.001 (-0.007, 0.006)	-0.013 (-0.025, -0.000)
P for interaction	0.991	0.877	0.060	0.304
Anemia				
Yes	-0.015 (-0.051, 0.021)	-0.022 (-0.054, 0.010)	-0.001 (-0.026, 0.024)	-0.060 (-0.109, -0.011)
No	-0.021 (-0.035, -0.006)	-0.022 (-0.033, -0.012)	-0.004 (-0.010, 0.003)	-0.013 (-0.024, -0.001)
P for interaction	0.770	0.978	0.831	0.064

Adjusted for age, gender, race, income poverty ratio, serum cotinine, vigorous physical activity, moderate physical activity, BMI, serum lead, albuminuria, and anemia, but not for the stratification variables themselves.
The bold values indicate significance (p<0.05).

associated with reduced BMD in adolescents, as indicated by ln-PFOA, ln-PFOS, and ln-PFNA all being negatively associated with total femur, femoral neck, and lumbar spine BMD, which is consistent with our previous hypothesis, the results of the stratified analysis showed that this association was mostly confined to females. Subsequent stratification analysis showed that the associations of ln-PFOS with femoral neck BMD and ln-PFNA with total femur BMD were more significant in overweight/obese, and those with anemia, respectively, while albuminuria conditions did not significantly modulate the association between PFAS and BMD.

Before our study, there were also NHANES studies that reported the association of PFASs exposure with reduced bone mineral density. In a survey of a population aged 8 years and older from 2005 to 2008, higher PFOS serum concentrations were found to be associated with reduced total lumbar spine BMD, primarily in premenopausal women, and no association was detected between serum PFOA, PFOS concentrations with femoral neck BMD (28). In addition, another report from 2009-2010 in a population aged 12-80 years found that serum PFOA, PFOS, PFHxS, and PFNA concentrations were associated with lower total femur and femoral neck BMD in women, while serum PFOA concentrations were associated with lower femoral neck BMD in men, but did not show any clear association between lumbar spine BMD and any PFAS (29).

In addition to the NHANES report, several other epidemiological studies have found an association between exposure to PFASs and reduced bone mineral density, including one study of overweight/obese adolescents aged 8 to 12 years finding that serum PFNA concentrations were significantly and negatively associated with skeletal parameters including broadband-ultrasound attenuation (BUA), the speed of sound waves (SOS), and the stiffness index (SI), which respond to higher bone health and higher BMD (30). Another similar study showed that PFAS exposure was significantly associated with reduced SI in young men (31). Also in a prospective study, PFOA and PFOS were associated with low BMD at several sites including spine, total hip, femoral neck, and hip rotor, and similar correlations were found for PFHxS, PFNA, and perfluorodecanoic acid (PFDA) in the intertransverse region of the hip (32). In addition to reports examining the relationship between a specific population's autologous PFAS exposure and bone mineral density, several studies have found a negative correlation between serum PFAS concentrations in women exposed prenatally to PFAS and their offspring's site-specific bone mineral density (14, 33, 34).

Not only epidemiological studies have uncovered the adverse effects of PFASs exposure on bone mineral density, but animal experiments have also reported a similar situation, in which PFOS was able to detectable in bone tissue of adult mice after 1-5 days of dietary exposure, and in addition, pregnant rats and mice exposed prenatally to PFOS showed fetal skeletal malformations as well as a decrease in bone mineral density (35–37), in addition, the results of human tissue examinations are similar to the findings of population studies, which have shown that PFASs can deposit in bone tissue and accumulate over time to exhibit some toxic effects, thereby affecting bone health (38–40).

The potential mechanisms of PFAS for adverse skeletal effects are not yet clear (11), and current studies have confirmed possible mechanisms that encompass several aspects, the first of which is the direct effect of PFAS on bone, current *in vivo* and *in vitro* studies on humans and animals have demonstrated that PFOA can take direct action on bone and bone marrow cells. In animal studies, for osteoblasts, the effects of different concentrations of PFOA on osteocalcin (OCN) expression and calcium secretion were dramatically different, as indicated by promotion at low concentrations and inhibition at high concentrations. In contrast, for osteoclasts, their number increased at all PFOA concentrations tested, but their resorption activity increased at low PFOA concentrations, decreased, and finally stopped at high concentrations (37). In terms of the effect of PFOA on osteoblasts, the results of human *in vitro* experiments were consistent with those of animal experiments, but PFOA did not interfere with osteogenic differentiation (40). PFOA also impairs the differentiation of hematopoietic stem cells and the stereotyping of bone marrow mesenchymal stem cells (41). Although very few studies have been conducted on osteoclast and osteoblast changes associated with PFAS exposure, PFOS, PFHxS, and PFOA have also been reported to affect multiple pathway targets (mRNA and protein of RUNX2), thereby inhibiting osteoblast differentiation (42). In addition, low concentrations of PFOS can achieve the same effect by decreasing the expression of the mRNAs for the osteoblast biomarkers bone bridging protein and bone junction protein (43), and PFOA and can also affect osteoblast function by significantly reducing alkaline phosphatase activity, collagen synthesis and mineralization in osteoblasts (44).

The second is that PFAS affects the skeleton through endocrine disruptive effects, mainly in both sex hormones and thyroid hormones (11), as both of them can significantly affect bone remodeling and bone health (45, 46). Both laboratory and epidemiological studies have now found a strong correlation between PFAS and sex hormones (47–53), for example, PFAS has a strong correlation with delayed puberty, early menopause, and serum estradiol concentration (54, 55), this may also explain the difference in the association between PFAS and BMD between the sexes in our study, and a large number of studies have confirmed that PFAS can also have significant effects on thyroid hormones (56–58), such as the association of PFAS with thyroxine (T4) and triiodothyronine (T3) levels (56).

Besides, recent *in vitro* evidence suggests that PFOA can interfere with the action of vitamin D by binding directly to hydroxyapatite crystals (59), along with epidemiological studies reporting that PFAS is associated with lower levels of total 25-hydroxyvitamin D (60), the latter is closely associated with bone health (61), so this could be another potential mechanism by

which PFAS affects bone. It has also been shown that PFAS can impair osteoblast formation by activating peroxisome proliferator-activated receptor-γ (PPARγ), thereby affecting bone health (62).

Another important finding of our study, in addition to reporting the above association, is that PFAS exposure was associated with higher BMD in adolescents, specifically, serum PFHxS was associated with higher BMD in the total femur and femoral neck, which seems to contradict our previous hypothesis. Although fewer studies have been reported to demonstrate that PFAS exposure is positively associated with BMD, there is still an NHANES study similar to our results, and their finding showed that PFOA, PFOS, PFHxS, and PFDE were negatively associated with proximal femoral BMD in premenopausal women, whereas PFOA, PFOS, PFHxS, and PFNA were positively associated with proximal femoral BMD in men (63). There are very few studies on the mechanisms underlying the positive effects of PFASs exposure on bone health. However, there is still experimental demonstration that some PFAS at low concentrations is associated with increased OCN expression and calcium secretion, which facilitates osteogenesis (37), and there are also studies showing that some PFAS are associated with increased FT4 (64), which may inhibit TSH, a decrease in which may contribute to osteoporosis. Therefore, the above speculations may explain this association.

Another interesting finding is that the association between PFAS and BMD is strengthened in those who are overweight/obese and have anemia, but not in those who have albuminuria the mechanisms of how PFAS and obesity interact remain unclear, but obesity can affect bone health through multiple pathways, and there may be a synergistic effect with PFAS in one of these pathways to affect bone health, such as hormone secretion (65, 66), and altered tissue distribution of PFAS in more obese populations may also influence its effect on bone health (67), all of these may help explain the enhanced effect of PFAS on BMD in overweight/obese populations. There are few studies on the interaction between albuminuria and PFAS, but some studies have shown that albuminuria is associated with reduced bone blood flow, which leads to a reduced rate of bone remodeling and the development of osteoporosis (68), and also that renal failure with albuminuria may lead to less renal reabsorption, which may have an impact on PFAS excretion, thus affecting serum PFAS levels (69). However, our study did not find that the association between PFAS and BMD was strengthened in the population with albuminuria. It has been suggested that anemia may affect serum PFAS levels (70), and have an effect on BMD (27), our study confirms such findings, but the exact mechanism is not explained by current studies, so more studies are needed to confirm these findings.

Some differences can be observed by comparing our study with previous NHANES reports. Our study found that in males, PFOA was negatively associated with femoral neck and lumbar spine BMD, PFOS was negatively associated with total femur, femoral neck, and lumbar spine BMD, PFHxS and PFNA were not associated with BMD at any of these three locations. In females, both PFOA and PFOS were associated with the reduced total femur and lumbar spine BMD, PFHxS and PFNA were positively and negatively associated with total femur and femoral neck BMD, respectively, but Lin's study reported that PFOS was not associated with femoral neck BMD (28), Khalil's study demonstrated that PFOA was associated with the reduced total femur and femoral neck BMD and not lumbar spine BMD in females, PFOS was also not associated with lumbar spine BMD, and PFHxS and PFNA were associated with the reduced total femur and femoral neck BMD in females (29). The above differences may be due to variations in NHANES survey period, survey sample size, age group, and different covariates.

Our study has some strengths, first, as far as we know, the correlation of autologous PFAS levels with bone mineral density in adolescents has never been explored separately, and this is the first study to do so. Second, we explored the role of the effect of different populations on the association between PFAS and BMD by stratifying the data for multiple comparisons. Third, we quantified the independent variables and performed trend tests, and also performed interaction tests after stratified analysis, which reduced the chances of data analysis and enhanced the robustness of the results.

However, our current analysis has some limitations. First, due to the cross-sectional nature of the study. We were unable to identify the causal relationship between serum PFOS levels and BMD. Second, in our analysis of subsequent stratification (BMI, proteinuria, and anemia), we did not fail to replicate the analysis for gender differences to derive differences in the effects of obesity, proteinuria, and anemia on PFAS and BMD by gender. Third, although potential confounding factors are considered, we cannot completely exclude residual and unmeasured confounding factors. Fourth, some of our covariate data, in spite of being collected by trained interviewers with standardized protocols, are still subject to self-report bias.

Conclusions

In conclusion, PFAO, PFOS, and PFNA were associated with lower BMD and PFAS with higher BMD in US adolescents aged 12-19 years, and these associations were mostly confined to females, and the negative effect of PFAS on BMD was more pronounced in those who were overweight/obese and had anemia however, additional laboratory and prospective epidemiological studies are needed to confirm these findings.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by NHANES. The patients/participants provided their written informed consent to participate in this study.

Author contributions

This study was designed by YG. XX extracted the associated data from NHANES. BC performed the statistical analysis. XX completed the composition of the manuscript, helped supervised the analysis, and revised and approved the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.980608/full#supplementary-material

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Effect of obesity status on adolescent bone mineral density and saturation effect: A cross-sectional study

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Background: The effect of obesity status on bone mineral density (BMD) in adolescents and whether there is a saturation effect is still insufficient. A cross-sectional study of adolescents aged 12–19 was conducted to investigate them.

Methods: Weighted multivariate linear regression models were used to assess the relationship between obesity status and BMD *via* datasets from the National Health and Nutrition Examination Survey 2011–2018. The nonlinear relationships and saturation values were ascertained by fitting smooth curves and analyzing saturation effects. At the same time, the subgroup stratified analysis was also performed.

Results: 4056 adolescents were included in this study. We found that body mass index (BMI) and waist circumference (WC) were significantly associated with total BMD, which remained significant in subgroups stratified by age, gender, standing height, and ethnicity. We also noticed an inverse correlation between left leg fat/lean mass and left leg BMD, which was only significant in males and other races. Fitting smooth curve and saturation effect analysis showed that BMI, WC, left leg fat/lean mass, and BMD had a specific saturation effect. There was a saturation effect on bone mineral density in adolescents with a BMI of 22 kg/m^2, a WC of 70.5 cm, or a left leg fat/lean mass of 0.2994.

Conclusions: We found a positive saturation effect of BMI and WC with BMD and a negative saturation effect of left leg fat/lean mass with BMD. Appropriate obesity status allows adolescents to have better bone mass development but not excessive obesity.

KEYWORDS

Bone mineral density, bone mineral content, body mass index, waist circumference, Fat/lean mass, NHANES, osteoporosis, Adolescents

Introduction

Osteoporosis (OP) is a degenerative disease of the bones that results in weakened bones, weakened microarchitecture, increased fragility, and increased fracture risk (1). According to an epidemiological survey, at least 200 million people globally suffer from OP, which is predicted to rise substantially over time (2). A new study predicts that more than 70 million more people in the United States will be diagnosed with OP, or bone loss, by 2030 (3). OP fractures will not only have a terrible psychological influence on the patient, but also place a significant financial strain on the entire family (4, 5). Bone mineral density (BMD) is one of the most critical diagnostic markers of OP, and obesity status is closely related to BMD. Both Ma et al. (6). and Y et al. (7). found a positive saturation effect between obesity status and BMD in people older than 50.

Adolescence is the most critical period to reach peak BMD (8). To our knowledge, however, existing studies examining the effects of obese status on adolescent BMD and the existence of a saturation effect are insufficient and contentious. Although Yujuan et al. (9). concluded that Body mass index (BMI) was positively associated with BMD in adolescents, their study did not adjust for some factors that have been shown to affect BMD in adolescents, such as regulating serum creatinine (10) and uric acid (11), and only considered BMI and did not consider indicators of other obesity conditions, such as waist circumference (WC) and body fat mass. Kátia et al. (12). found that obesity negatively impacts skeletal development in adolescents, leading to underdevelopment of bone mass. In a cross-sectional study, Yin et al. (13). found a negative correlation between WC and lumbar BMD among people aged 8 to 18. A survey of 982 Korean young people aged 12–19 found a negative relationship between body fat mass and total-body-less-head BMD in males (14). A study of 795 adolescent participants by Hee-Cheol et al. (15). found no association between body fat mass and BMD after adjusting for lean body mass.

In this study, we used the NHANES database to conduct a cross-sectional analysis to explore the effect of several indicators of obesity status (BMI, WC, and fat/lean mass) on adolescent BMD and whether saturation effects exist. This study's results can be used as a guide for therapy, which can allow adolescents to have better bone mass development but not excessive obesity.

Methods

Data source and study population

Our cross-sectional analysis was supported by data from the NHANES 2011–2018. The survey is aimed at patients from all backgrounds of life in America. All of the subjects were subjected to a battery of tests, consisting of BMI, WC, Standing height, lab tests, and standardized questionnaires concerning their age, gender, race/ethnicity, moderate activity, and household income-to-poverty ratio. This data was utilized in the evaluation of the prevalence and severity of a wide variety of diseases, as well as in the formulation of public health policies and the provision of medical care.

The participants in the study ranged in age from 12 to 19. From a total of 39,156 participants between 2011 and 2018, we excluded 11,324 children under the age of 12 and 22,617 adults over the age of 19; 255 subjects with missing BMI information; 153 subjects with missing WC information; 656 subjects with missing total BMD information, as well as 89 subjects with missing other BMD; and 6 subjects with missing fat and lean. Following the aforesaid screening, data from 4056 participants was included (Figure 1).

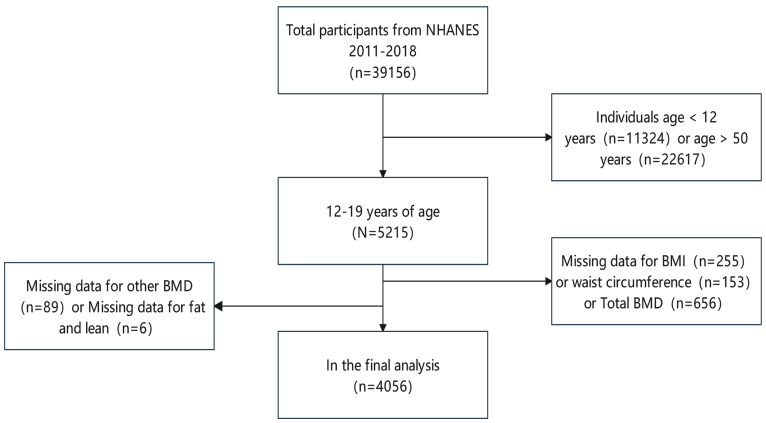

FIGURE 1
Flowchart of participant selection.

Ethics statement

The NHANES required every individual who took part in the survey to sign an informed consent form, which was then reviewed and authorized by the National Center for Health Statistics Ethics Review Board. The data can now be accessed by the general public following privacy-preserving. It is already possible to transform data into a form that can be analyzed. All statistics would be used for data analysis and all studies will be done in compliance with applicable laws and standards provided we comply with the study's data usage guidelines.

Covariates

Self-reporting of completed questions included information on age, gender, race/ethnicity, moderate activity, and the percentage of household income in poverty. Professional physical examination using conventional procedures, including assessment of weight, height, and WC. The height and weight of the respondents were measured by first reminding them to take off their shoes and any heavy clothing. Afterward, their BMI was measured by dividing their total body weight by the square of their height. Drawing the right midaxillary line by drawing a horizontal line above the highest lateral border of the right ilium, and positioning a tape measure at the intersection of the two lines are all necessary steps in the process of measuring the WC. During the process of the research project, measurements were taken of things like serum alkaline phosphatase, serum calcium, serum phosphorus, uric acid, total cholesterol, triglyceride, blood urea nitrogen, serum creatinine, and urine albumin-to-creatinine ratios. This took place at the scheduled visit.

Outcome variable

Whole-body scans using dual-energy X-ray absorptiometry (DXA) were performed on all of the subjects. To calculate the BMC and BMD, a qualified and certified radiographer used a QDR-4500A fan-beam densitometer, DXA images from Hologic, Inc. in Bedford, Massachusetts, and Hologic APEX (version 4.0) software. For more information on how to collect covariate data and how to measure WC, BMI, fat, lean, BMC, and BMD, among other things, go to www.cdc.gov/nchs/nhanes/.

Statistical analysis

We used EmpowerStats (http://www.empowerstats.com) and R (3.4.4 version) software for statistical analysis. This study conducted these analyses to see whether categorical and continuous variables differed significantly. This was accomplished through the utilization of multivariate linear regression models to calculate the β value and 95% confidence interval (CI). According to the Strengthening the Reporting of Observational Studies in Epidemiology guidelines, all covariates were adjusted in all models.

Result

Characteristics of participants

The weighted distribution of the basic information of the population in this investigation is shown in Table 1. The number of individuals was 2151 males and 1905 females. There were no highly relevant differences between male and female participants in terms of age, the ratio of household income to poverty, WC, pelvis BMD, and thoracic BMC. Moderate activity, alkaline phosphatase, serum calcium, serum phosphorus, serum uric acid, triglycerides, blood urea nitrogen, serum creatinine, standing height, total BMD, left leg BMD, left arm BMD, trunk BMD, left rib BMD, total BMC, lumbar spine BMC, left leg BMC, left arm BMC, left rib BMC, pelvis BMC, trunk BMC baseline in male participants were higher than female in terms of bone mineral resources and lower than females in terms of total cholesterol, urinary albumin creatinine ratio, BMI, WC, total fat lean, trunk fat lean, left leg fat lean, left arm fat lean, lumbar spine BMD, thoracic BMD, head BMD, and head BMC, and these differences were statistically significant.

The association between BMI and BMD

Table 2 presents three distinct weighted multiple linear regression models. All variables were adjusted, and there was a statistically significant positive correlation between BMD and BMI in all three models. When stratifying BMI by quartile and using the lowest quartile as a reference point, the trend analysis was statistically significant (*P* for trend < 0.001). In subgroups stratified by gender, age, standing height, and race, the positive associations for total BMD, left leg BMD, and BMI remained significant. Notably, in the age-stratified subgroup, this association of BMI with lumbar spine BMD was not observed among adolescents aged 12, 13, and 17 - 19 years. Figures 2A, B are the forest plots of each body part's BMD or BMC and BMI, respectively, and each body part's BMD or BMC and BMI has significant correlations. Figures 2C, D are the smooth curve fitting graphs of total BMD or total BMC and BMI, respectively. When we smooth curve fit the revised model, there is a saturating effect for total BMD and BMI (Figure 2C). We conducted a saturation effect model analysis to determine the BMI tipping point and determined that the BMI saturation effect

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TABLE 1 Weighted characteristics of the study sample.

	Male (n = 2151)	Female (n = 1905)	*P* value
Age (years)	15.36 ± 2.26	15.40 ± 2.25	0.527
Race/ethnicity (%)			0.046
Mexican American	436 (20.27%)	412 (21.63%)	
Other Hispanic	201 (9.34%)	225 (11.81%)	
Non-Hispanic White	590 (27.43%)	497 (26.09%)	
Non-Hispanic Black	533 (24.78%)	428 (22.47%)	
Other race - including multi-racia	391 (18.18%)	343 (18.01%)	
Ratio of family income to poverty (%)	2.08 ± 1.45	2.02 ± 1.48	0.221
Moderate activities (%)			0.003
No	632 (29.38%)	655 (34.38%)	
Yes	1167 (54.25%)	955 (50.13%)	
No record	352 (16.36%)	295 (15.49%)	
Alkaline phosphatase (u/L)	168.72 ± 102.53	102.28 ± 56.09	<0.001
Serum calcium (mmol/L)	2.41 ± 0.07	2.38 ± 0.07	<0.001
Serum phosphorus (mmol/L)	1.43 ± 0.22	1.36 ± 0.18	<0.001
Serum uric acid (umol/L)	329.98 ± 67.62	266.78 ± 55.46	<0.001
Total cholesterol (mmol/L)	3.97 ± 0.71	4.11 ± 0.74	<0.001
Triglyceride (mmol/L)	1.13 ± 0.79	1.03 ± 0.62	<0.001
Blood urea nitrogen (mmol/L)	4.18 ± 1.18	3.71 ± 1.06	<0.001
Serum creatinine (umol/L)	68.54 ± 14.92	58.16 ± 11.24	<0.001
Urinary albumin creatinine ratio (mg/g)	19.14 ± 98.89	34.80 ± 138.16	<0.001
Standing height (cm)	169.23 ± 9.37	159.95 ± 6.87	<0.001
Body mass index (kg/m^2)	23.84 ± 6.01	24.34 ± 6.13	0.009
Waist circumference (cm)	82.00 ± 15.52	81.71 ± 14.30	0.532
Total fat/lean mass	0.36 ± 0.16	0.57 ± 0.17	<0.001
Trunk fat/lean mass	0.30 ± 0.16	0.48 ± 0.18	<0.001
Left Leg fat/lean mass	0.44 ± 0.20	0.73 ± 0.19	<0.001
Left Arm fat/lean mass	0.38 ± 0.22	0.70 ± 0.25	<0.001
Head fat/lean mass	0.36 ± 0.01	0.36 ± 0.01	<0.001
Total bone mineral density (g/cm^2)	1.05 ± 0.13	1.02 ± 0.10	<0.001
Lumbar Spine Bone Mineral Density (g/cm^2)	0.94 ± 0.17	1.00 ± 0.14	<0.001
Left Leg Bone Mineral Density (g/cm^2)	1.14 ± 0.16	1.07 ± 0.12	<0.001
Left Arm Bone Mineral Density (g/cm^2)	0.74 ± 0.10	0.67 ± 0.06	<0.001
Trunk Bone Mineral Density (g/cm^2)	0.87 ± 0.14	0.85 ± 0.10	<0.001
Pelvis Bone Mineral Density (g/cm^2)	1.18 ± 0.20	1.18 ± 0.16	0.404
Thoracic Bone Mineral Density (g/cm^2)	0.74 ± 0.12	0.75 ± 0.10	<0.001
Head Bone Mineral Density (g/cm^2)	1.75 ± 0.32	1.92 ± 0.34	<0.001
Left Rib Bone Mineral Density (g/cm^2)	0.64 ± 0.10	0.61 ± 0.07	<0.001
Total Bone Mineral Content (g)	2173.59 ± 530.31	1897.75 ± 355.95	<0.001
Lumbar Spine Bone Mineral Content (g)	50.82 ± 15.92	48.47 ± 11.00	<0.001
Left Leg Bone Mineral Content (g)	431.73 ± 107.02	350.36 ± 70.20	<0.001
Head Bone Mineral Content (g)	415.73 ± 87.97	424.25 ± 85.83	0.002
Left Arm Bone Mineral Content (g)	157.50 ± 48.72	127.14 ± 28.49	<0.001
Left Rib Bone Mineral Content (g)	80.15 ± 21.00	70.87 ± 15.59	<0.001
Thoracic Bone Mineral Content (g)	94.78 ± 28.37	94.05 ± 21.42	0.36
Pelvis Bone Mineral Content (g)	263.45 ± 91.20	223.62 ± 58.35	<0.001
Trunk Bone Mineral Content (g)	566.30 ± 165.39	506.35 ± 109.18	<0.001

Continuous variables are presented as Mean ± SD, P-value was calculated by a weighted linear regression model. Categorical variables are presented as %, P-value was calculated by chi-square test.

TABLE 2 Association between body mass index (kg/m^2) and bone mineral density (g/cm^2).

Exposure	Total BMDβ (95% CI)	Lumbar Spine BMD	β (95% CI)	Left Leg BMD	β (95% CI)
Quintiles of body mass index (kg/m2)					
< 18.5	reference	reference		reference	
>= 18.5, < 25	0.0493 (0.0416, 0.0569)	0.0577 (0.0471, 0.0683)		0.0645 (0.0555, 0.0735)	
>= 25, < 30	0.0708 (0.0615, 0.0801)	0.0663 (0.0535, 0.0792)		0.0984 (0.0875, 0.1094)	
>= 30	0.1012 (0.0906, 0.1117)	0.0815 (0.0669, 0.0961)		0.1450 (0.1326, 0.1574)	
P for trend	< 0.001	< 0.001		< 0.001	
Stratified by gender					
Male	0.0048 (0.0040, 0.0055)	0.0044 (0.0035, 0.0054)		0.0065 (0.0056, 0.0074)	
Female	0.0048 (0.0041, 0.0055)	0.0031 (0.0021, 0.0041)		0.0078 (0.0071, 0.0086)	
Stratified by age (years old)					
12	0.0031 (0.0019, 0.0043)	-0.0412 (-0.1757, 0.0932)		0.0069 (0.0055, 0.0083)	
13	0.0045 (0.0033, 0.0056)	-0.0463 (-0.1734, 0.0808)		0.0067 (0.0053, 0.0082)	
14	0.0049 (0.0035, 0.0063)	0.1571 (0.0141, 0.3001)		0.0068 (0.0053, 0.0084)	
15	0.0058 (0.0044, 0.0072)	0.2323 (0.0737, 0.3910)		0.0074 (0.0058, 0.0090)	
16	0.0050 (0.0037, 0.0063)	0.1580 (0.0142, 0.3018)		0.0076 (0.0061, 0.0091)	
17	0.0049 (0.0036, 0.0063)	0.1060 (-0.0453, 0.2573)		0.0073 (0.0057, 0.0088)	
18	0.0023 (0.0012, 0.0035)	0.0639 (-0.0710, 0.1987)		0.0049 (0.0036, 0.0062)	
19	0.0032 (0.0018, 0.0046)	-0.0447 (-0.1906, 0.1013)		0.0049 (0.0033, 0.0066)	
Stratified by standing height (cm)					
Q1 (132.9-160.3)	0.0056 (0.0048, 0.0064)	0.0042 (0.0030, 0.0053)		0.0094 (0.0084, 0.0103)	
Q2 (160.4-169)	0.0043 (0.0034, 0.0052)	0.0029 (0.0017, 0.0041)		0.0065 (0.0054, 0.0076)	
Q3 (169.1-190.9)	0.0047 (0.0038, 0.0056)	0.0044 (0.0031, 0.0056)		0.0061 (0.0050, 0.0072)	
Stratified by race					
Mexican American	0.0044 (0.0033, 0.0055)	0.0031 (0.0017, 0.0045)		0.0068 (0.0055, 0.0081)	
Other Hispanic	0.0060 (0.0045, 0.0076)	0.0066 (0.0044, 0.0088)		0.0088 (0.0069, 0.0106)	
Non-Hispanic White	0.0050 (0.0040, 0.0060)	0.0034 (0.0021, 0.0048)		0.0075 (0.0064, 0.0087)	
Non-Hispanic Black	0.0037 (0.0027, 0.0047)	0.0039 (0.0025, 0.0053)		0.0057 (0.0046, 0.0069)	
Other race	0.0052 (0.0040, 0.0064)	0.0042 (0.0025, 0.0059)		0.0065 (0.0051, 0.0079)	

Adjusted for all confounding factors (age, gender, standing height, race, ratio of family income to poverty, moderate activities, alkaline phosphatase, serum calcium, serum phosphorus, serum uric acid, total cholesterol, triglyceride, blood urea nitrogen, serum creatinine, urinary albumin creatinine ratio).
The model is not adjusted for the stratification variable itself in the subgroup analysis.

value was 22 kg/m^2. When BMI < 22 kg/m^2, BMD increased by 0.0136 g/m^2 for per unit increase in BMI; for BMI > 22 kg/m^2, BMD increased by 0.0027 g/m^2. In addition, when stratified by age, we discovered that the BMI of teenagers at each age had a saturation effect, as shown in Table 3. Likewise, when we separated the data by gender, we found that both males and females had BMI saturation values.

The association between WC and BMD

Table 4 presents three weighted multiple linear regression models. All variables were adjusted, and there was a statistically significant positive correlation between BMD and WC in all three models. Trend analysis was statistically significant (P for trend < 0.05) when BMI was stratified by quartile and the lowest quartile was used as a reference point. The positive associations for total BMD, left leg BMD, and WC remained significant in subgroups stratified by gender, age, standing height, and ethnicity. Likewise, in age-stratified subgroups, this association of WC with lumbar spine BMD was not observed in 12, 13, and 17 - 19 years adolescents. Figures 3A, B are forest plots of BMD or BMC and WC for each body part, respectively, and BMD or BMC and WC for each body part have a statistically positive correlation. Figures 3C, D are the smooth curve fitting graphs of Total BMD or Total BMC and WC, respectively. When we smooth curve-fit the revised model, there is a saturation effect for total BMD and WC (Figure 3C). We also conducted saturation effect model research to determine the WC tipping point and determined that the WC saturation effect value was 70.5 cm. When WC was less than 70.5 cm, BMD increased by 0.0054 g/m^2 for each unit increase in WC. However, when WC was greater than 70.5 cm, BMD increased by just 0.0010 g/m^2 for each unit increase in WC. As shown in Table 3, the WC saturation effect values for teenagers of different ages were different in the subgroups that were split up by age. Likewise,

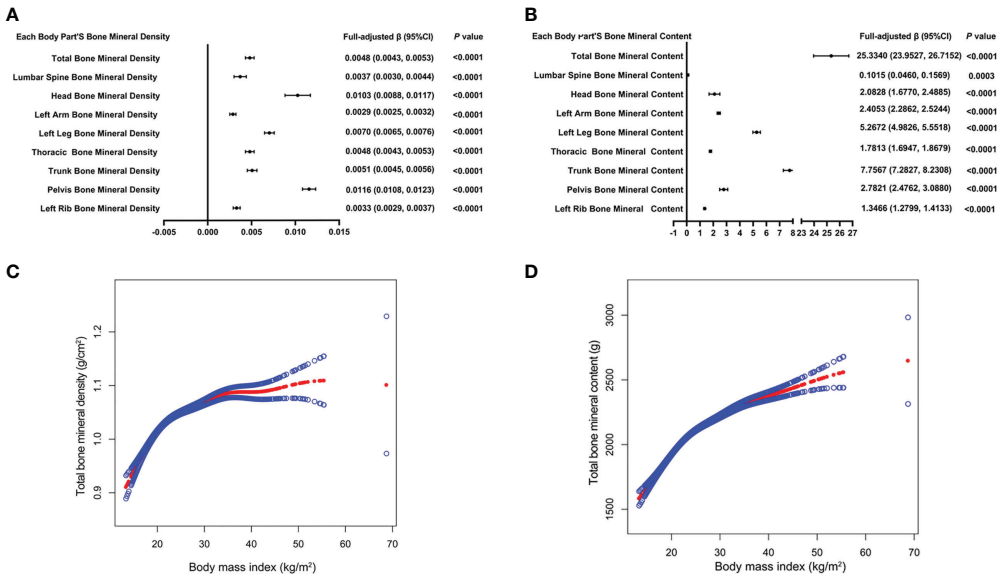

FIGURE 2
The forest plots of each body part's bone mineral density or bone mineral content and body mass index, respectively **(A, B)**. Association of total bone mineral density and bone mineral content with body mass index **(C, D)**. The solid red line represents the smooth curve fit between variables. Blue bands represent the 95% confidence interval from the fit. All confounding factors were adjusted.

when we separated the data by gender, we found that both males and females had WC saturation values.

Association between fat/lean mass and BMD of corresponding parts of the body

Table 5 presents the fully corrected models for total body, trunk, and left leg fat/lean body mass and BMD, respectively. When stratified by quartile for fat/lean mass and using the lowest quartile as the reference point, this trend analysis was statistically significant in the model for only left calf fat-lean mass and BMD (P for trend < 0.05), and there is a significant negative correlation. In subgroups stratified by gender, this negative association was only found in male. This negative association was not statistically significant in subgroups stratified by age and standing height. Among the subgroups stratified by race, however, only the other race had a statistically significant negative correlation between left leg fat/lean mass and BMD. Figures 4A, B are forest plots of BMD or BMC and fat/lean mass for each body part, respectively. There is a statistically significant negative correlation between left leg BMD and fat/lean mass, and BMC for each body part is associated with fat There was a significant positive correlation with lean body mass. Figures 4C, D are fitted with smooth curves drawn in the revised model, there is a saturation effect for left leg fat/lean mass and left leg BMD. The saturation effect value of left leg fat/lean mass was calculated to be 0.2994 using a saturation effect model analysis to determine the turning point for left leg fat/lean mass. When the fat/lean mass ratio of the left leg is smaller than 0.2994, the BMD drops by 0.3185g/m^2 for each unit of left leg fat/lean mass. When left leg fat/lean mass is more than 0.2994, the BMD drops by 0.0096 g/m^2 for every unit of increase in left leg fat/lean mass. Table 3 shows that a turning point of left leg fat/lean mass was only found in teenagers aged 14 to 17 years. And when stratified by gender, we found a saturated value of left leg fat/lean mass in males.

Discussion

Statistics from the NHANES were used to analyze the connection between obesity status and total BMD in teens ages 12 to 19. In this cross-sectional study of adolescents, a significantly positive connection was observed between BMI, WC, and total BMD. These conclusions appear to be consistent with previous studies (16–20). However, the association between left leg fat/lean mass and left leg BMD was negative. Moreover, through fitting smooth curve and saturation effect model analysis, it was shown that the saturation effect values of BMI, WC, and left leg fat/lean mass were 22 kg/m^2, 70.5 cm, and 0.2994, respectively. As BMI and WC surpassed this effective value, the degree of the rise in total BMD diminished. The magnitude of the increase in total BMD decreased when BMI and WC exceeded this effect size. After the fat/lean mass of the left leg was lower than the effect value, the BMD of the left leg

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TABLE 3 Saturation effect analysis of obesity status and bone mineral density (g/cm^2).

Bone mineral density (g/cm^2)	Model: saturation effect analysis		
	Body mass index(kg/m^2)	Waist circumference (cm)	Left leg fat/lean mass
Turn point	**22**	**70.5**	**0.2994**
< Turn point, effect1	0.0136 (0.0120, 0.0152)	0.0054 (0.0041, 0.0067)	-0.3185 (-0.4569, -0.1802)
> Turn point, effect2	0.0027 (0.0021, 0.0033)	0.0010 (0.0007, 0.0012)	-0.0096 (-0.0282, 0.0091)
Stratified by gender			
Turn point of males	**22**	**78**	**0.2893**
< Turn point, effect1	0.0135 (0.0113, 0.0158)	0.0032 (0.0023, 0.0042)	-0.2582 (-0.4292, -0.0872)
> Turn point, effect2	0.0026 (0.0017, 0.0035)	0.0005 (0.0002, 0.0009)	-0.0341 (-0.0631, -0.0051)
Turn point of females	**21.5**	**102**	**0.8016**
< Turn point, effect1	0.0141 (0.0116, 0.0166)	0.0020 (0.0016, 0.0024)	-0.0049 (-0.0440, 0.0343)
>Turn point, effect2	0.0031 (0.0024, 0.0039)	-0.0001 (-0.0009, 0.0008)	0.0482 (-0.0007, 0.0971)
Stratified by age			
Turn point of 12 years old	**17.6**	**64.5**	**0.3082**
< Turn point, effect1	0.0222 (0.0130, 0.0314)	0.0053 (0.0014, 0.0093)	-0.4383 (-1.0353, 0.1588)
>Turn point, effect2	0.0026 (0.0011, 0.0040)	0.0005 (-0.0001, 0.0011)	-0.0166 (-0.0545, 0.0213)
Turn point of 13 years old	**20**	**69.5**	**0.7613**
< Turn point, effect1	0.0138 (0.0084, 0.0191)	0.0042 (0.0014, 0.0070)	-0.0573 (-0.1166, 0.0020)
> Turn point, effect2	0.0029 (0.0013, 0.0046)	0.0010 (0.0004, 0.0016)	0.0891 (-0.0194, 0.1977)
Turn point of 14 years old	**19.3**	**71.4**	**0.2624**
< Turn point, effect1	0.0313 (0.0231, 0.0396)	0.0109 (0.0075, 0.0142)	-1.1514 (-1.8918, -0.4110)
> Turn point, effect2	0.0031 (0.0016, 0.0047)	0.0005 (-0.0002, 0.0012)	-0.0012 (-0.0540, 0.0516)
Turn point of 15 years old	**21.5**	**65.7**	**0.7883**
< Turn point, effect1	0.0197 (0.0144, 0.0251)	0.0188 (0.0065, 0.0310)	-0.0721 (-0.1371, -0.0070)
> Turn point, effect2	0.0035 (0.0017, 0.0052)	0.0016 (0.0009, 0.0022)	0.0579 (-0.0664, 0.1823)
Turn point of 16 years old	**23.9**	**84.7**	**0.8027**
< Turn point, effect1	0.0149 (0.0113, 0.0185)	0.0044 (0.0030, 0.0058)	-0.0890 (-0.1594, -0.0186)
> Turn point, effect2	0.0030 (0.0011, 0.0049)	0.0007 (-0.0002, 0.0016)	0.2030 (0.0467, 0.3592)
Turn point of 17 years old	**24.5**	**79.7**	**0.2111**
< Turn point, effect1	0.0124 (0.0084, 0.0164)	0.0041 (0.0020, 0.0062)	-4.2717 (-6.1958, -2.3475)
> Turn point, effect2	0.0014 (-0.0007, 0.0034)	0.0004 (-0.0005, 0.0012)	0.0386 (-0.0184, 0.0955)
Turn point of 18 years old	**21.8**	**78**	**0.2019**
< Turn point, effect1	0.0145 (0.0081, 0.0209)	0.0029 (0.0005, 0.0053)	1.6428 (0.3547, 2.9309)
> Turn point, effect2	0.0020 (0.0005, 0.0035)	0.0003 (-0.0004, 0.0010)	-0.0144 (-0.0718, 0.0429)
Turn point of 19 years old	**33.9**	**117**	**0.3562**
< Turn point, effect1	0.0076 (0.0056, 0.0096)	0.0019 (0.0011, 0.0027)	-0.2356 (-0.5011, 0.0300)
> Turn point, effect2	-0.0039 (-0.0079, 0.0002)	-0.0024 (-0.0052, 0.0004)	0.0186 (-0.0559, 0.0930)

Adjusted for all confounding factors.
The model is not adjusted for the stratification variable itself in the subgroup analysis.
BMI and WC VS total BMD. Left leg fat/lean VS left leg BMD.

decreased accordingly. As previously stated (21, 22)when the body fat rate is lower than 33%, the body fat content is positively correlated with bone density, reducing the risk of fractures to a certain extent, but when the body fat rate is higher than 33%, body fat content in most skeletal areas is inversely correlated with BMD. Our findings are in line with these conclusions.

Obesity status and OP have emerged as major public health issues that are receiving increased attention. Obesity status and OP, however, they are controversial subjects. According to a Tartu cross-sectional study (23), obese boys showed greater BMD values than their normal-weight classmates. In a similar study, the researchers evaluated the BMD of female teenagers and separated them into two groups: the fat group and the normal group. Significantly increased BMD was found in the obese group compared to the normal group (24). A meta-analysis and systematic review include 27 studies found that people who are overweight or obese have much higher BMD than people who are a healthy weight (25). Nevertheless, there is research that has produced results that are contradictory (26, 27). The WC is frequently used as an indicator of abdominal

TABLE 4 Association between waist circumference (cm) and bone mineral density (g/cm^2).

Exposure	Total BMDβ (95% CI)	Lumbar Spine BMD β (95% CI)	Left Leg BMD β (95% CI)
Stratified by quintiles of Waist circumference (cm)			
Q1 (54.6-71)	reference	reference	reference
Q2 (71.1-78.2)	0.0195 (0.0120, 0.0270)	0.0221 (0.0119, 0.0322)	0.0284 (0.0196, 0.0373)
Q3 (78.3-88.9)	0.0304 (0.0224, 0.0383)	0.0248 (0.0140, 0.0356)	0.0453 (0.0359, 0.0547)
Q4 (89-163.3)	0.0464 (0.0378, 0.0551)	0.0180 (0.0063, 0.0297)	0.0777 (0.0675, 0.0879)
P for trend	< 0.001	0.048	< 0.001
Stratified by gender			
Male	0.0010 (0.0007, 0.0013)	0.0006 (0.0002, 0.0010)	0.0017 (0.0013, 0.0021)
Female	0.0015 (0.0012, 0.0018)	0.0004 (-0.0001, 0.0008)	0.0028 (0.0025, 0.0032)
Stratified by age (years old)			
12	0.0006 (0.0002, 0.0011)	0.0002 (-0.0005, 0.0008)	0.0021 (0.0015, 0.0027)
13	0.0012 (0.0007, 0.0017)	0.0004 (-0.0002, 0.0010)	0.0021 (0.0015, 0.0027)
14	0.0011 (0.0006, 0.0017)	0.0011 (0.0003, 0.0018)	0.0018 (0.0011, 0.0025)
15	0.0016 (0.0010, 0.0022)	0.0010 (0.0002, 0.0018)	0.0021 (0.0014, 0.0028)
16	0.0014 (0.0008, 0.0019)	0.0008 (0.0001, 0.0015)	0.0023 (0.0017, 0.0030)
17	0.0014 (0.0008, 0.0020)	0.0008 (-0.0000, 0.0016)	0.0024 (0.0017, 0.0031)
18	0.0004 (-0.0001, 0.0009)	-0.0001 (-0.0008, 0.0006)	0.0015 (0.0010, 0.0021)
19	0.0007 (0.0001, 0.0013)	-0.0004 (-0.0012, 0.0004)	0.0014 (0.0007, 0.0021)
Stratified by Standing height (cm)			
Q1 (132.9-160.3)	0.0019 (0.0015, 0.0022)	0.0008 (0.0003, 0.0014)	0.0036 (0.0032, 0.0040)
Q2 (160.4-169)	0.0011 (0.0007, 0.0015)	0.0003 (-0.0002, 0.0008)	0.0019 (0.0015, 0.0024)
Q3 (169.1-190.9)	0.0013 (0.0009, 0.0016)	0.0008 (0.0003, 0.0013)	0.0019 (0.0014, 0.0023)
Stratified by Race			
Mexican American	0.0012 (0.0007, 0.0016)	0.0004 (-0.0002, 0.0009)	0.0022 (0.0016, 0.0027)
Other Hispanic	0.0017 (0.0011, 0.0024)	0.0016 (0.0006, 0.0025)	0.0029 (0.0022, 0.0037)
Non-Hispanic White	0.0012 (0.0008, 0.0016)	0.0002 (-0.0004, 0.0008)	0.0022 (0.0017, 0.0027)
Non-Hispanic Black	0.0010 (0.0005, 0.0014)	0.0006 (-0.0000, 0.0012)	0.0017 (0.0012, 0.0022)
Other race	0.0014 (0.0009, 0.0019)	0.0005 (-0.0001, 0.0012)	0.0018 (0.0013, 0.0024)

Adjusted for all confounding factors.
The model is not adjusted for the stratification variable itself in the subgroup analysis.

obesity. The linear regression analysis revealed a markedly adverse connection between WC and BMD in a sample of 271 adolescents, including those with and without metabolic syndrome (MS). Among the components of MS, the connection between increased WC and decreased BMD is the strongest (28). Although obesity benefits BMD, numerous studies show that having a high BMI significantly raises one's personal risk of developing type 2 diabetes, pre-diabetes, dyslipidemia, non-alcoholic fatty liver disease, and heart conditions, the underpinning mechanisms involved include inflammation, oxidative stress, and mitochondrial dysfunction (29–35). Faienza et al. (36) believe that these mechanisms of obesity can increase the possibility of osteoporosis and brittle fractures.

Presently, the mechanism between Obesity and OP is uncertain. There were multiple mechanisms may exist. to begin with, excess body fat deposition and significantly high obesity result in increased static mechanical compliance (37, 38), which causes static mechanical pressures on bone and a series of changes in bone structure. Secondly, obesity increases the number and metabolic rate of adipocytes in the bone marrow. The bone marrow has cells called bone mesenchymal stem cells (BMSCs). These cells can change into osteoblasts and adipocytes. Obesity can stimulate the development of BMSCs into adipocytes, a result of the increase of bone marrow adipocytes as well as a reduction of bone marrow osteoblasts (39). The inappropriate buildup of bone marrow adipocytes in the skeletal portion will lead to an imbalance in osteocyte activity and a reduction in bone turnover. It can easily result in the start of OP at an earlier age (40). Thirdly, obesity contributes to inflammation. The proliferation of adipocytes in the microenvironment of bone marrow will hasten the release of pro-inflammatory and immunoregulatory substances. In addition to accelerating the production and activation of osteoclasts, these inflammatory substances also limit the release of osteoprotegerin, diminish the differentiation of osteoblasts, and induce osteoclasts (41). Fourth, people who are overweight or obese have a higher synthesis and release of

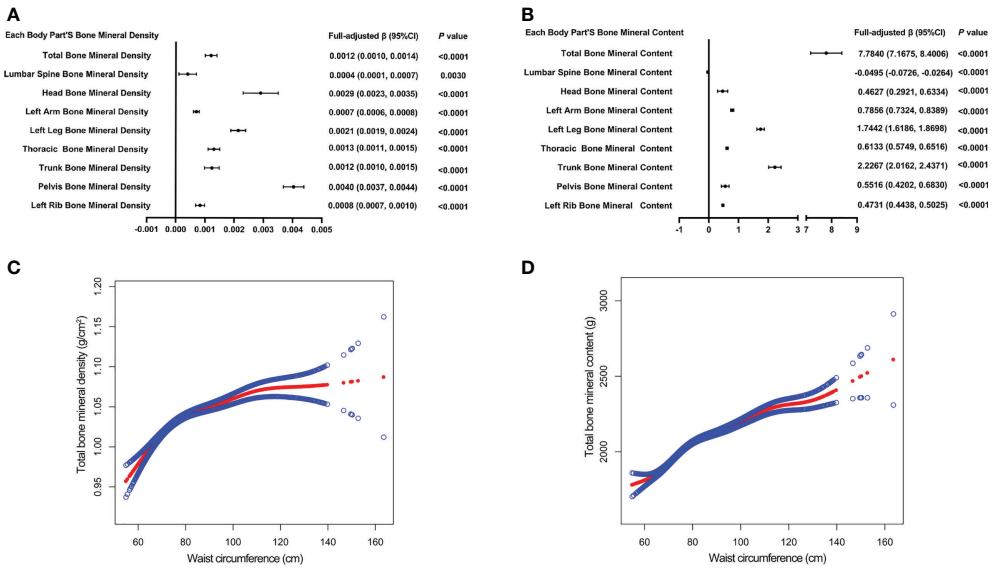

FIGURE 3
The forest plots of each body part's bone mineral density or bone mineral content and waist circumference, respectively **(A, B)**. Association of total bone mineral density and bone mineral content with waist circumference **(C, D)**. The solid red line represents the smooth curve fit between variables. Blue bands represent the 95% confidence interval from the fit **(C, D)**. All confounding factors were adjusted.

endocrine hormones, including estrogen, insulin, leptin, etc. These hormones inhibit bone resorption and bone remodeling and thus exert a beneficial influence on BMD (42–46). Fifth, obesity alters genes connected to obesity. For example, the Pro10 allele of tumor necrosis factor-1 (47), the leptin gene (48), and the fourth receptor gene for melanocorticoid (49). Studies show that these gene mutations make people more likely to be overweight and hurt their bones.

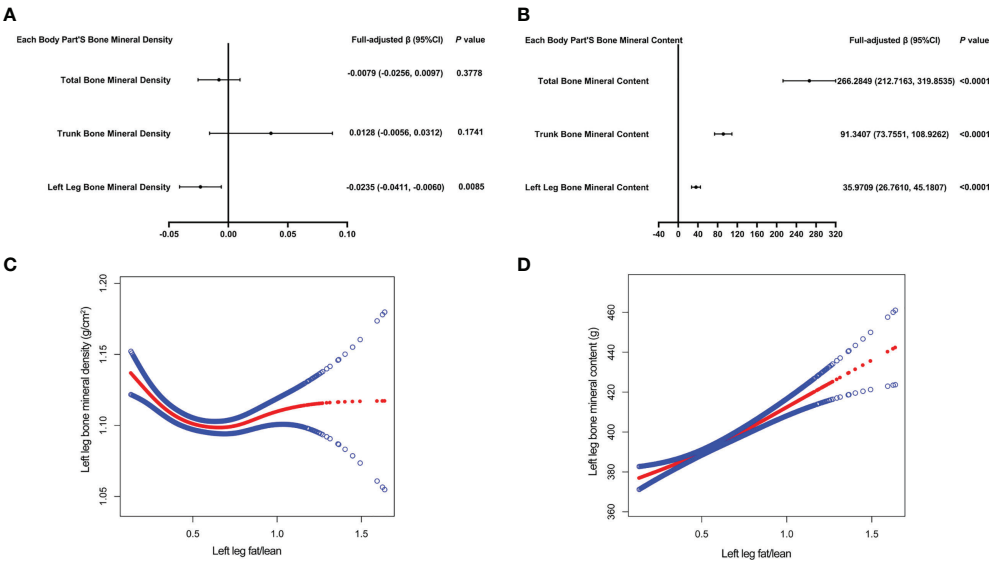

FIGURE 4
The forest plots of each body part's bone mineral density or bone mineral content and left leg fat/lean mass, respectively **(A, B)**. Association of left leg bone mineral density and bone mineral content with left leg fat/lean mass **(C, D)**. The solid red line represents the smooth curve fit between variables. Blue bands represent the 95% confidence interval from the fit **(C, D)**. All confounding factors were adjusted.

TABLE 5 Association between fat/lean mass and bone mineral density (g/cm^2) of corresponding parts of the body.

Exposure	Total, β (95% CI)	Trunk, β (95% CI)	Left Leg, β (95% CI)
Stratified by quintiles of fat/lean mass			
Lowest quartiles	reference	reference	reference
2nd	-0.0100 (-0.0180, -0.0021)	-0.0034 (-0.0117, 0.0049)	-0.0165 (-0.0260, -0.0070)
3rd	-0.0157 (-0.0243, -0.0071)	-0.0040 (-0.0130, 0.0049)	-0.0154 (-0.0260, -0.0047)
4th	-0.0092 (-0.0189, 0.0006)	0.0021 (-0.0080, 0.0122)	-0.0185 (-0.0302, -0.0069)
P for trend	0.053	0.683	0.009
Stratified by gender			
Male	-0.0440 (-0.0706, -0.0174)	-0.0306 (-0.0600, -0.0013)	-0.0493 (-0.0756, -0.0230)
Female	0.0289 (0.0047, 0.0531)	0.0510 (0.0276, 0.0745)	0.0174 (-0.0066, 0.0415)
Stratified by age (years old)			
12	-0.0500 (-0.0875, -0.0126)	-0.0279 (-0.0682, 0.0123)	-0.0241 (-0.0604, 0.0123)
13	0.0039 (-0.0378, 0.0456)	0.0157 (-0.0283, 0.0596)	-0.0144 (-0.0569, 0.0282)
14	0.0100 (-0.0392, 0.0593)	0.0383 (-0.0116, 0.0882)	-0.0242 (-0.0752, 0.0268)
15	-0.0040 (-0.0581, 0.0501)	0.0216 (-0.0324, 0.0756)	-0.0394 (-0.0925, 0.0137)
16	0.0394 (-0.0178, 0.0966)	0.1002 (0.0434, 0.1570)	-0.0246 (-0.0821, 0.0330)
17	0.0240 (-0.0341, 0.0820)	0.0084 (-0.0498, 0.0665)	0.0183 (-0.0391, 0.0756)
18	-0.0438 (-0.0990, 0.0115)	-0.0358 (-0.0947, 0.0231)	0.0016 (-0.0547, 0.0578)
19	0.0066 (-0.0577, 0.0710)	0.0193 (-0.0474, 0.0859)	-0.0100 (-0.0771, 0.0571)
Stratified by Standing height (cm)			
Q1 (132.9-160.3)	0.0120 (-0.0156, 0.0396)	0.0379 (0.0099, 0.0659)	0.0004 (-0.0270, 0.0278)
Q2 (160.4-169)	-0.0059 (-0.0365, 0.0247)	0.0197 (-0.0109, 0.0503)	-0.0304 (-0.0612, 0.0003)
Q3 (169.1-190.9)	-0.0096 (-0.0459, 0.0267)	-0.0048 (-0.0446, 0.0350)	-0.0192 (-0.0565, 0.0181)
Stratified by Race			
Mexican American	-0.0229 (-0.0617, 0.0158)	0.0049 (-0.0344, 0.0443)	-0.0271 (-0.0669, 0.0127)
Other Hispanic	0.0122 (-0.0436, 0.0680)	0.0527 (-0.0042, 0.1096)	-0.0052 (-0.0610, 0.0505)
Non-Hispanic White	-0.0125 (-0.0469, 0.0220)	0.0082 (-0.0279, 0.0442)	-0.0298 (-0.0633, 0.0037)
Non-Hispanic Black	0.0016 (-0.0358, 0.0391)	0.0095 (-0.0304, 0.0493)	-0.0054 (-0.0431, 0.0324)
Other race	-0.0155 (-0.0584, 0.0275)	0.0098 (-0.0348, 0.0545)	-0.0447 (-0.0875, -0.0020)

Adjusted for all confounding factors.
The model is not adjusted for the stratification variable itself in the subgroup analysis.

Nevertheless, we must equally acknowledge that our findings have many drawbacks. Because of the study's cross-sectional methodology, there could not be a causative connection established between lower BMD and being overweight or obese. Secondly, we could not gather full-scale data of participants regarding their living habits, eating habits, prescription data, bone metabolism indicators, and endocrine hormones that regulate bone metabolism. Our findings suggest that there are statistically significant differences between men and women in indicators such as moderate exercise, blood biochemical markers, and body composition, which may be partly explained by shifts in hormone levels during puberty, as well as differences in exercise patterns, venues, etc. It may lead to differences in sun exposure time, exposed parts, etc., thereby affecting calcium and phosphorus metabolism. Additionally, we were unable to gather menstrual histories from female participants. Finally, we could not identify participants with a history of fractures, osteoarthritis, premature infants, anorexia, etc. Previous studies have reported that these factors affect bone health during adolescence (50, 51). In addition, this study investigated the association between obesity status and BMD, which, although most important for bone health, does not 100% represent bone health, as Longhi et al. and Vibha et al. both found an increased risk of bone fractures in the extremities of adolescents with excess obesity compared with average weight, which may be related to reduced bone strength due to reduced cross-sectional and cortical areas of the skeleton (27, 52).

Our study included a sizable and geographically representative sample pool to draw from. Similar to what was reported in previous studies, our weighted multiple linear regression analysis demonstrated that obesity status was favorably associated with enhanced BMD. When we conducted smooth curve fitting in a model which adjusted for all the variables, we found that the effect sizes between BMI, WC, fat/lean mass, and BMD, respectively, were saturated. According to our findings, the total BMD reached saturation when the BMI of adolescents was 22kg/cm^2 and the WC was 70.5cm. At the same time, when fat/lean body mass < 0.2994, left leg BMD and

fat/lean mass were significantly negatively correlated, and we noticed that there was a correlation with age. As a result, we recommend that adolescents keep their BMI, WC, and fat/lean mass close to the saturation effect value in order to allows adolescents to have better bone mass development but not excessive obesity.

Data availability statement

Publicly available datasets were analyzed in this study. The survey data can be found here: https://www.cdc.gov/nchs/nhanes/.

Ethics statement

The studies involving human participants were reviewed and approved by The NHANES required every individual who took part in the survey to sign an informed consent form, which was then reviewed and authorized by the National Center for Health Statistics Ethics Review Board. The data can now be accessed by the general public following privacy-preserving. It is already possible to transform data into a form that can be analyzed. All statistics would be used for data analysis and all studies will be done in compliance with applicable laws and standards provided we comply with the study's data usage guidelines. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

G-XW and Z-BF contributed equally to this study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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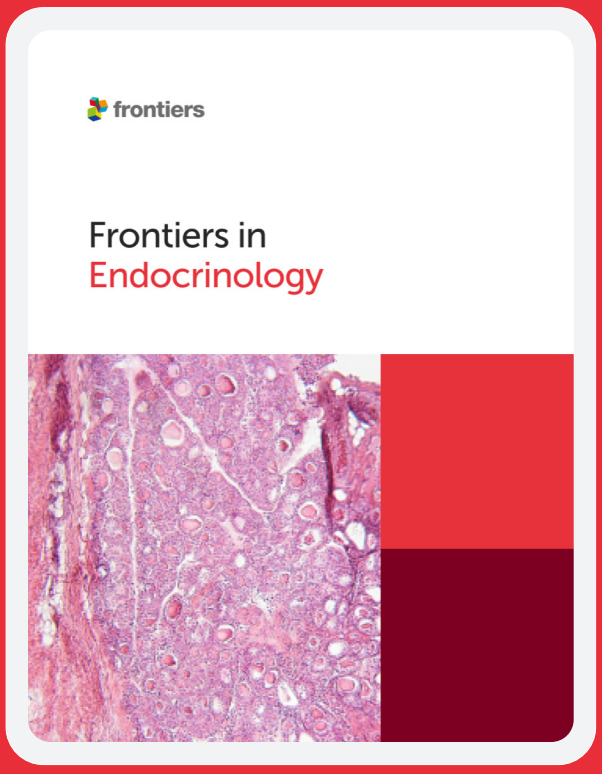

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