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ORIGINAL RESEARCH

Stanozolol Decreases Bone Turnover Markers, Increases Mineralization, and Alters Femoral Geometry in Male Rats

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Abstract Stanozonol (ST) is a synthetic derivative of testosterone; it has anabolic/androgenic activity, increasing both the turnover of trabecular bone and the endocortical apposition of bone. The present study aimed to examine the effects of ST on bone status in rats by bone mineral content, markers of formation and resorption, bone density, and structural and microarchitectural parameters. Twenty male Wistar rats were randomly distributed into two experimental groups corresponding to placebo or ST administration, which consisted of weekly intramuscular injections of 10 mg/kg body weight of ST. Plasma parameters were analyzed by immunoassay. Bone mineral content was determined by spectrophotometry. Bone mineral density (BMD) and structural parameters were measured by peripheral quantitative computed tomography, and trabecular and cortical microarchitecture by microcomputed tomography. Plasma Ca, Mg, and alkaline phosphatase were higher, and urinary Ca excretion, corticosterone, and testosterone concentrations lower in the ST group. Femur Ca content was higher and P content was lower in the ST, whereas osteocalcin, aminoterminal propeptides of type I procollagen, and C-terminal telopeptides of type I collagen were lower. Total cross-sectional, trabecular, and cortical/subcortical areas were lower in the ST. No differences were observed on BMD and area parameters of the diaphysis as well as on trabecular and cortical microarchitecture. The use of ST increases bone mineralization, ash percentage, and Ca and Mg content in femur. In spite of an absence of changes in BMD, geometric metaphyseal changes were observed. We conclude that ST alters bone geometry, leads to low bone turnover, and thus may impair bone quality.

Keywords Stanozolol · Bone properties · μCT · pQCT · Bone mineral density · Bone mineral content

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Introduction

Anabolic androgenic steroids (AAS) are widely used in both professional and recreational sports, and the potential health risks derived from this practice are of great scientific concern [1]. The potential effects of AAS in different tissues are modulated at the cellular level by tissue-specific steroid converting enzymes [2]. It is well established that androgens have effects on skeletal development [3] and on the maintenance of bone mass, stimulating bone formation and inhibiting bone resorption, likely through multiple mechanisms that involve both androgen and estrogen-mediated processes [2].

Anabolic androgenic steroid administration disturbs the regular endogenous production of testosterone and gonadotrophins that may persist for months after drug withdrawal [4]. AAS stimulate erythropoietin synthesis and red cell production as well as bone formation but counteract bone breakdown [4]. Androgens are important in the maintenance of a healthy skeleton. They have been shown to stimulate bone formation in the periosteum [2] but reduce formation on the endosteal surface in cortical and in trabecular bone [2]. Thus, androgens increase radial growth in cortical bone through periosteal apposition. Evidence suggests that androgens act directly on the osteoblasts, and it appears that exposure time is an important variable. The effect of androgens on osteoblasts has been shown to be biphasic, with enhancement following short or transient treatment but significant inhibition following longer treatment [5]. On the other hand, mounting evidence suggests that chronic androgen treatment increases neither osteoblast number nor viability in the mature bone compartment. It is tempting to speculate that, given the strong androgen-mediated stimulation at periosteal surface, the inhibitory action by androgen in osteoblasts at the endosteum is important for the maintenance of cortical width [2].

AAS have been used in the treatment of established osteoporosis in human patients, and it has been demonstrated that, at least in part, AAS increase the bone density by either stimulating bone formation or having anti-catabolic effects [6]. In rat models of bone loss from hindlimb unweighting, administration of either testosterone or the synthetic androgen nandrolone blocked most of the immobilization-related decrease in bone mineral density (BMD) [7].

Stanozolol is a synthetic non-aromatizable [8] derivative of testosterone; its properties include anabolic/androgenic activity [9], probably associated with its affinity for androgenic and, at lower doses, glucocorticoid receptors [10]. Since stanozolol is not converted to dihydrotestosterone, it might be less potent on the skeleton than testosterone. Stanozolol has been suggested to increase both, the turnover of trabecular bone and the endocortical apposition of bone [11]. The long-term use of stanozolol has shown its effectiveness in established postmenopausal osteoporosis increasing the net total bone mass above pretreatment levels [12].

Despite some species differences (e.g., the lack of osteonal cortical remodeling in rodents [13]), the extrapolation of rodent studies on bone metabolism and structure to humans is widely found in the literature [14]. Furthermore, the use of growing rodent experimental models is especially useful on bone metabolism, because years, not weeks, are required to assess BMD changes in humans.

Therefore, the present study aimed to examine the effects of AAS administration on bone status in rats. To the best of our knowledge, this is the first comprehensive study concomitantly analyzing the effects of AAS on bone status in rats by bone mineral content (BMC), specific bone markers of formation and resorption, parameters of bone density and structure by peripheral quantitative computed tomography (pQCT), and 3D outcomes for trabecular and cortical bone microarchitecture by micro-computed tomography (μCT).

Materials and Methods

Animals and Experimental Design

A total of 20 albino male Wistar rats were allocated into two groups ($n = 10$ each) divided into the AAS administration group or placebo group for an experimental period of 12 weeks. The animals, aged 8 weeks and with an initial body weight of 170 ± 8 g, were located in a well-ventilated thermostatically controlled room (21 ± 2 °C), with relative humidity ranging from 40 to 60 %. Throughout the experimental period, all rats had free access to distilled water and the animals consumed the diets ad libitum. Experimental diet (Table 1) was formulated to meet the nutrient requirements of rats based on the AIN-93M formulation described by Reeves et al. [15] but was slightly modified regarding to the protein source and content and the oil source (olive oil). The protein content was established at 10 % according to the American Institute of Nutrition (AIN-93M) [15]. Commercial soy protein isolate was used as the source of protein since this protein is widely available and used by athletes. One week prior to the experimental period, the rats were allowed to adapt to the diet and experimental conditions.

Body weight was measured weekly in all animals on the same day and hour and after a fasting period of 12 h, and the amount of food consumed by each rat was registered daily.

Table 1 Composition of the experimental diet

Nutritional composition (g/100 g DM)	Amount (g)
Soy protein supplement	13.1
Mineral mix (AIN-93M-MX)	3.5
Vitamin mix (AIN-93-VX)	1
Fat (olive oil)	4
Choline chloride	0.50
Cellulose	5
Starch	62.4
Methionine	0.5
Sucrose	10

DM dry matter

On day 74, a 12-h urine sample from each animal was collected for biochemical analysis. During these 12 h, located in the dark cycle, in which the animals are more active, water and food were removed in order to avoid interferences with urine collection. This period in the absence of any water being provided does not cause any effect of clinical dehydration, as demonstrated by Bekkevold et al. [16]. Urine volumes were recorded and samples were transferred into graduated centrifuge tubes for pH, Ca, and citrate analysis.

At the end of the experimental period, and after 8 h of fasting, the animals were anesthetized with ketamine-xylazine and sacrificed by cannulation of the abdominal aorta. Blood was collected (with heparin as anticoagulant) and centrifuged at 3500 rpm for 25 min to separate plasma, which was frozen in liquid N and stored at −80 °C for subsequent biochemical analysis. Femurs were defleshed. The left femur was fixed in formalin and stored in 70 % ethanol for BMD analysis, and the right femur was frozen in liquid N for femur ash analysis.

All experiments were undertaken according to Directional Guides Related to Animal Housing and Care [17], and all procedures were approved by the Animal Experimentation Ethics Committee of the University of Granada (Spain).

Anabolic Androgenic Steroids Administration

Following similar studies performed in rats, the animals received 10 mg/kg body weight of stanozolol once a week by intramuscular injection in the gluteus (alternating the lateral side each week). This dose is comparable to the dose that has been reported as being frequently used by athletes (600 mg/week or approximately 8 mg/Kg/week) [18]. We used a commercially available stanozolol solution of 50 mg/ml (Winstrol Depot, Desma Pharma group) that was diluted in saline solution to appropriate concentrations in order to maintain constant the volume of injection. The control group was injected with identical volumes of saline solution as placebo.

Chemical Analyses

Total nitrogen (N) content of the protein isolates and diets was determined according to Kjeldahl's method. Crude protein was calculated as N × 6.25. Femurs were weighed after drying them. Bone ash was prepared by calcination at 500 °C to a constant weight. Ca, Mg, and Zn content in bone were determined by atomic absorption spectrophotometry using a Perkin Elmer Analyst 300 spectrophotometer (Perkin Elmer, Wellesley, MA, USA). Analytical results were validated by standard references certified reference material (CRM)-189 (wholemeal starch; Community Bureau of Reference, Geel, Belgium). P content in bone was determined using the methodology described by Chen et al. [19].

Urinary pH was analyzed using a bench pH-meter (Crison, Barcelona, Spain). Urinary citrate was measured using a commercial kit (Spinreact, S.A. Gerona, Spain). Plasma urea, Ca, and alkaline phosphatase were measured using an autoanalyzer (Hitachi-Roche p800, F. Hoffmann-La Roche Ltd. Switzerland).

Plasma testosterone concentrations were measured in a subsample by radioimmunoassay using a commercially available TESTO-CTK I-125 Kit (Dia Sorin, Italy) without modification. All samples were assayed in duplicate and in the same assay. The intra-assay coefficient of variation was 5.1 %, and the sensitivity was 0.02 ng/mL.

Bone Turnover Markers

Osteocalcin was determined in the Rat-MID™ Osteocalcin enzymeimmunoassay (Immunodiagnostics System Ltd, Boldon, UK) from serum samples. The absorbencies of the stopped reactions were read at 450 nm using a Bio-Rad microplate reader (Bio-Rad Laboratories Inc., California, USA). The absorbance level is inversely related to the concentration of osteocalcin in the sample.

Aminoterminal propeptides of type I procollagen (PINP) were determined in the Rat/Mouse PINP enzymeimmunoassay (Immunodiagnostics System Ltd, Boldon, UK) from serum samples. The absorbances of the stopped reactions were read at 450 nm using a Bio-Rad microplate reader (Bio-Rad Laboratories Inc., California, USA). Color intensity developed was inversely proportional to the concentration of PINP.

Degradation products from C-terminal telopeptides of type I collagen were measured using a RatLaps™ enzymeimmunoassay (Immunodiagnostics System Ltd, Boldon, UK) from serum samples. The absorbance was read using a Bio-Rad microplate reader (Bio-Rad Laboratories Inc., California, USA), which is inversely related to concentration of RatLaps antigens in the sample.

Tartrate-resistant acid phosphatase (TRACP 5b) was measured in rat serum using the RatTRAP™ assay (Immunodiagnostics System Ltd., Boldon, UK) which uses a highly characterized, specific monoclonal antibody prepared using baculovirus generated recombinant rat TRACP as antigen [20]. The absorbance of the reactions was read at 405 nm in a microplate reader (Bio-Rad Laboratories Inc., California, USA). Color intensity was directly proportional to the activity of TRACP 5b present in the sample.

Positive controls for the analyzed bone turnover biomarkers (osteocalcin, PINP, C-terminal telopeptides of type I collagen and TRACP 5b) were assayed together with samples, and their concentration was found to be within the manufactureŕs quality control concentration limits.

Bone Mineral Density and Structure Measurements

Volumetric BMD of the left femur was measured by pQCT using a XCT Research M + pQCT machine (Stratec Medizintechnik, Pforzheim, Germany) as described Schneider et al. [21]. One slice (0.2 mm thick) in the mid-diaphysis of the femur as a cortical bone site, and 3 slices in the distal femoral metaphysis located 1.5, 2, and 2.5 mm proximal to the articular surface of the knee joint as a site rich in trabecular bone were measured. BMD values of the distal femoral metaphysis were calculated as the mean over three slices. A voxel size of 0.070 mm and a threshold of 710 mg/cm^3 were used for calculation of cortical BMD. Trabecular BMD was calculated using a threshold of 450 mg/cm^3.

µCT Analyses of Selected Bone Sites

Bone microarchitecture parameters of the distal femora were analyzed by µCT using a µCT-35 device (Scanco Medical AG, Switzerland). The micro-tomographic imaging system was equipped with a 0.5 mm focal spot X-ray tube as a source. The long axis of the biopsies was oriented along the rotation axis of the scanner. The X-ray tube was operated at 55 kVp with an intensity of 145 µA. Scan parameters were set with a voxel size of 3.5 µm and a 7.2 mm field of view resulting in, at best, a 5.2 µm spatial resolution at 10 % modulation transfer function (manufacturer specifications). Importantly, voltage, current, and scan parameters were adjusted to balance adequate contrast. A total of 345 slices in the mid-diaphysis (midshaft) were obtained as a cortical bone site located by selecting the reference line in the midshaft (50 % of the length of the femur) and then analyzed 4.14 mm distal. To get the trabecular parameters in the metaphysis, 231 slices were obtained by selecting the knee joint as a start point (reference line) and calculating the relative position of the first slice to the reference line at 1.5 mm, and then analyzed for 1.39 mm distal.

Statistical Analysis

The results are presented as mean and standard error of the mean. A homogeneity test was employed to determine equal or unequal variance. Differences between placebo and AAS groups were analyzed using the Student's t test with final body weight, food intake, urine, plasma, and bone parameters as dependent variables. All analyses were conducted using the Statistical Package for Social Sciences (SPSS, version 19.0 for Windows; SPSS Inc., Chicago, IL), and the level of statistical significance was set at $P \leq 0.05$.

Results

Body Weight, Plasma, and Urinary Parameters

The effect of AAS on final body weight, plasma (biochemical and hormone levels), and urinary parameters is presented in Table 2. Final body weight was a 6 % lower and the urinary Ca excretion was a 37 % lower in the AAS-administered group (both, $P \leq 0.01$). Plasma Ca, Mg, and alkaline phosphatase were higher (22, 32, and 99 %, respectively), and corticosterone and testosterone concentrations were lower in the AAS-administered group (−26 and −37 %, respectively) (all, $P \leq 0.05$).

Femur Dry and Ash Weight, Length, Mineral Content, and Bone Turnover Markers

The effect of the AAS on femur dry, ash weight, length of femur, BMC, and turnover markers is presented in Fig. 1 and Table 3, respectively. Femur Ca content was a 7 % higher, and P content a 5 % lower in the AAS-administered group (both, $P \leq 0.01$), whereas osteocalcin, PINP and C-terminal telopeptides of type I collagen were lower (−34, −53, and −34 %, respectively) (all, $P \leq 0.001$).

Bone Density Parameters and Area of Femur Metaphysis and Diaphysis

The effect of AAS on parameters of femur metaphysis density and area is presented in Fig. 2 and Table 4, respectively. Total cross-sectional, trabecular, and cortical/subcortical areas were lower in the AAS-administered group (−5, −6, and −5 %, respectively) (all, $P \leq 0.05$).

The effect of AAS on parameters of femur diaphysis density and area is presented in Table 4. No significant differences between groups were observed.

Trabecular and Cortical Bone Microarchitecture

The effect of AAS on trabecular and cortical bone microarchitecture in femur metaphysis and diaphysis is presented in Fig. 3 and Table 5, respectively. No differences between groups were observed in femur trabecular and cortical bone (all, $P > 0.05$).

Discussion

The main findings of this study were (i) AAS administration increased bone mineralization and led to higher ashes percentage and Ca and Mg content. Although no changes in BMD were observed, geometric metaphyseal changes, such

Table 2 Effects of anabolic androgenic steroids (AAS) on final body weight, plasma, and urinary parameters

	Placebo	AAS	%	*P*
Final body weight (g)	336.1 (4.4)	317.0 (4.0)	−5.7	0.002
Food intake (g/day)	16.3 (0.2)	14.8 (0.3)	−9.2	≤0.001
Testicles weight (g)	1.6 (0.03)	1.1 (0.02)	−34.8	≤0.001
Plasma parameters				
Ca (mg/dL)	10.3 (0.7)	12.5 (0.6)	21.9	0.011
Mg (mg/dL)	2.1 (0.2)	2.8 (0.3)	31.8	0.029
P (mg/dL)	6.4 (0.3)	6.9 (0.3)	8.0	0.214
Alkaline phosphatase (UI/L)	74.5 (4.5)	148.2 (5.6)	99.0	≤0.001
Urea (mg/dL)	31.9 (1.4)	30.3 (1.2)	−5.0	0.303
Serum total proteins (g/dL)	5.5 (0.08)	5.4 (0.05)	−2.9	0.084
Corticosterone (ng/mL)	941.9 (26.8)	693.3 (43.2)	−26.4	≤0.001
Testosterone (ng/mL)	2.3 (0.2)	1.4 (0.4)	−37.4	0.050
Urinary parameters				
Ca (mg/L)	3.4 (0.9)	2.1 (0.3)	−37.2	≤0.001
Ca (mg/day)	1.0 (0.10)	0.6 (0.05)	−46.1	0.003
Citrate (g/L)	1.3 (0.5)	1.5 (0.5)	18.3	0.351
pH	6.5 (0.17)	6.6 (0.15)	1.4	0.250

Values expressed as mean (standard error of the mean)

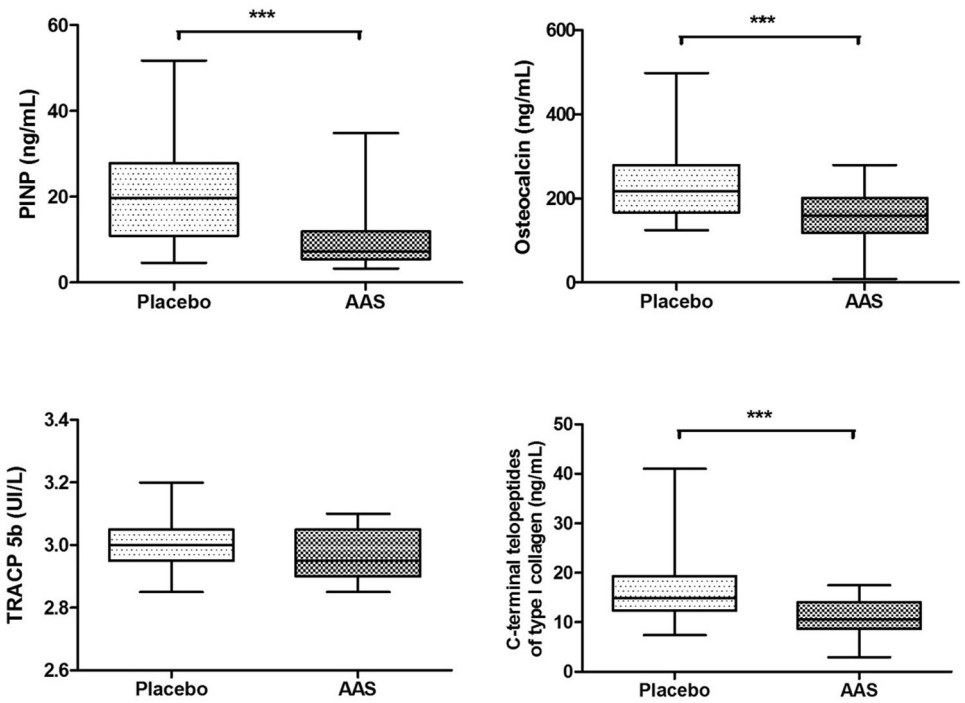

Fig. 1 Effect of anabolic androgenic steroids (AAS) on specific bone turnover markers. PINP, amino terminal propeptides of type I procollagen; TRACP 5b, tartrate-resistant acid phosphatase ***$P \leq 0.001$

as a smaller total cross-sectional, trabecular, and cortical/subcortical areas, were found in the AAS-administered group. (ii) AAS were also associated with a lower rate of bone turnover.

The administration of AAS reduced food intake and led to a smaller increase of body weight, which concurs with other authors [22, 23]. AAS promoted higher levels of Ca and Mg in plasma, a reduction of the urinary Ca excretion, which contributed to the retention of water [4, 24]. On the other hand, in the study performed by Benéton et al. [11], the treatment with a higher dose of stanozolol (5 mg/day for 1 year) was associated with no changes in the plasma concentrations of Ca and phosphate. The high concentration of minerals in plasma may have promoted bone

Table 3 Effects of anabolic androgenic steroids (AAS) on bone mineral content

	Placebo	AAS	%	*P*
Femur length (cm)	3.5 (0.04)	3.5 (0.03)	−0.3	0.188
Femur dry weight (g)	0.5763 (0.0073)	0.5595 (0.0057)	−2.9	0.069
Femur ash weight (g)	0.3736 (0.0049)	0.3725 (0.0040)	−0.3	0.801
Bone mineral content				
Ca (mg/g dry femur)	224.5 (3.0)	239.3 (1.9)	6.6	≤0.001
Ca (mg/g ash)	346.1 (4.3)	361.3 (3.0)	4.4	0.004
Mg (mg/g dry femur)	4.4 (0.09)	4.8 (0.06)	7.2	0.004
Mg (mg/g ash)	6.9 (0.14)	7.2 (0.10)	4.7	0.064
P (mg/g dry femur)	110.9 (3.0)	105.1 (1.8)	−5.3	0.008
P (mg/g ash)	171.2 (4.7)	158.2 (2.8)	−7.6	0.017
Zn (μg/g dry femur)	311.8 (10.6)	312.8 (6.7)	0.3	0.485
Zn (μg/g ash)	481.9 (17.1)	470.5 (10.0)	−2.4	0.164

Values expressed as mean (standard error of the mean)

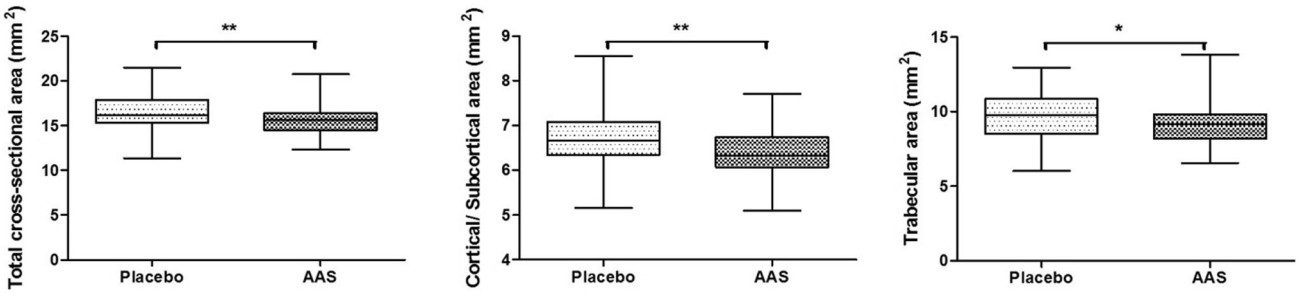

Fig. 2 Effect of anabolic androgenic steroids (AAS) on bone parameters of the metaphysis $*P \leq 0.05$, $**P \leq 0.01$

Table 4 Effects of anabolic androgenic steroids (AAS) on selected pQCT parameters of bone density and area in femur (metaphysis and diaphysis)

	Placebo	AAS	%	*P*
Metaphysis				
Total BMD metaphysis (mg/cm^3)	594.0 (5.2)	603.9 (5.6)	1.7	0.101
Cortical/subcortical BMD (mg/cm^3)	1062.8 (4.3)	1067.1 (4.4)	0.4	0.341
Trabecular BMD (mg/cm^3)	242.9 (7.8)	255.7 (7.1)	5.3	0.208
Diaphysis				
Total BMD diaphysis (mg/cm^3)	895.4 (5.6)	905.3 (6.4)	1.1	0.256
Total cross-sectional area (mm^2)	9.8 (0.13)	9.7 (0.08)	−1.6	0.279
Cortical BMD (mg/cm^3)	1388.5 (1.4)	1389.6 (1.7)	0.1	0.517
Cortical area (mm^2)	6.02 (0.07)	5.95 (0.05)	−1.2	0.440
Cortical thickness (mm)	0.67 (0.005)	0.67 (0.004)	0.0	0.926
Periosteal perimeter (mm)	11.09 (0.07)	11.01 (0.05)	−0.7	0.318
Endocortical perimeter (mm)	6.9 (0.07)	6.8 (0.05)	−1.0	0.315

Values expressed as mean (standard error of the mean). BMD, bone mineral density. Additional parameters are shown in Fig. 2

mineralization [25]. Femurs had a higher content of absolute values of Ca and Mg but, possibly due to the decreased P content, it was not reflected in an increased of volumetric BMD. However, our results assessed by the pQCT indicate that, although there were no changes on femur length, we observed alterations in the metaphysis, but not in the diaphysis. The differences on metaphyseal geometry (lower total cross-sectional area, trabecular area, and cortical/subcortical area) potentially could result in an alteration of the mechanical properties of bone [26–28].

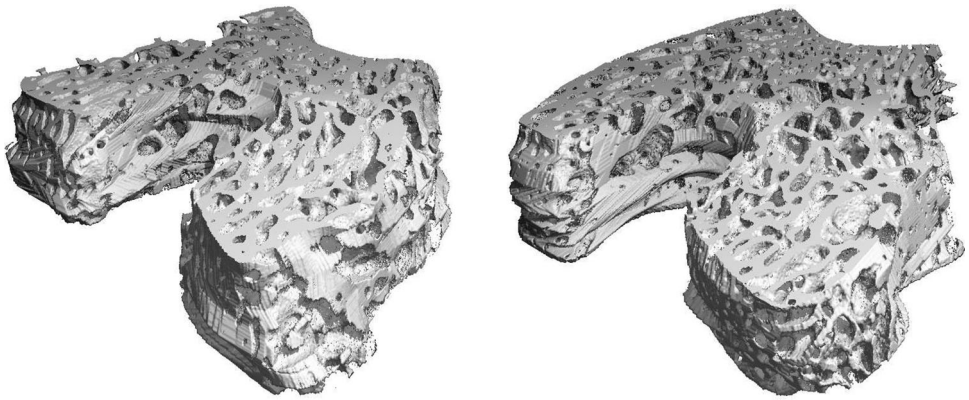

Fig. 3 Representative microCT images of placebo **a** and anabolic androgenic steroids (AAS) **b** of bone metaphysis

Table 5 Effects of anabolic androgenic steroids (AAS) on 3D outcomes for trabecular and cortical bone microarchitecture

	Placebo	AAS	%	*P*
Metaphysis (trabecular)				
TV (mm^3)	28.5 (0.5)	29.3 (0.4)	2.9	0.194
BV (mm^3)	11.8 (0.2)	12.0 (0.2)	1.6	0.538
BV/TV (1)	0.4 (0.006)	0.4 (0.005)	−2.4	0.402
Conn. D ($1/mm^3$)	541.5 (53.2)	441.2 (22.1)	−18.5	0.088
SMI (1)	3.3 (0.3)	3.3 (0.2)	−0.6	0.955
Tb.N (1/mm)	11.0 (0.5)	10.6 (0.3)	−2.9	0.566
Tb.Th (mm)	0.087 (0.001)	0.086 (0.0006)	−1.2	0.236
Tb.Sp (mm)	0.111 (0.007)	0.110 (0.004)	−0.9	0.871
Mean density of TV (mg HA/ccm)	380.9 (7.7)	375.6 (5.2)	−1.4	0.558
Mean density of BV (mg HA/ccm)	866.0 (4.5)	869.1 (3.3)	0.4	0.579
Diaphysis (cortical)				
TV (mm^3)	40.6 (0.7)	40.0 (0.4)	−1.6	0.453
BV (mm^3)	26.7 (0.5)	26.6 (0.3)	−0.7	0.761
BV/TV (1)	0.66 (0.004)	0.66 (0.005)	0.0	0.383
Mean density of TV (mg HA/ccm)	705.3 (4.9)	712.9 (5.7)	1.1	0.312
Mean density of BV (mg HA/ccm)	1066.3 (1.4)	1069.7 (1.8)	0.3	0.129

Values expressed as mean (standard error of the mean)

TV total volume, *BV* bone volume, *BV/TV* bone volume density, *Conn. D* connectivity density, *SMI* structure model index, *Tb.N* trabecular number, *Tb.Th* trabecular thickness, *Tb.Sp* trabecular spacing, *HA* hydroxyapatite

Furthermore, our results obtained by μCT indicated that there were no modifications in the bone volume at the metaphysis level. In contrast to our findings in intact young male rats, in the study performed by Benéton et al. [11] in patients with osteoporosis, stanozolol increased both the turnover of trabecular bone and the endocortical apposition of bone. Stanozolol could prevent the decrease of the BMD and biomechanical properties of the femur and the 5th lumbar vertebra in osteoporotic rats [29].

AAS have been shown to promote absorption of Ca from intestine and increase BMC in women [30], stimulate endosteal bone formation in both humans [31] and animals [32], and block bone loss by inhibition of bone resorption in ovariectomized rats with osteopenia [33].

Under our experimental conditions, the administration of stanozolol increased the levels of alkaline phosphatase. This enzyme is a non-specific indicator of bone formation [34]. Our results are in agreement with previous findings [5, 35]. However, stanozolol also induced a decrease in two specific bone formation markers (osteocalcin and PINP) and the specific resorption marker (C-terminal telopeptides of type I collagen), which reflects a general inhibition of bone turnover, as described by several authors [36, 37] for another AAS, nandrolone decanoate. Aerssens et al. [36] concluded that nandrolone has the unique ability to decrease bone turnover, characterize by decreased serum and bone osteocalcin levels, and to stimulate some aspects of osteoblast activity, expressed by high serum alkaline

phosphatase levels, and increased IGF-I concentrations in the bone matrix.

The reduction in bone turnover markers in this growing rat model transitioning from rapid growth to skeletal maturity supports the concept that there is an alteration in the levels of these formation and resorption markers throughout the life span of the animals. According to Baron et al. [38], large variations occur during skeletal maturation. It should be considered that rats were young adults when they were sacrificed [39]. This could explain that after the postpubertal stage, which is characterized by highly increased bone turnover markers [40], a pronounced decrease occurs in a later stage of development, but we observed this decrease in our rats even when they had not yet concluded the adult/skeletal maturation phase. This finding supports the fact that stanozolol could have accelerated skeletal maturation and thereby decreased bone turnover markers [38].

We must also consider the treatment duration. Indeed, the effect of AAS on osteoblasts is biphasic: its activity is increased when the treatment is short term and inhibited in prolonged treatments [32] (as in our study). This inhibition of osteoblastic activity is reflected by the drop of the specific markers of osteoblasts formation and the alteration of metaphyseal geometry.

Huang et al. [41] found that bones of ovariectomized monkeys showed a lower total carbonate content than controls. Moreover, they observed that the ovariectomized animals had higher bone acid phosphate content than controls (+12 %). Because the overall carbonate/matrix ratio increased with nandrolone treatment, this observation suggests that nandrolone treatment results in an increased formation of apatite with carbonate occupying the phosphate position of hydroxyapatite. Our results are in agreement with the study of Huang et al. [41] since we observed an increase of the Ca/P ratio and a decrease in the total bone P content. On the other hand, Burnell et al. [42] found a decreased Ca/P ratio and increased Mg content in iliac crest biopsies from postmenopausal osteoporotic females.

Plasma levels of AAS were high enough to block the production of FSH and LH to bind to the receptors of the hypothalamic-pituitary axis [43, 44]. We found a decrease in plasma testosterone levels, and testicular atrophy, according to Spitz et al. [45]. Moreover, we observed that AAS administration reduced plasma corticosterone levels probably due to the inhibition of the hypothalamic-pituitary axis which occurs in long-term treatments with nandrolone, as described by Schlussman et al. [46]. Furthermore, according to Alsio et al. [47], there is a general inhibition of the enzymes involved in the synthesis of corticosterone. The changes in the levels of testosterone and corticosterone lead to a lower corticosterone/testosterone ratio in rats injected with AAS, which explains the main effects of the steroid.

The effect of AAS in decreasing urinary Ca excretion likely contributed to bone mineralization. In agreement with our results in intact young male rats, Benéton et al. [11] observed a decrease in the fasting urinary excretion of Ca in patients with osteoporosis treated with a higher dose of stanozolol.

The results of our investigation in rodents may be of relevance for the understanding of adverse effects of androgenic steroids abuse on bone health in humans. Probably, as a consequence of low bone turnover, metaphyseal dimensions were decreased in the treated rats. When extrapolating these findings to humans, we would predict impaired bone growth in adolescents with steroids abuse. Moreover, we should need to emphasize that in several pathologic conditions (e.g., glucocorticoid-induced osteoporosis or adynamic renal bone disease) low bone remodeling is associated with poor bone quality and high risk of fractures [48, 49].

Limitation and Strengths

The present study has some limitations that need to be mentioned. First, the current physiological results obtained in growing rodents must be confirmed in young adult human subjects. Second, an alternative approach to assess bone remodeling might have been the use of dynamic histomorphometry. Third, only one dose of stanozolol was employed since a similar dose has been reported to be frequently used by athletes [18]. Mechanistic studies were beyond the scope of the current manuscript. Since stanozolol binds to the androgen receptor, it is generally believed that the actions of stanozolol on bone are androgen receptor mediated. On the other hand, we employed several specific and independent markers of bone formation and resorption. Moreover, bone parameters were analyzed with pQCT and μCT devices, which are the most advanced and innovative techniques nowadays, and we have described a large number of structural parameters. The μCT method provides a 3D outcome to simultaneously investigate the bone structure as well as the degree of mineralization in a non-destructive manner using a high resolution. It is important to highlight the great importance of the pQCT and μCT devices together with the different methodological approaches used to determine the bone markers and the structural properties of the bone. A further important advantage of the present study is that mineral content or the exact amount of Ca, P, Mg, and Zn present in the samples was measured directly. Furthermore, it should be emphasized that our study for the first time found an alteration in the geometry of the femur metaphysis and in the total bone P content after treatment with AAS.

Conclusion

An anabolic steroid has significant effects on bone metabolism in male rats. Specifically, stanozolol alters the metaphyseal geometry and mineralization of the bone, leads to low bone turnover, and thus may impair bone quality.

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Authors' Contributions EN was involved organizing this research, the acquisition of data, analysis and interpretation of data, and drafting the manuscript. She is guarantor. VAA was involved in the conception, planning, and designing this study, and the acquisition, analysis, and interpretation of data. DC was involved in the acquisition of data. RM was involved in the acquisition of data. RGE was involved in the analysis and interpretation of data. GK was involved in the acquisition of data. CS was involved in the analysis and interpretation of data. CT was involved in planning and designing this study. JMP was involved in the analysis, the acquisition and interpretation of data, and revising the manuscript. MLJ was involved in the conception, planning, and designing this study, and drafting and revising the manuscript. PA was involved in the conception, planning, and designing this study, and the acquisition, analysis, and interpretation of data. PP was involved in the analysis and interpretation of the data and revising the manuscript. All authors revised the paper critically for intellectual content and approved the final version. All authors agree to be accountable for the work and to ensure that any questions relating to the accuracy and integrity of the paper are investigated and properly resolved.

Compliance with Ethical Standards

Conflict of Interest E. Nebot, V. A. Aparicio, D. Camiletti-Moirón, R. Martinez, R. G. Erben, G. Kapravelou, C. Sánchez-González, C. De Teresa, J. M. Porres, M. López-Jurado, P. Aranda, P. Pietschmann declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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