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Full title: Early effects of androgen deprivation on bone and mineral homeostasis in adult men: a prospective cohort study

Short title: Androgen deprivation and mineral homeostasis

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ABSTRACT

Objective: Long-term androgen deprivation therapy (ADT) negatively influences bone. The short-term effects on bone and mineral homeostasis are less known. Therefore, we aimed to investigate the early effects of ADT on calcium/phosphate homeostasis and bone turnover.

Design: Prospective cohort study

Methods: Eugonadal adult male sex offenders, who were referred for ADT to the endocrine outpatient clinic, received cyproterone acetate. Changes in blood markers of calcium/phosphate homeostasis and bone turnover between baseline and first follow-up visit were studied.

Results: Of 26 screened patients, 17 were included. The median age was 44 (range 20-75) years. The median time interval between baseline and first follow-up was 13 (6-27) weeks. Compared to baseline, an 81% decrease was observed for median total testosterone (to 3.4 nmol/L (0.4-12.2); P<0.0001) and free testosterone (to 0.06 nmol/L (0.01-0.18); P<0.0001). Median total estradiol decreased 71% (to 17.6 pmol/L (4.7-35.6); P<0.0001). Increased serum calcium (P<0.0001) and phosphate (P=0.0016) was observed, paralleled by decreased PTH (P=0.0156) and 1,25-dihydroxyvitamin D_3 (P=0.0134). The stable calcium isotope ratio ($\delta^{44/42}Ca$) decreased (P=0.0458), indicating net calcium loss from bone. Bone-specific alkaline phosphatase and osteocalcin decreased (P<0.0001 and P=0.0056, respectively), periostin tended to decrease (P=0.0500) whereas sclerostin increased (P<0.0001), indicating suppressed bone formation. Serum bone resorption markers (TRAcP5b, CTX) were unaltered.

Conclusions: In adult men, calcium release from the skeleton occurs early following sex steroid deprivation, reflecting early bone resorption. The increase of sclerostin and reduction of bone formation markers, without changes in resorption markers, suggests a dominant negative effect on bone formation in the acute phase.

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INTRODUCTION

In men, testosterone (T) and estradiol (E2) are not only important regulators of bone mass acquisition during puberty; they are also essential for the maintenance of adult bone (1). It is well known that male hypogonadism is associated with bone loss. Accordingly, in prostate cancer patients androgen deprivation therapy (ADT) induces bone loss, thereby increasing the risk for osteoporosis and fractures (2–9). However, in case of metastatic prostate cancer the impact of ADT *per se* on bone homeostasis could be obscured. ADT is also used in male sex offenders with paraphilic or hypersexual disorder (10), either by using androgen receptor (AR) antagonists or GnRH agonists or antagonists. In contrast to prostate cancer patients, this patient group is generally younger, has less comorbidities, and is expected to need long-term treatment with ADT (11,12). However, research on adverse health effects of ADT is limited in these patients and only a few studies included data on bone health (3–6,8). Moreover, while the long-term effects of androgen deprivation on bone have been extensively investigated, mainly in prostate cancer patients, studies on the short-term impact of changes in sex steroids on bone and mineral homeostasis are limited.

In patients treated with ADT, serum bone turnover markers (BTMs) can be used to detect changes in bone homeostasis, thereby enabling the assessment of both bone formation and resorption. Classical formation markers include N-terminal propeptide of type 1 collagen (P1NP), which is released during formation of type I collagen; bone alkaline phosphatase (BAP), a protein found on the surface of osteoblasts reflecting their activity; and osteocalcin, produced by osteoblasts and incorporated into the bone matrix. Bone resorption markers include C-or N-terminal telopeptide of type I collagen (CTX or NTX), degradation products of type I collagen; and tartrate-resistant acid phosphatase 5b (TRAcP5b), an enzyme produced by osteoclasts that reflects their number (13,14). These BTMs are blood-based and relatively easy to measure, and can therefore be repeatedly assessed in the same patient (13). However, BTMs have disadvantages as well. They can be affected by sex, age, body

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weight, circadian rhythm, food intake, exercise, renal or liver function, recent bone fracture and lifestyle (13).

Several studies have shown that long-term sex steroid deprivation with GnRH agonists in adult men increases both bone resorption and formation markers, by stimulating bone remodeling, with a dominant effect on resorption, leading to a negative bone mineral balance (15,16). In the acute phase of ADT, however, the group of Khosla revealed decreased osteocalcin and P1NP levels 3 weeks following GnRH agonist treatment combined with an aromatase inhibitor, suggesting suppressed bone formation. Furthermore, supplementation with either T and/or E2 showed that estradiol is mainly involved in the suppression of bone resorption, while testosterone mainly stimulates bone formation (17).

In this study, we aimed to better understand the early effects of ADT on bone and mineral homeostasis. Therefore, we studied the serum calcium/phosphate response, and the change in calciotropic hormones and bone turnover markers in a prospective cohort of adult male sex offenders referred for initiation of ADT.

We hypothesized that in the early phase of androgen deprivation bone resorption is increased whereas bone formation is reduced, causing a negative bone balance, which subsequently influences mineral metabolism.

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MATERIALS AND METHODS

Participants and study design

Between July 2012 and June 2016, male sex offenders aged ≥ 18 years were asked to participate in an observational study at the University Hospitals Leuven, Belgium, department of endocrinology. All men were referred by their treating psychiatrist for medical evaluation before and during ADT as adjuvant therapy to psychotherapy. ADT consisted of daily oral intake of cyproterone acetate (CPA, 50 mg/d), in line with the guidelines for management of male sex offenders (18,19). CPA is an AR antagonist that also functions as a progestagen, thus also decreasing LH and FSH and suppressing endogenous sex steroid production.

The following clinical and biochemical data were extracted from the medical files: height, weight, smoking status, history of diabetes mellitus, albumin, hemoglobin, renal function (eGFR, cystatin C), liver function (alkaline phosphatase (ALP); aspartate aminotransferase (AST); alanine transaminase (ALT); gamma-glutamyl transferase (GGT)), and maintenance therapy. Patients under bone anti-resorptive treatment or medication interfering with sex steroid metabolism or calcium/phosphate homeostasis, except for calcium and/or vitamin D supplements, were excluded. Men with hypogonadism prior to the start of ADT (defined by a total T ≤7 nmol/L, measured between 8 AM and 10 AM) were also excluded, as well as men without decrease in testosterone under CPA treatment, suggestive for non-compliance. As shown in figure 1, 26 participants were screened and 17 participants were eligible for final analysis. The ethical committee of the University Hospitals Leuven approved the study (ClinicalTrials.gov number NCT02434562). All participants gave written informed consent.

Blood sampling and laboratory measurements

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A morning blood sample was taken prior to treatment initiation and at first clinical follow-up 2-4 months post-initiation as part of standard clinical care. All measurements were performed in single-assay runs.

Gonadal axis

Total T and total E2 were measured by liquid chromatography-tandem mass spectrometry as previously described (20,21). SHBG was measured using the Modular E170 platform electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany). Free T was calculated using the Vermeulen formula (22). LH and FSH were assessed by an electrochemiluminescence immunoassay (HITACHI/Roche - COBAS 8000).

Calcium and phosphate homeostasis

Calcium, phosphate, and albumin were measured on a HITACHI/Roche - COBAS c702. Calcium was corrected for albumin using the following formula: serum calcium + 0.8 * (4 - serum albumin). 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 were measured by liquid chromatography-tandem mass spectrometry (23,24). Vitamin D-binding protein (DBP) was measured by single radial immunodiffusion, as previously described (25). Free vitamin D was calculated using the Vermeulen formula (22). Third generation PTH and intact fibroblast growth factor 23 (FGF23) were measured with the Liaison automate (Diasorin, Saluggia, Italy).

Bone turnover markers

BAP, osteocalcin, P1NP, and TRAcP5b were measured with the IDS iSYS platform (Boldon, UK). Measurement of sclerostin (TECO medical, Sissach, Switzerland) and periostin (Biomedica, Vienna, Austria) was performed by ELISA. CTX was assessed by an electrochemiluminescence immunoassay (HITACHI/Roche - COBAS 8000).

Stable calcium isotopes

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Stable calcium isotope measurements were performed on a MC-ICP-MS (Neptune plus, Thermo Fisher Scientific, Bremen, Germany) at the mass spectrometer facilities of the GEOMAR Helmholtz Centre for Ocean Research Kiel in Germany, as previously described (26). NIST SRM 915a was used as a reference material. The isotopic composition is reported as $\delta^{44/42}$Ca in parts per thousand (‰). Calcium consists of six stable natural isotopes, with a mass from 40 to 48 atomic mass units. During bone formation, the lighter isotopes are preferentially incorporated into bone, while there is no isotope preference during bone resorption. Thus, in case of a positive bone mineral balance, a serum shift towards heavier values will occur, while lighter isotopes become more abundant in case of a negative bone mineral balance (27). Due to sample availability, the stable calcium isotope ratio was determined on 12 out of 17 patients.

Statistical analysis

Values are expressed as median (range). To analyze changes in biochemical parameters after initiation of ADT, normality was first assessed with the D'Agostino & Pearson normality test. If data were normally distributed, a paired *t*-test was applied, otherwise the Wilcoxon matched-pairs signed rank test was used to determine statistical significance (two-tailed $P<0.05$). All analyses were performed using GraphPad Prism (version 6.07, La Jolla California USA).

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RESULTS

Effect of androgen deprivation on general parameters and on the gonadal axis

At baseline, the median age of the cohort was 44 years (20-75), with a median BMI of 24.6 kg/m^2 (17.9-31.7). Eleven patients (65%) were active smokers or had a history of smoking. None of the patients had diabetes mellitus. The average time interval between the start of therapy and the first follow-up visit was 13 weeks (6-27). No changes were observed in BMI, kidney function, or liver function. A decrease in albumin and hemoglobin, a proxy for androgen deficiency, as well as a decrease of total ALP was observed (table 1).

At the first follow-up visit, androgen deprivation caused an 81% decrease in total T as well as free T on average. Estradiol concentrations decreased 71% and SHBG decreased 30% on average. Gonadotropins were lowered as well (table 1 and figure 2).

Effect of androgen deprivation on serum calcium and phosphate homeostasis

Albumin-corrected serum calcium and phosphate concentrations increased after ADT (table 2 and figure 3). PTH and 1,25-dihydroxyvitamin D_3 decreased, while 25-hydroxyvitamin D_3 increased. However, serum DBP also increased, leading to similar free 25-hydroxyvitamin D_3 under ADT, while free 1,25-dihydroxyvitamin D_3 decreased to a greater extent. 24,25-dihyroxyvitamin D_3 and 25D:24,25D ratio did not change significantly. No change was observed for serum FGF23 (table 2).

Effect of androgen deprivation on bone homeostasis

The serum levels of the bone resorption markers TRAcP5b and CTX were not affected by ADT. The serum levels of the bone formation markers BAP and osteocalcin were decreased (table 2 and figure 4), while P1NP was not altered. Serum sclerostin clearly increased, while periostin levels tended to decrease following ADT. Finally, a decrease in the calcium isotope ratio was observed, indicating a negative bone mineral balance (table 2 and figure 4).

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DISCUSSION

We observed that ADT with CPA in adult males is associated with a negative bone mineral balance, releasing calcium and phosphate from the skeleton already after approximately 3 months of treatment, with a compensatory decline in serum PTH and 1,25-dihydroxyvitamin D_3. Accordingly, the stable calcium isotope ratio, allowing the sensitive detection of early calcium loss from bone, decreased even while the bone resorption markers CTX and TRAcP5b were unaltered. This mild bone resorption was paralleled by a clear suppression of bone formation in the early phase of androgen deprivation. This pattern of bone imbalance contrasts with the increased bone resorption and subsequently increased formation observed in chronic ADT.

In this study, serum samples were taken from eugonadal male sex offenders referred to the endocrine clinic by their treating psychiatrist for initiation of chemical castration. Androgen deprivation with the AR antagonist CPA significantly decreased total T and E2. However, treatment with GnRH analogues might have suppressed T and E2 more prominently (17,28).

Long-term sex steroid deprivation is characterized by increased bone formation markers at 6 months (15) and 1 year (16) following ADT in prostate cancer patients, as a result of coupling of formation to resorption. In addition, prostate cancer patients receiving GnRH analogues for a mean duration of 41 months also exhibited higher BAP levels than patients without ADT (3). In our study exploring short-term effects of ADT on bone homeostasis, bone resorption did not appear to be increased, as indicated by unaltered TRAcP5b and CTX concentrations. However, bone formation was reduced, as illustrated by the increase in sclerostin and decrease in BAP, osteocalcin and periostin. Sclerostin is produced by mature osteocytes, directly inhibiting bone formation and indirectly promoting bone resorption by enhancing osteoclast differentiation (29). Our data are in agreement with others, who described increased serum sclerostin after ADT in prostate cancer patients as well (30). In addition, young hypogonadal men have been shown to have increased serum sclerostin (31). Periostin, an extracellular matrix protein expressed by periosteal osteoblasts and osteocytes, promotes bone

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formation (32). It exerts it function by inhibiting sclerostin expression and stimulating the Wnt signaling pathway (33,34). Accordingly, periostin knockout mice exhibit both cortical and trabecular bone loss, leading to diminished bone strength (33). Serum periostin appears to be age-dependent, with higher concentrations in early adulthood than at older age (35). Moreover, serum concentrations have been associated with fracture risk in postmenopausal women (36). To our knowledge, we are the first to show that serum periostin may decrease early following ADT. This observation needs confirmation in a larger cohort.

Our results are in agreement with others who previously reported low bone formation in the early phase following ADT as well (17,37). Interestingly, compared to studies in patients treated with GnRH analogues, the patients in our study were not severely estrogen-deficient, which could blunt the increase in bone resorption, known as being mainly estrogen-driven (17,28). As such, CPA treatment might represent a model of mild androgen deprivation with more pronounced testosterone relative to estrogen deficiency. The coupling effect with bone formation, as generally seen with prolonged androgen and estrogen deficiency, may therefore not be present to the same extent in our study. As such, the observed acute impairment in bone formation could represent a dominant effect of testosterone. P1NP was not affected in our study. Discrepancies have been observed, with both unaltered (6) and increased (38) P1NP levels being reported following ADT. Timing could play a role, as others have described a decrease in serum P1NP after 4 weeks of ADT in healthy men, which normalized again by 12 weeks (37). Again, the extent of decrease in testosterone and estradiol could play a role. Finkelstein *et al.* also showed that ADT with a GnRH analog in healthy men only affected P1NP when T was decreased below 3.5 nmol/L (100 ng/dL) and E2 below 18 pmol/L (5 ng/L) (28). In our study, approximately half of the men were above this threshold. Although P1NP and osteocalcin are both considered bone formation markers, they are expressed during different stages of osteoblast maturation (17). Therefore, as illustrated in this study, they may show a different response during the early stages of sex steroid deficiency.

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Currently used bone resorption and formation markers may be influenced by formation and resorption, respectively, due to coupling. In contrast, the stable calcium isotope ratio reflects the net bone balance. We observed a shift toward lighter calcium isotopes, confirming the calcium flux from the skeleton. This marker has previously been shown to sensitively reflect a negative bone mineral balance in postmenopausal women with osteoporosis (26) as well as in response to skeletal unloading during long-term bed rest in males and females (27). Our data suggest that this novel calcium-based indicator of net bone mineral balance may also be used in male osteoporosis and represents a more sensitive marker for bone resorption in the early phase of ADT compared to CTX and TRAcP5b. Early markers of bone resorption could be useful to identify patients who have a high risk of bone loss, such as prostate cancer patients and hypersexual men receiving ADT.

The increase in serum calcium and phosphate and concomitant decrease in PTH and 1,25-dihydroxyvitamin D_3 are indicative for an acute net release of calcium and phosphate from the bone, consistent with the calcium isotope data. We failed to observe a compensatory increase of FGF23, probably due to a lack of power. Similar effects on calcium and phosphate homeostasis were reported following treatment with GnRH analogs in healthy adult men and prostate cancer patients after 12 weeks (39) and 6 months (7), respectively, with a secondary decrease in PTH (7,39). Conversely, young adult and older hypogonadal men treated with androgen replacement showed decreased serum calcium (40). We observed a significant increase in DBP after androgen deprivation, thereby altering free vitamin D concentrations. In hypogonadal men on the other hand, T treatment has been shown to lower DBP after 4 to 12 weeks (41). In our study, free 1,25-dihydroxyvitamin D_3 decreased to a greater extent than total 1,25-dihydroxyvitamin D_3 and free 25-hydroxyvitamin D_3 was unaffected. The 24,25-dihyroxyvitamin D_3 levels and 25D:24,25D ratio were not changed either, indicative for a direct inhibitory effect of the decreased PTH on the 1α-hydroxylase enzyme activity, thereby lowering the production of 1,25-dihydroxyvitamin D_3.

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Our study has several strengths. First, we prospectively investigated the effects of androgen deprivation in male sex offenders, which represents a younger patient group with less comorbidities compared to prostate cancer patients. Second, we explored the early effects of ADT on bone and calcium/phosphate homeostasis, including a wide range of BTMs and calcium-regulating hormones. Third, we assessed the serum calcium isotope ratio, a novel non-invasive method to sensitively detect early net calcium loss from the skeleton. Our study has several limitations as well, including the small number of participants, the absence of a control group, and the differences in timing of the follow-up visit. Replication of these findings is warranted in a larger and controlled clinical trial. Information on dietary calcium and phosphate intake and urine samples were not available, and blood samples were not systematically collected in a fasting state.

In summary, we explored the early effects of gonadal steroid withdrawal by CPA on the adult male skeleton and serum calcium and phosphate homeostasis. We observed that calcium is released from the skeleton, as detected by a novel stable calcium isotope technique and supported by a decrease of PTH and 1,25-dihydroxyvitamin D_3. Concomitantly, serum sclerostin increased along with a reduction of bone formation markers but without significant changes in bone resorption markers, suggesting that the negative calcium balance may be explained by an acute impairment of bone formation. This study helps in the understanding of the pathophysiology of acute hypogonadal bone loss in males.

In conclusion, the effects of short-term CPA treatment on skeletal dynamics in men are mediated largely, if not completely, by suppression of bone formation.

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DISCLOSURE

The authors have no interests to disclose. This work was supported by the KU Leuven [grant GOA/15/017], the Research Foundation Flanders (FWO) [grant G0D2217N] and Klinische onderzoeks- en opleidingsraad (KOOR) University Hospitals Leuven [S54034].

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FIGURE LEGENDS

Figure 1. Study flow diagram.

Figure 2. Change in sex steroids and SHBG after ADT by CPA. Each line represents one patient.

Figure 3. Change in mediators of calcium and phosphate homeostasis after ADT by CPA. Each line represents one patient.

Figure 4. Change in bone turnover markers and stable calcium isotope ratio ($\delta^{44/42}Ca$) after ADT by CPA. Each line represents one patient.

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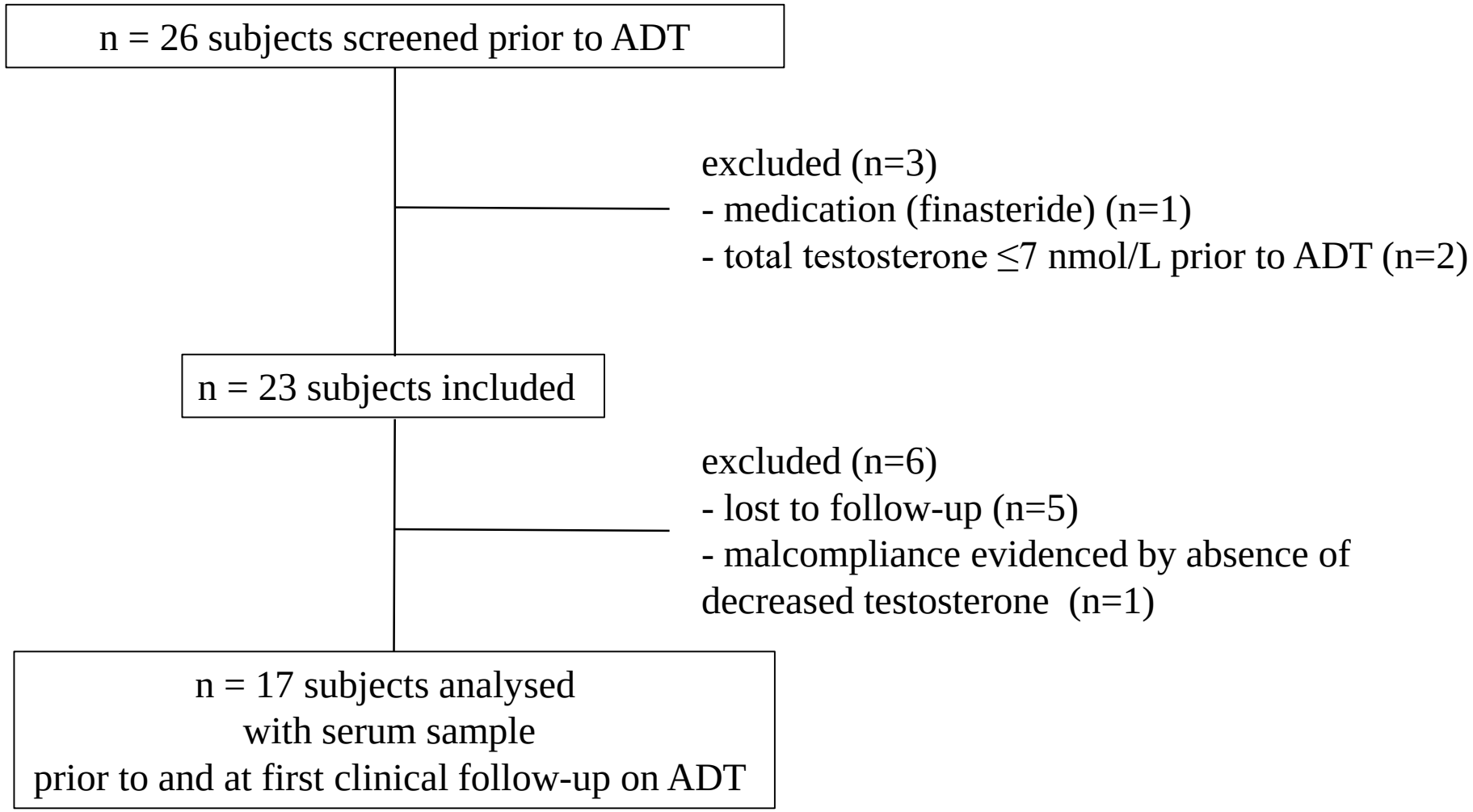

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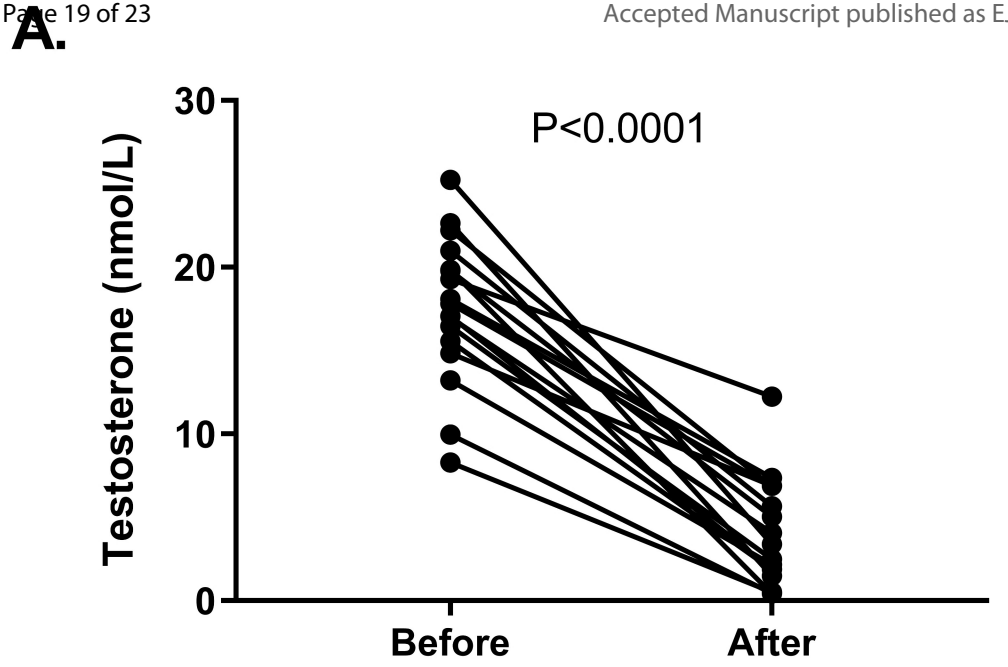

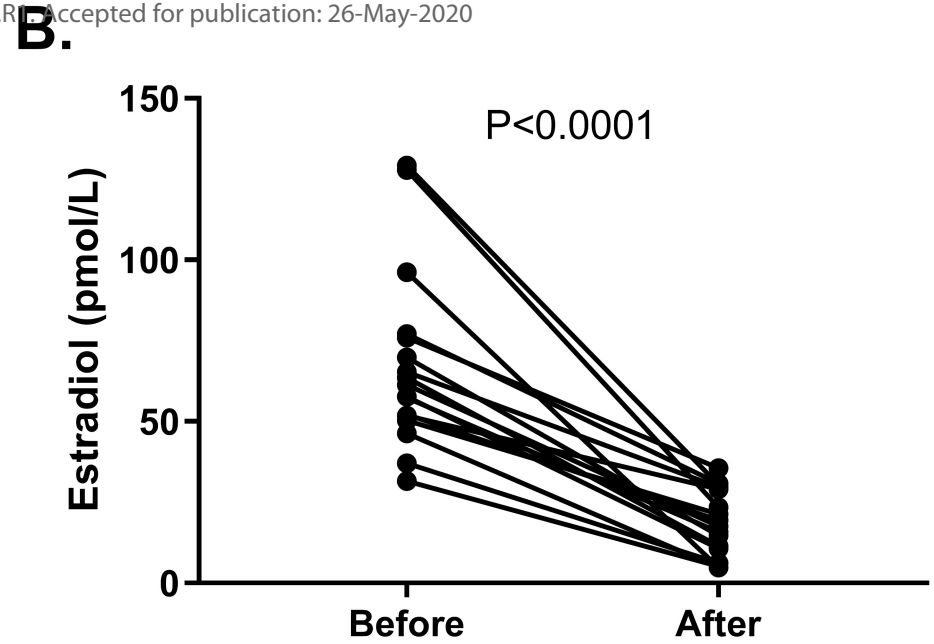

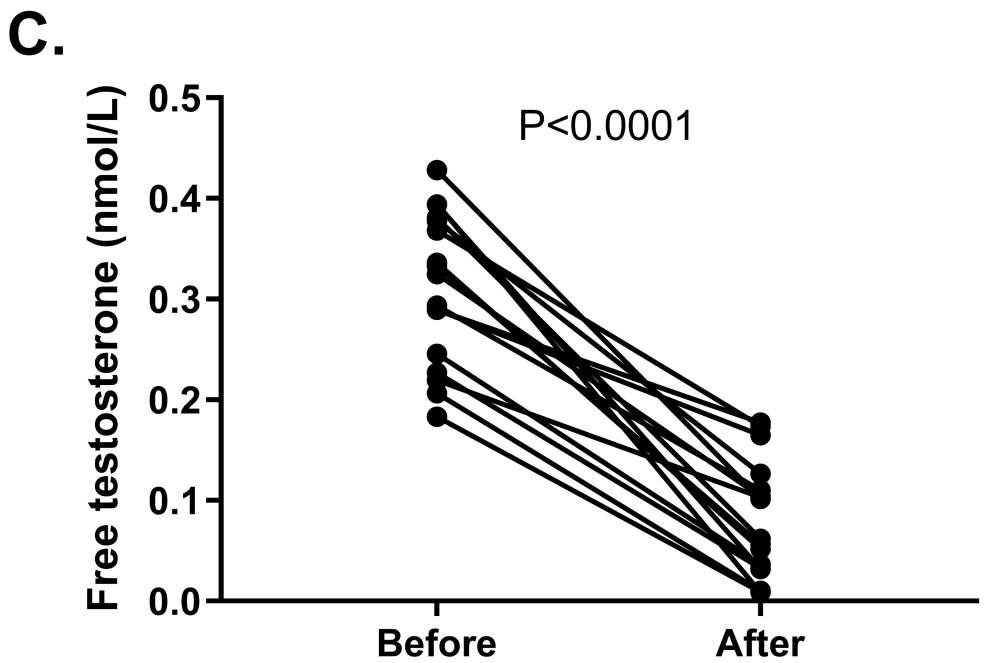

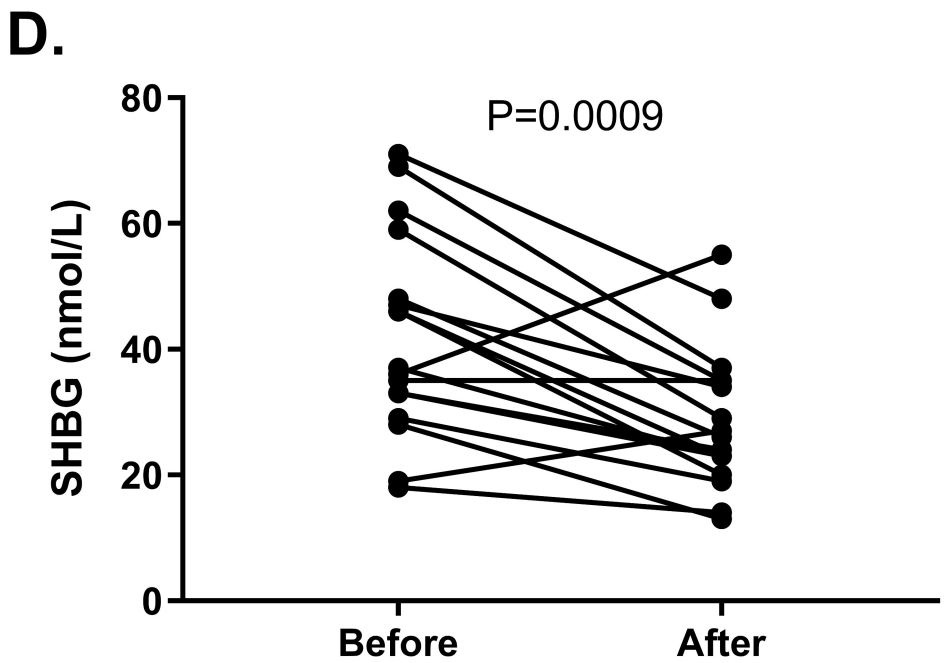

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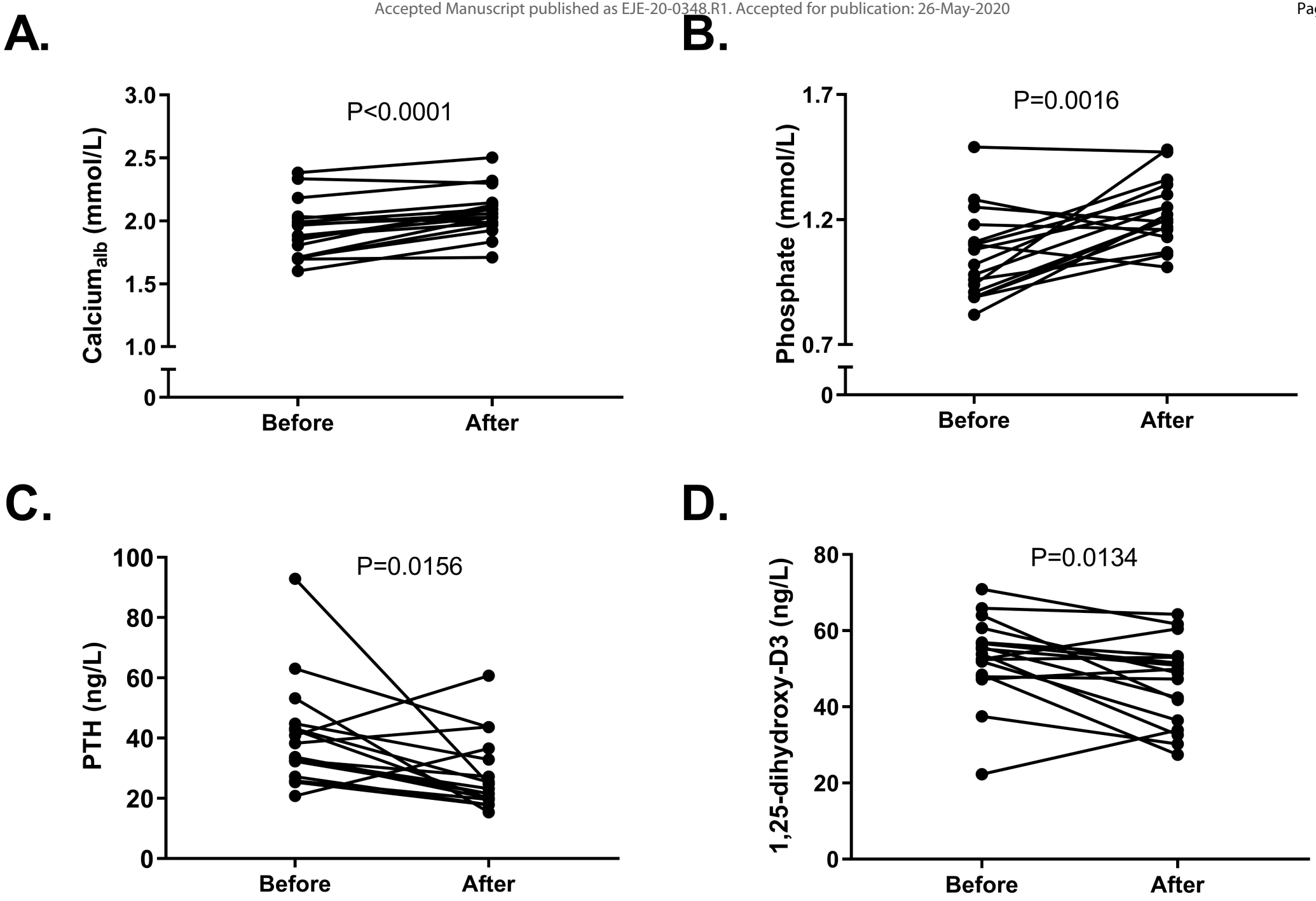

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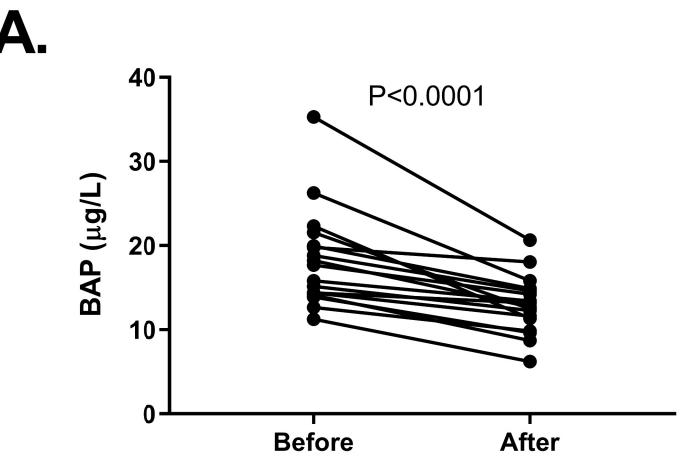

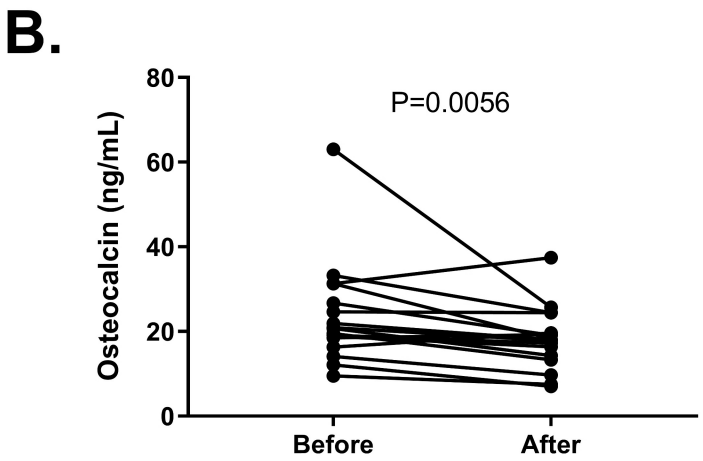

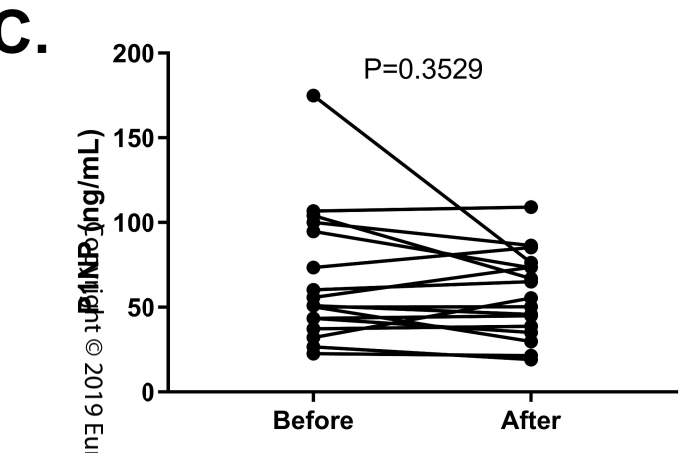

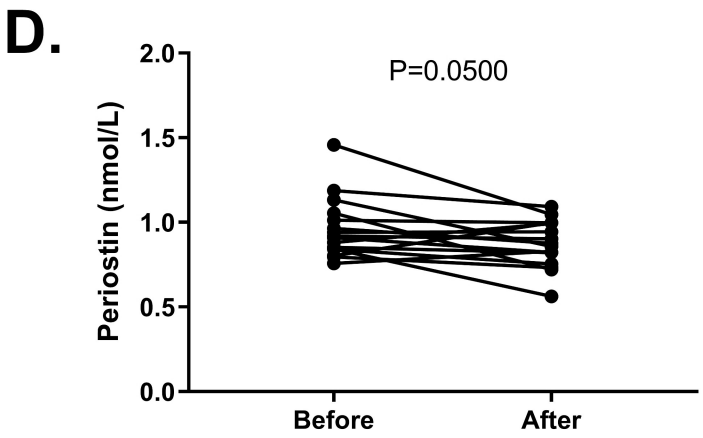

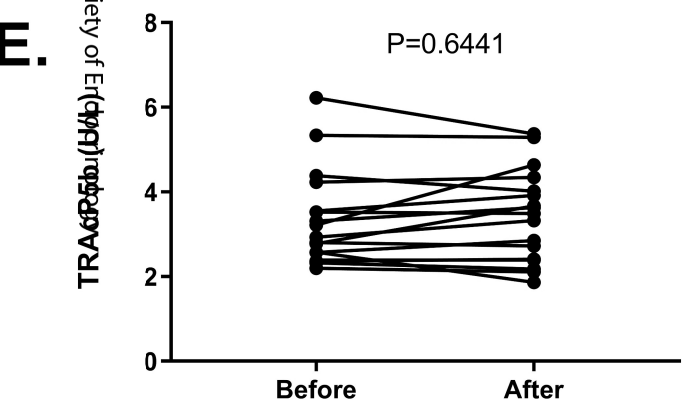

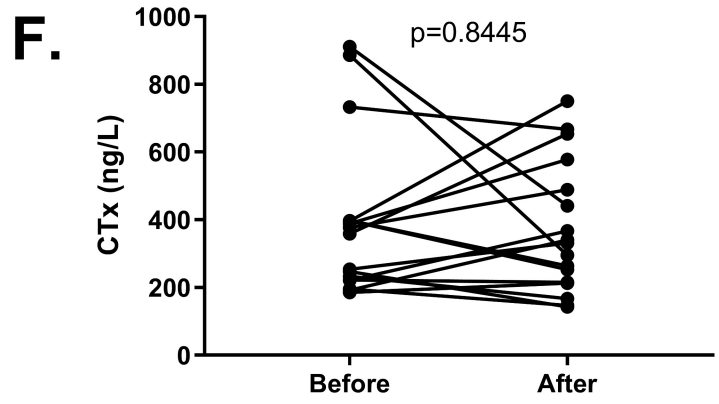

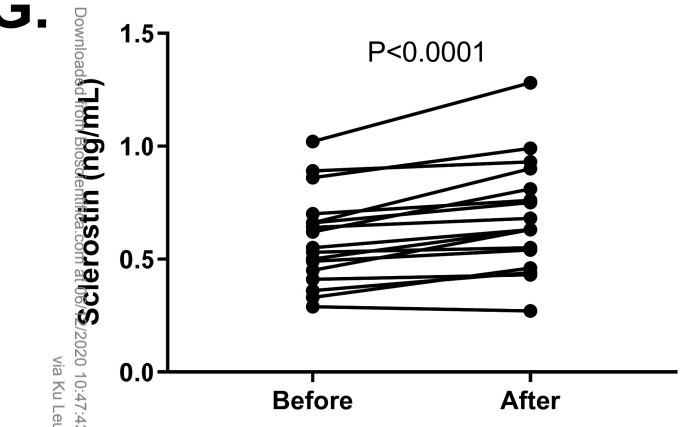

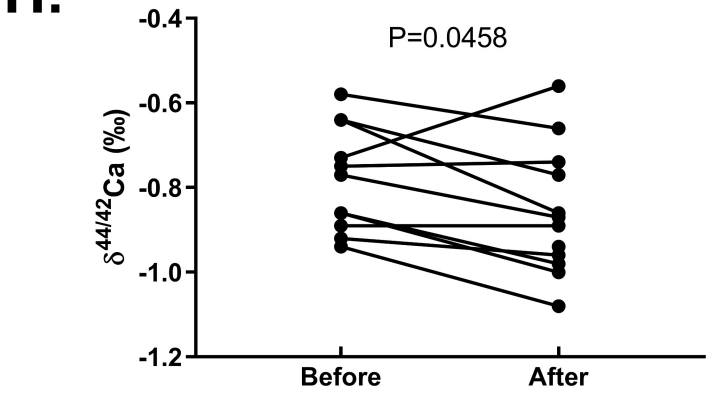

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Table 1 Effect of androgen deprivation on general parameters and gonadal axis. Data are presented as median (range). Statistical significance was determined by a paired *t*-test if data were normally distributed, otherwise the Wilcoxon matched-pairs signed rank test was used (two-tailed p<0.05).

	Before ADT	**After ADT**	***P*-value**
General parameters			
BMI (kg/m^2)	24.6 (17.9-31.7)	24.1 (18.2-31.7)	0.6166
Albumin (g/L)	46.0 (41.2-50.3)	44.9 (40.6-50.5)	0.0022
Hemoglobin (g/dL)	15.1 (13.1-16.3)	13.5 (11.3-15.8)	0.0001
eGFR (mL/min/1.73 m^2)	101 (63-131)	101 (64-130)	>0.9999
Cystatin C (mg/L)	0.87 (0.77-1.27)	0.83 (0.71-1.26)	0.1697
ALP (IU/L)	68 (54-109)	59 (39-97)	<0.0001
AST (IU/L)	21 (12-28)	17 (10-42)	0.2885
ALT (IU/L)	21 (13-53)	21 (8.0-74)	0.7050
GGT (IU/L)	15 (10-71)	21 (14-38)	0.0591
Gonadal axis			
Total T**S** (nmol/L)	17.8 (8.3-25.2)	3.4 (0.4-12.2)	<0.0001
SHBG (nmol/L)	37 (18-71)	26 (13-55)	0.0009
Free T**S** (nmol/L)	0.32 (0.18-0.43)	0.06 (0.01-0.18)	<0.0001
E2 (pmol/L)	61.3 (31.6-129.2)	17.6 (4.7-35.6)	<0.0001
LH (IU/L)	5.4 (2.3-26.5)	3.5 (0.2-7.9)	0.0040
FSH (IU/L)	3.6 (1.9-53.5)	2.6 (0.8-22.9)	0.0025

TS, testosterone; E2, estradiol; eGFR, estimated glomerular filtration rate; ALP, total alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase.

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Table 2 Effect of androgen deprivation on calcium/phosphate homeostasis and bone turnover. Data are presented as median (range). Statistical significance was determined by a paired *t*-test if data were normally distributed, otherwise the Wilcoxon matched-pairs signed rank test was used (two-tailed p<0.05).

	Before ADT	**After ADT**	***P*-value**
Calcium/phosphate homeostasis			
$Calcium_{alb}$ (mmol/L)	1.88 (1.60-2.38)	2.03 (1.71-2.50)	<0.0001
Phosphate (mmol/L)	1.02 (0.82-1.49)	1.20 (1.01-1.48)	0.0016
PTH (ng/L)	33.7 (20.8-92.9)	23.2 (15.4-60.7)	0.0156
Total 1.25D (ng/L)	53.8 (22.3-70.9)	48.9 (27.4-64.3)	0.0134
Total 25D (µg/L)	18.5 (6.0-35.4)	23.0 (11.7-43.7)	0.0496
Total 24.25D (ng/mL)	1.65 (0.16-4.02)	2.04 (0.74-4.53)	0.0694
25D:24.25D ratio	13.2 (8.8-38.8)	10.4 (7.0-15.8)	0.0785
DBP (µg/mL)	290 (239-359)	314 (269.-393)	0.0168
Free 1.25D (pg/L)	252 (94-371)	205 (118-283)	0.0041
Free 25D (ng/L)	4.60 (1.43-9.64)	5.90 (2.66-10.3)	0.1133
FGF23 (pg/mL)	44.7 (24.5-65.2)	45.4 (25.8-78.5)	0.4737
Bone turnover			
BAP (µg/L)	17.7 (11.3-35.3)	12.7 (6.2-20.7)	<0.0001
Osteocalcin (ng/mL)	20.7 (9.5-63.0)	18 (7.0-37.4)	0.0056
P1NP (ng/mL)	50.9 (22.6-175)	55.4 (19.2-109)	0.3529
Periostin (nmol/L)	0.91 (0.76-1.46)	0.87 (0.56-1.09)	0.0500
TRAcP5b (U/L)	2.93 (2.19-6.22)	3.49 (1.86-5.37)	0.6441
CTX (ng/L)	359 (185-911)	331 (143-750)	0.8445
Sclerostin (ng/mL)	0.55 (0.29-1.02)	0.63 (0.27-1.28)	<0.0001
$\delta^{44/42}$Ca (‰)	-0.77 (-0.94- -0.58)	-0.88 (-1.08- -0.56)	0.0458

1.25D, 1,25-dihydroxyvitamin D_3; 25D, 25-hydroxyvitamin D_3; 24.25D, 24,25-dihydroxyvitamin D_3; DBP, vitamin D binding protein; FGF23, fibroblast growth factor 23; TRAcP5b, tartrate-resistant acid phosphatase 5b; CTX, C-terminal telopeptide of type I collagen; $\delta^{44/42}$Ca, stable calcium isotope ratio; BAP, bone alkaline phosphatase; P1NP, N-terminal propeptide of type 1 collagen.

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