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Effect of a single-dose denosumab on mineral homeostasis in infertile men: insights from a pilot intervention study and a randomized controlled trial

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Abstract

Background Denosumab, a drug that inhibits RANKL to reduce bone resorption in osteoporotic postmenopausal women, has been shown to improve semen quality in a subgroup of infertile men. This study aimed to investigate the effects of denosumab on mineral homeostasis in young infertile men.

Methods Secondary data from two clinical trials designed to test the effect on semen quality were used: (1) a pilot intervention study with 12 men receiving a single-dose of 60 mg denosumab and (2) a single-center, double-blinded, randomized clinical trial, where 100 infertile men were randomized 1:1 to receive denosumab 60 mg once sc. or placebo. A linear mixed model for repeated measures was employed to analyze data from follow-up samples.

Results In the pilot intervention study, denosumab treatment induced a decrease in ionized calcium 5, 20, 40, and 80 days after treatment compared with baseline (all $p < 0.05$). Serum phosphate decreased on all time points up to and including day 40 (all $p < 0.05$), while alkaline phosphatase was only lowered at 40 days and onwards ($p = 0.014$). Serum PTH increased significantly at all time points up to and including day 80 ($p = 0.026$). One hundred eighty days after treatment, all reported analyses were comparable to baseline levels. The observed temporal changes were confirmed in the RCT with differences in serum calcium ($p < 0.001$) and phosphate ($p < 0.001$) on day 14, PTH ($p < 0.002$), and alkaline phosphatase ($p < 0.001$) on days 80 and 160. Denosumab treatment had no significant effect on vitamin D status, renal function, or serum albumin concentration after 80 and 160 days.

Conclusions Small but significant changes in mineral homeostasis and bone mineral content were observed but the changes were transient and normalized after treatment cessation. A single injection of denosumab in infertile men appears to have no major long-term impact on bone or mineral homeostasis.

Trial registration ClinicalTrials.gov NCT03030196. Registered January 24, 2017.

Keywords Denosumab, Calcium, PTH, Vitamin D, Phosphate, Randomized controlled trials

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Background

The delicate balance between bone resorption and bone formation is strongly influenced by the RANKL system, which explains why the receptor activator of the nuclear factor κB ligand (RANKL) inhibitor denosumab is effectively used to treat osteoporosis. The soluble or transmembrane isoform of RANKL binds to its receptor, RANK (1,2), and stimulates osteoclast precursor maturation and proliferation through phosphorylation and inactivation of inhibitory kinases [1]. In addition to the ligand and receptor, the RANKL system includes an endogenous soluble decoy receptor, osteoprotegerin (OPG), which has an important role in modulating the interaction between RANKL and RANK. It binds to RANKL and thereby inhibits the activation of RANK [2]. By doing so, OPG effectively prevents osteoclast differentiation and activation, consequently leading to a reduction in bone resorption and promoting bone formation [3–5]. This knowledge prompted the development of denosumab, a human monoclonal antibody, which targets RANKL by acting as an inhibitor mimicking the effect of OPG.

Denosumab was the first approved biologic agent for the treatment of osteoporosis. It is a potent antiresorptive drug leading to a significant reduction in the risk for hip, vertebral, and non-vertebral fractures in postmenopausal osteoporosis [6]. Although the effects of denosumab on bone health have been extensively studied, the data originates mainly from older people, i.e., post-menopausal women or men with prostate cancer. Discontinuation of denosumab treatment has caused some concern after repeated usage in osteoporotic patients induces a risk of a rebound increase in bone turnover, which may lead to an increased risk of fractures [7, 8]. This rebound effect occurs more often if the medication is discontinued abruptly after several years of use [8, 9].

The reduced bone resorption also induces a calcium-lowering effect of denosumab that occasionally may induce milder adverse effects but has also proven beneficial in a wide array of malignancies, including breast, prostate, colorectal, lung, bladder, and gastric cancer [10, 11]. Moreover, the indication for the use of denosumab could be expanding in the coming years, as the RANKL system is expressed in other sites than the bone [12–16]. RANKL is expressed in the Sertoli cells of the testis, and signals to RANK in the germ cells, and inhibition of testicular RANKL increases sperm production in mice models and germ cell proliferation in human testicular tissue models with none or mild testicular dysgenesis [12, 17, 18]. With the possible repurposing of denosumab for other indications, it is important to gather data on its safety and efficacy in these patient populations. While denosumab has been extensively studied in postmenopausal women and elderly individuals for its efficacy in reducing bone loss and fracture risk [19–24], its impact on the bone health of younger individuals remains understudied. Recent studies have suggested that the RANKL system plays a role in male infertility, testis cancer [12, 17], and skeletal muscle [25], including new clinical and preclinical studies that have suggested that even only a single injection of denosumab may be used to stimulate spermatogenesis and improve male fertility potential in a sub-population of infertile men [17, 18, 26].

The objective of this study was to investigate the impact of denosumab on mineral homeostasis in infertile men. The study is based on two separate interventional trials. Initially, an intervention study involving 12 infertile men to explore preliminary effects. Subsequently, a randomized controlled trial (RCT) involving a cohort of 100 infertile men (receiving either denosumab or placebo) was used to validate and generalize the findings.

Methods

Study population

The study consisted of two separate cohorts. Both studies were carried out at the Department of Growth and Reproduction, Copenhagen University Hospital—Rigshospitalet, Copenhagen. In the pilot cohort (NCT02422108), a total of 22 infertile men were screened, and 12 men were included. All 12 participants received a one-time subcutaneous injection of 60 mg of denosumab. The men were followed and requested to deliver semen and fasted blood samples before the intervention and on days 5, 20, 40, 80, 120, 180, and 270 after treatment. To prevent denosumab induced hypocalcemia, all participants received daily vitamin D_3 (25 μg) and calcium (400 mg) supplementation during the treatment period. The primary endpoint (sperm concentration) of the intervention has been published in 2021 [17].

The RCT (NCT03030196) was a single-center, double-blinded, randomized clinical trial. All men eligible for inclusion were part of an infertile couple whose semen analysis had shown impaired semen quality and the men were referred for further investigation at our andrological center. The infertile men delivered two semen samples for routine analyses and blood samples. All included men were randomized 1:1 to receive either a single-dose subcutaneous denosumab 60 mg injection or a placebo injection with saline. The selection of the study population in the trial is shown as a prism diagram in Fig. 1. In the trial, all men also received 400 mg of calcium and 25 μg vitamin D_3 supplementation from treatment and during follow-up. The main results of the randomized clinical trial have been published recently [18].

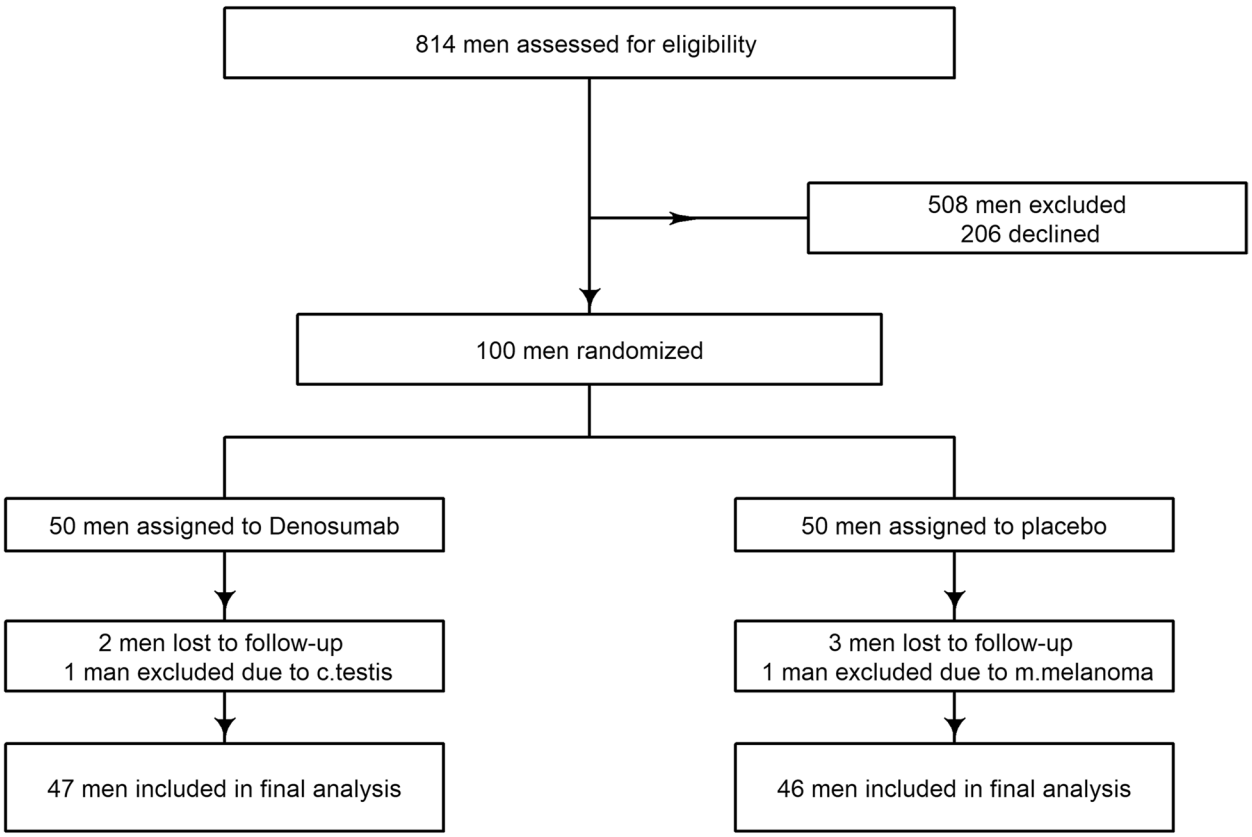

Fig. 1 Selection of the study population in the randomized controlled trial. Flowchart of selection of study population showing exclusions

Biochemical analysis

In serum, total calcium (coefficient of variation (CV) 2.5%), parathyroid hormone (PTH) (CV < 4%), alkaline phosphatase (CV < 10%), albumin (CV 4.5%), creatinine (CV < 5%), and phosphate (CV < 3%) were measured using Cobas 8000, whereas ionized calcium (CV 3%) was measured on ABL837. Serum vitamin D levels were measured on liquid chromatography–mass spectrometry (LC–MS) with a CV of 9% for 25-hydroxyvitamin D (25(OH)D) and 18% for 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). Bone markers CTX (CV 8%) and PINP (CV 8%) were measured with chemiluminescence immunoassays on i10 (IDS Plc, Tyne and Wear, France). Whole body and specific bone mineral content (BMC) was measured in grams hydroxyapatite, bone size expressed as anterior–posterior projected bone area (BA) measured in square centimeters, and bone mineral density (BMD) measured as BMC/BA was determined by dual-energy X-ray absorption (DXA) scans performed on Hologic CDR 1000/W densitometer (Hologic, Inc., Bedford, MA, USA).

Statistical analysis

Descriptive statistics were calculated for all variables and presented as means with standard deviation (SD) for continuous variables in Tables 1 and 2, and additionally as numbers with percentages for categorical variables (Table 3). A linear mixed model analysis was conducted to investigate changes in mean ionized calcium, PTH, 25(OH)D, phosphate, alkaline phosphatase, and eGFR over time [27]. This is similar to a one-way ANOVA model (with time as the categorical covariate), but with correlated data due to the repeated measurements and allows the inclusion of patients with an incomplete follow-up [28]. Before this, the distribution of the data was checked for normality. In a few of the variables, there was a slightly right-skewed distribution, however, linear mixed models are quite robust, and even though the pilot study cohort's size was rather small (12 subjects), this did not compromise the results of the analysis, as a log-transformation of these data gave the same conclusions. To account for multiple comparisons, post hoc adjustments were conducted

Table 1 Baseline characteristics of 12 infertile men included in the pilot study

Variable	Reference interval	*N*	Mean	SD
Age (years)		12	33.4	3.3
BMI (kg/m^2)		12	26.3	3.3
Ionized calcium (mmol/L)	(1.18–1.32)	12	1.24	0.05
Total calcium (mmol/L)	(2.15–2.51)	12	2.40	0.07
eGFR (mL/min/1.73 m^2)	(> 90)	12	90	6
Phosphate (mmol/L)	(0.71–1.53)	12	0.91	0.19
25(OH)D (nmol/L)	(> 50)	12	72	18
PTH (pmol/L)	(2.0–8.5)	12	3.3	1.2
1,25(OH)$_2$D$_3$ (pmol/L)	(60–160)	12	76.5	20.8
Alkaline phosphatase (U/L)	(35–105)	12	61.4	16.8
Albumin (g/L)	(36–48)	12	41	3

Baseline characteristics of all included men in the pilot study with denosumab. All data presented as mean and SD

Abbreviations: *BMI*, body mass index; *eGFR*, estimated glomerular filtration rate; *PTH*, parathyroid hormone; *1,25(OH)$_2$D$_3$*, 1,25-dihydroxyvitamin D$_3$; *25(OH)D*, 25-hydroxyvitamin D

using Dunnett's test, which allowed for the identification of statistically significant differences between the groups while controlling for the type I error rate. Independent *t*-tests were used to compare differences between groups (Figs. 3 and 4). A significance level of $p < 0.05$ was considered statistically significant. All statistical calculations were conducted using the statistical software R, version 3.4.1 (R Core Team 2021. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org).

Results

Pilot study cohort: baseline characteristics

The included study population consisted of 12 men (Table 1). The mean age of the patients was 33.4 years (SD 3.3), and the mean body mass index (BMI) was 26.3 (SD 3.3). At baseline, the study population was normocalcemic with an average serum ionized calcium of 1.24 mmol/L (SD 0.05). The average total calcium concentrations were 2.40 mmol/L (SD 0.07), and serum PTH concentrations were on average 3.3 pmol/L (SD 1.2). Kidney function was also normal with an average eGFR of 90.2 mL/min/1.73 m^2 (SD 6.1). The mean baseline level of 25(OH)D was 72 nmol/L (SD 18), which was expected as vitamin D deficiency was an exclusion criterion prior to treatment start. Baseline phosphate levels were also normal at 0.91 mmol/L (SD 0.19). Other variables measured at baseline included 1,25(OH)$_2$D$_3$, alkaline phosphatase, and albumin, which were all normal (Table 1).

Pilot study cohort: changes in serum calcium and PTH

Table 2 displays the average concentrations of the examined variables over 270 days, showing the changes observed during the treatment period. Ionized calcium concentrations gradually declined following the injection with denosumab before restoring to baseline levels at the end of the experimental period. Baseline average ionized calcium decreased from 1.24 mmol/L (SD 0.05) to 1.17 mmol/L (SD 0.03) on day 5 ($p = 0.044$), 1.18 mmol/L (SD 0.03) on day 20 ($p = 0.017$), and 1.17 mmol/L (SD 0.03) on day 40 ($p < 0.001$). The normalized plot for changes in serum ionized calcium is shown in Fig. 2A. The most striking decline was seen in one individual who dropped ~ 15% on day 5 and ~ 20% on day 120 before slowly normalizing. The average values remained low but stable until day 80 when a slight increase was observed reaching 1.19 mmol/L (SD 0.03), although still significantly lower than baseline ($p = 0.047$). On day 120 and forwards, the average calcium ion levels rose again and did not differ from baseline on day 180 ($p = 0.990$) with concentration at 1.23 mmol/L (SD 0.07). Total calcium concentrations followed a similar pattern as was observed for ionized calcium and decreased from baseline 2.40 mmol/L (SD 0.08) to 2.31 mmol/L (SD 0.11) on day 5 ($p < 0.0001$) and remained low until day 40. Here, a gradual increase was observed, with levels reaching

Table 2 Changes in serum levels during the pilot study

	Reference interval	Baseline	Day 5	Day 20	Day 40	Day 80	Day 120	Day 180	Day 270
Ionized calcium	(1.18–1.32)	1.24 (0.05)	1.17 (0.03)	1.18 (0.03)	1.17 (0.03)	1.19 (0.03)	1.20 (0.05)	1.23 (0.07)	1.21 (0.03)
Total calcium	(2.15–2.51)	2.40 (0.08)	2.31 (0.11)	2.30 (0.07)	2.31 (0.09)	2.36 (0.08)	2.33 (0.11)	2.37 (0.09)	2.40 (0.08)
eGFR	(> 90)	90 (6)	89 (5)	90 (3)	88 (5)	89 (5)	90 (4)	88 (6)	93 (6)
Phosphate	(0.71–1.53)	0.91 (0.20)	0.78 (0.13)	0.80 (0.15)	0.76 (0.15)	0.8 (0.18)	0.74 (0.22)	0.84 (0.12)	0.82 (0.14)
25(OH)D	(> 50)	72 (18)	73 (14)	67 (11)	68 (15)	70 (17)	65 (15)	68 (22)	68 (27)
PTH	(2.0–8.5)	3.3 (1.2)	5.6 (2.2)	6.4 (1.9)	6.2 (3.6)	4.7 (1.2)	5.7 (3.1)	4.1 (2.0)	3.6 (0.9)
Alkaline phosphatase	(35–105)	61.4 (17.5)	62.4 (18.7)	59.9 (18.8)	51.8 (12.7)	44.0 (10.3)	46.1 (12.0)	45.3 (11.9)	50.4 (14.1)
Albumin	(36–48)	41 (3)	41 (4)	41 (4)	40 (3)	40 (3)	41 (3)	40 (2)	42 (4)

Changes of mean of all 12 men included in the pilot study with denosumab. All data presented as mean (SD)

Abbreviations: *eGFR*, estimated glomerular filtration rate; *PTH*, parathyroid hormone; *25(OH)D*, 25-hydroxyvitamin D

Table 3 Baseline characteristics of the randomized controlled trial

Variable	Denosumab			Placebo			All		
	N	Mean	SD	*N*	Mean	SD	*N*	Mean	SD
Age (years)	49	34.3	5.9	49	34.2	6.4	98	34.4	6.2
Height (cm)	48	183.8	8.5	48	183.9	6.2	96	183.8	7.5
Weight (kg)	48	90.2	13.4	48	87.9	13.9	96	89.1	13.7
BMI (kg/m^2)	48	26.8	4.1	48	26.0	3.6	96	26.4	3.8
Ionized calcium (mmol/L)	49	1.21	0.03	48	1.21	0.03	97	1.20	0.04
Total calcium (mmol/L)	49	2.41	0.05	48	2.41	0.09	97	2.41	0.07
Phosphate (mmol/L)	49	0.89	0.14	48	0.90	0.14	97	0.90	0.14
25(OH)D (nmol/L)	49	69	25	48	72	24	97	70	25
PTH (pmol/L)	49	4.2	1.1	48	4.0	0.9	97	4.1	1.0
Alkaline phosphatase (U/L)	49	62	16	48	65	16	97	63	17
Albumin (g/L)	49	42	2	48	42	2	97	42	2

Baseline characteristics of all included men in the randomized clinical trial. All data presented as mean with SD

Abbreviations: *BMI*, body mass index; *PTH*, parathyroid hormone; *25(OH)D*, 25-hydroxyvitamin

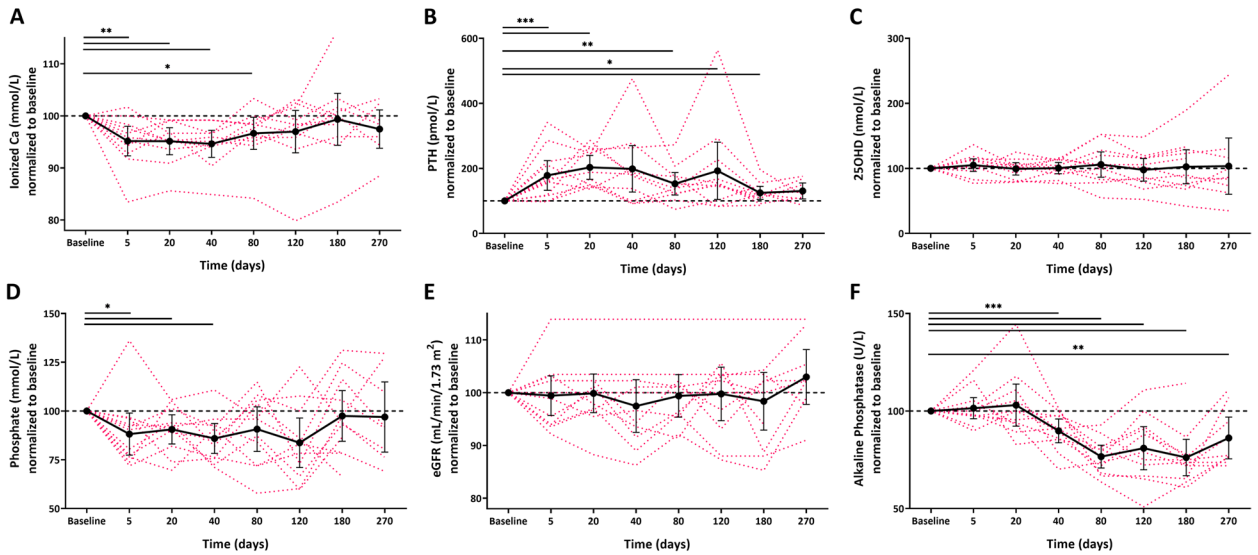

Fig. 2 Changes in minerals in the pilot study. **A**–**F** Normalized plots for serum concentrations of ionized calcium (Ca), parathyroid hormone (PTH), 25-hydroxyvitamin D (25(OH)D), phosphate, estimated glomerular filtration rate (eGFR), and alkaline phosphatase respectively, in patients who underwent intervention with denosumab, at baseline and after 5, 20, 40, 80, 120, 180, and 270 days. Changes in mean (SD) were compared by a linear mixed model for repeated measurements. *, **, and *** indicate p values < 0.05, < 0.01, and 0.001, respectively

2.36 mmol/L (SD 0.08) on day 80 ($p = 0.012$), 2.37 mmol/L (SD 0.09) on day 180 ($p = 0.443$), and 2.40 mmol/L (SD 0.08) on day 270 ($p = 0.991$). As expected, serum PTH concentrations increased in response to the decrease in calcium. Initially, with a rise from 3.3 pmol/L (SD 1.2) at baseline to 5.6 pmol/L (SD 2.2) on day 5 ($p = 0.005$) its peak average level at 6.4 pmol/L (SD 1.9) on day 20 ($p < 0.0001$). The peak was followed by a constant phase until day 40 equal to 6.2 pmol/L (SD 3.6) and a subsequent start of restoration back towards baseline levels by reaching 4.7 pmol/L (SD 1.2) on day 80 ($p = 0.027$). The average PTH levels then fluctuated but dropped to 3.6 pmol/L (SD 0.9) on day 270 ($p = 0.966$), which corresponds to baseline levels. The normalized plot for changes in serum PTH is shown in Fig. 2B.

Pilot study cohort: changes in serum phosphate, alkaline phosphatase, 25(OH)D, and renal function

Serum 25(OH)D levels fluctuated slightly during the study period, but no significant changes were observed (Fig. 2C and Table 2). Serum phosphate levels also gradually dropped during the treatment period

before normalizing at the end. The normalized plot for changes in serum phosphate is in Fig. 2D. Levels decreased from 0.91 nmol/L (SD 0.20) at baseline to 0.78 nmol/L (SD 0.13) on day 5 ($p=0.041$), followed by some fluctuations and further decreases to 0.76 nmol/L (SD 0.15) and 0.74 nmol/L (SD 0.22) on day 40 ($p=0.036$) and 120 ($p=0.063$), respectively. However, the levels increased after this, reaching 0.84 nmol/L (SD 0.12) and 0.82 nmol/L (SD 0.14) on day 180 ($p=0.667$) and day 270 ($p=0.992$), respectively. The eGFR was stable over time with minor variations but no significant changes (Fig. 2E). Serum levels of alkaline phosphatase levels also experienced a gradual decline but with a delayed start. Changes were not seen initially, with the concentrations varying from 61.4 U/L (SD 17.6) at baseline to 62.4 U/L (SD 18.7) and 59.9 U/L (SD 18.8) at day 5 ($p=0.974$) and day 20 ($p=0.990$), respectively. After this, the levels dropped to 51.8 U/L (SD 12.7) on day 40 ($p=0.002$), 44.0 U/L (SD 10.3) on day 80 ($p<0.0001$), 46.1 U/L (SD 12.0) on day 120 ($p=0.005$), and 45.3 U/L (SD 11.9) on day 180 ($p<0.001$). The normalized plot for changes in serum alkaline phosphatase is shown in Fig. 2F.

The randomized controlled trial: baseline characteristics

In total, 100 men were included and randomized; however, two men unexpectedly met an exclusion criterion after inclusion and were subsequently excluded due to malignant disease. Therefore, there are 98 men included and presented with baseline characteristics (Table 3), with 49 men in the treatment group receiving denosumab and 49 men in the control group receiving placebo treatment (placebo group). The participant's age, height, weight, and BMI were similar in both groups (Table 3). At baseline, the study population was comparable with no differences between the groups in serum total and ionized calcium, phosphate, PTH, and serum 25(OH)D.

The randomized controlled trial: changes after 14, 80, and 160 days

Tables 4 and 5 show the changes after the intervention on day 80 and day 160, respectively, while Fig. 3 highlights some of the important changes in the serum concentrations of minerals and calciotropic hormones. Total calcium levels dropped after 14 days (Fig. 3A), from 2.41 mmol/L (SD 0.05) to 2.34 mmol/L (SD 0.09) ($p<0.0001$). At baseline, ionized calcium levels

Table 4 Calciotropic hormones 80 days after denosumab injection

	Denosumab			Placebo			Difference	Dmab vs. placebo
	N	Mean	SD	*N*	Mean	SD	%	*p* value
25(OH)D (nmol/L)	45	74	22	46	77	22	−4.3%	0.483
Total calcium (mmol/L)	47	2.38	0.09	46	2.38	0.07	−0.2%	0.768
Ionized calcium (mmol/L)	47	1.19	0.04	46	1.19	0.03	−0.2%	0.709
PTH (pmol/L)	47	5.7	2.0	46	4.2	1.1	35.4%	**<0.0001**
Phosphate (mmol/L)	47	0.75	0.16	46	0.90	0.17	−17.0%	**<0.0001**
Alkaline phosphate (U/L)	47	45.4	12.4	46	63.0	16.2	−28.0%	**<0.0001**
Albumin (g/L)	47	41	2	46	41	2	0.1%	0.898

Changes in mean over 80 days in men included in the randomized clinical trial. All data presented as mean with SD. *P*-values less than 0.05 are significant and displayed in bold text

Abbreviations: *BMD*, bone mineral density; *Dmab*, denosumab; *PTH*, parathyroid hormone; *25(OH)D*, 25-hydroxyvitamin D

Table 5 Calciotropic hormones 160 days after denosumab injection

	Denosumab			Placebo			Difference	Dmab vs. placebo
	N	Mean	SD	*N*	Mean	SD	%	*p* value
25(OH)D (nmol/L)	47	72	22	46	73	21	−2.4%	0.688
Total calcium (mmol/L)	47	2.37	0.08	46	2.39	0.09	−0.8%	0.257
Ionized calcium (mmol/L)	47	1.20	0.03	46	1.21	0.04	−0.4%	0.539
PTH (pmol/L)	47	5.0	1.8	46	3.9	1.2	27.1%	**0.002**
Phosphate (mmol/L)	47	0.79	0.14	46	0.91	0.13	−13.2%	**<0.0001**
Alkaline phosphate (U/L)	47	46.1	13.0	46	62.3	17.1	−26.1%	**<0.0001**
Albumin (g/L)	47	41	2	46	42	2	−0.9%	0.412

Changes in mean over 160 days in men included in the randomized clinical trial. All data presented as mean with SD. *P*-values less than 0.05 are significant and displayed in bold text

Abbreviations: *BMD*, bone mineral density; *Dmab*, denosumab; *L*, lumbar spine; *PTH*, parathyroid hormone; *25(OH)D*, 25-hydroxyvitamin D

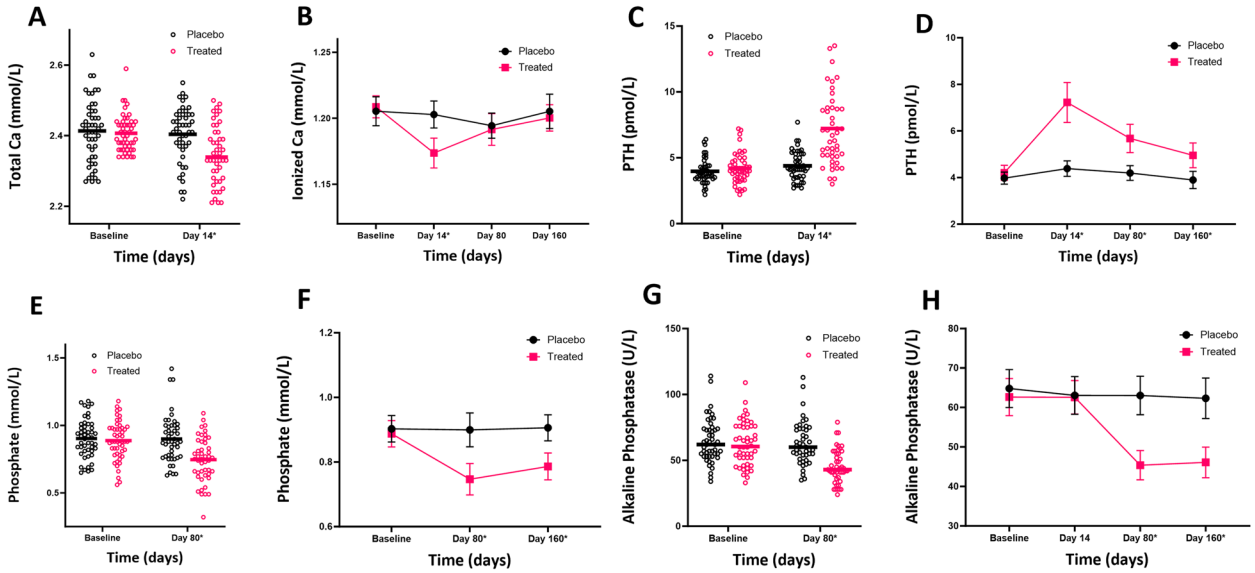

Fig. 3 Changes in minerals in the randomized controlled trial. **A**–**H** Plots for serum levels of total calcium, ionized calcium, parathyroid hormone (PTH), phosphate, and alkaline phosphatase respectively, in patients who underwent intervention with denosumab compared to placebo. * indicates *p* values < 0.05 when comparing denosumab treated with placebo

were 1.21 mmol/L (SD 0.03) in the denosumab group, which also dropped to 1.17 mmol/L (SD 0.04) on day 14 ($p < 0.0001$) (Fig. 3B). No changes were observed in the placebo group for ionized calcium ($p = 0.960$) or total calcium ($p = 0.739$), compared to baseline. Furthermore, 80 days after injection of denosumab there was no difference between the denosumab group and the placebo group as ionized and total calcium levels did not differ (1.19 vs. 1.19 mmol/L; $p = 0.709$, and 2.38 vs. 2.38 mmol/L; $p = 0.768$). Calcium content was also comparable between groups 160 days after denosumab injection (Table 5). The initial decline in serum calcium induced a compensatory increase in serum PTH levels in the denosumab treated men, which remained high as the denosumab group had a 35% higher serum PTH concentration compared with the placebo group (5.7 vs. 4.2 pmol/L; $p < 0.0001$) at day 80. The difference was smaller at day 160 but remained 27% higher (5.0 vs. 3.9 pmol/L; $p = 0.002$). The increase in serum PTH in the denosumab group resulted in a significant change in serum phosphate levels, which was 17% lower than in placebo treated men (0.75 vs. 0.90 mmol/L; $p < 0.0001$) at day 80 and 13% lower at day 160 (0.79 vs. 0.91 mmol/L; $p < 0.0001$). Figure 3E and F shows the changes in serum phosphate levels. Furthermore, as shown in Fig. 3G and H, the denosumab group had a decrease in serum alkaline phosphatase compared to the placebo group, with a substantial reduction of 28% (45.4 vs. 63.0 U/L; $p < 0.0001$) at day 80 and 26% at day 160 (46.1 vs. 62.3 U/L; $p < 0.0001$). At day 80, the denosumab group showed no difference in serum 25(OH)D levels compared to the placebo group (73.6 vs. 76.9 nmol/L; $p = 0.483$), and at day 160, the difference was also negligible at −2.4% in the intervention group (71.6 vs. 73.4 nmol/L; $p = 0.688$) (Tables 4 and 5). Lastly, there were no differences in serum albumin levels between the denosumab group and the placebo group on either day 80 or day 160 (Tables 4 and 5).

The randomized controlled trial: changes in bone mass density and bone markers

The changes in bone markers and bone mass density presented here have been previously published in tabular form [18]. Procollagen type I N-terminal propeptide (PINP) was lower at day 80 (17 µg/L) compared with baseline (72 µg/L) in the denosumab treated group, while it remained stable in the placebo group (69 µg/L vs. 71 µg/L). Type I collagen cross-linked C-telopeptide (CTX) was undetectable in all denosumab treated men on day 80 (dropping from baseline value of 203 ng/L), while it increased in the placebo group (from 207 ng/L at baseline to 241 ng/L on day 80). There were some increases in total bone mineral density (BMD) (1.32 vs. 1.31 g/cm^2; $p = 0.010$), spine BMD (1.27 vs. 1.23 g/cm^2; $p < 0.001$), and hip BMD (1.11 vs. 1.03 g/cm^2; $p = 0.033$) (Fig. 4A–D) in the denosumab treated men on day 160 compared to baseline. Despite the increases, no differences were seen between the denosumab and placebo group on day 160 (all $p > 0.05$). There were also no differences between groups in total *Z*-score (1.2 vs. 1.0 $p = 0.592$), spine *Z*-score (0.4 vs. 0.2, $p = 0.400$), hip *Z*-score (0.2 vs. 0.2, $p = 0.946$), or neck of the hip (0.3 vs. 0.2, $p = 0.928$).

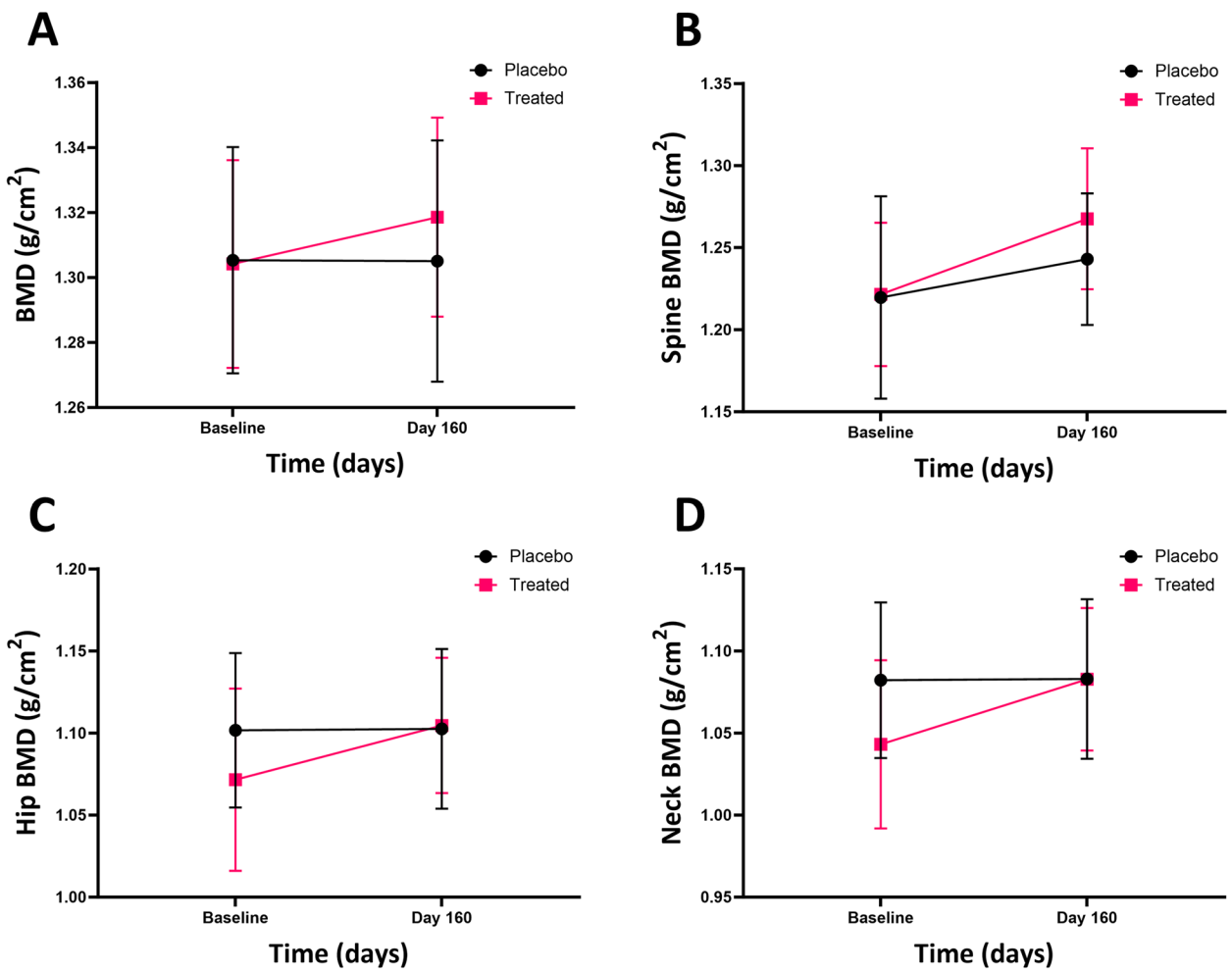

Fig. 4 Changes in bone mineral density in the randomized controlled trial. Plots for changes in bone mineral density (BMD) for **A** total, **B** spine, **C** hip, and **D** neck in patients who underwent intervention with denosumab compared to placebo

Discussion

This study shows that a single injection of denosumab in young infertile men induces a transient decrease in serum calcium and a subsequent compensatory increase in serum PTH, which lowers serum phosphate. All the observed changes are relatively subtle although the initial decrease in serum calcium may transiently induce symptoms of hypocalcemia in some men which can be treated with vitamin D_3 and calcium supplementation as the men also received in this study. The observed decline in ionized and total calcium is consistent with the expected mechanism-of-action of denosumab, which is to inhibit osteoclast activity and reduce bone resorption [29]. Comparable effects of denosumab were found in the young infertile men with normal BMD in the present study as previously reported in postmenopausal women with osteoporosis [30]. The initial decline in calcium was fully reversible and calcium concentration returned to baseline values at day 180, which is the duration of a single-dose denosumab 60 mg subcutaneous injection. However, serum PTH was not normalized after 180 days, which highlights that the increase in PTH could still be secondary to the changes in skeletal metabolism despite no significant differences in BMD in the bone of these infertile but otherwise healthy males after 180 days. In the first intervention study, calcium homeostasis was also assessed 270 days after treatment and here serum PTH was normalized back to baseline despite that alkaline phosphatase activity was not fully normalized, which supports normalization of calcium homeostasis shortly after treatment stop in these young infertile men with normal BMD.

Our findings align with previous studies reporting similar reductions in ionized calcium following denosumab treatment [31]. Calcium changes typically reflect a more advanced stage of imbalance, often occurring

after prolonged disruptions in phosphate or PTH levels. The rise in PTH in particular suggests a compensatory response to low calcium levels, which could have implications for bone metabolism and overall mineral balance. Similarly, we observed a comparable trend in serum phosphate levels, where a temporary decrease occurred during the initial stages of denosumab treatment probably due to the rise in PTH. Overall, the net effect of increased PTH on serum phosphate levels depends on the balance between the inhibitory effect on phosphate reabsorption in the kidneys and the stimulatory effect on intestinal phosphate absorption through the activation of vitamin D [32]. Moreover, the regulation of serum phosphate is not solely controlled by PTH with other factors, such as fibroblast growth factor 23 (FGF23), and calcitonin also playing important roles in phosphate homeostasis [33]. As bone resorption is diminished, less phosphate is released into the bloodstream, contributing to the observed decline. The subsequent normalization of phosphate levels as the treatment progressed is suggestive of a compensatory mechanism, e.g., an increased renal reabsorption of phosphate to maintain overall phosphate homeostasis [34, 35].

Alkaline phosphatase concentrations also decreased during denosumab treatment. During bone remodeling, osteoblasts actively deposit new bone matrix, and this process is associated with the release of alkaline phosphatase into the bloodstream [36]. As denosumab suppresses bone resorption and reduces bone remodeling, the activity of osteoblasts is also affected. We found a reduction in alkaline phosphatase activity at the outset of treatment, which gradually returned to baseline levels as the treatment period advanced. However, the change occurred later than the changes in calcium and phosphate. This delayed decrease in alkaline phosphatase activity is in line with the suppressed bone remodeling process attributed to denosumab's action on osteoclast inhibition and has previously been shown after the use of denosumab [37, 38]. Moreover, these men have only been injected with denosumab once, and the rebound phenomenon is generally not considered to happen after 2 injections in osteoporotic patients.

A link between mineral homeostasis, particularly calcium and phosphate, and reproductive function has received more attention in recent years [39]. Calcium supports sperm motility, acrosome reaction, and fertilization, while phosphate aids ATP production for energy-demanding processes and may be important for sperm maturation [40]. Dysregulation of these minerals can impair gamete function and fertility, potentially influenced by regulators such as PTH. Our study cohort consisted of young, infertile men, and while infertility, in general, can be attributed to various factors, including hormonal imbalances, testicular dysfunction, and genetic abnormalities, there is a possible relationship between impaired fertility and disturbances in mineral homeostasis, primarily calcium and vitamin D [41]. The male infertility cohort was heterogeneous, as some participants suffered from conditions like varicocele and cryptorchidism, contributing to the variability in fertility status that is also influenced by the high intraindividual variation in semen quality variables. Therefore, understanding the effects of denosumab on mineral homeostasis in infertile men may offer preliminary insights into the complex interplay between reproductive function and skeletal and mineral homeostasis. Currently, denosumab is tested in a randomized clinical trial as a potential treatment for male infertility by inducing an increase in sperm production [26]. Therefore, an increased understanding of the multiple effects of denosumab on bone health and male fertility is a necessity if a potential multifaceted therapeutic use is considered.

This study has several strengths that enhanced the reliability and validity of the findings. First, the combination of an intervention study and an RCT with a placebo group allowed us to confirm the findings in different ways and account for potential confounding factors. The intervention study provided valuable insights into the changes in biochemical markers and bone mineral metabolism induced by denosumab treatment, and each participant provided blood samples 8–10 times during the study period. This ensured a detailed follow-up description of the serum markers. The RCT and placebo controlled design allowed for a direct comparison between a denosumab treated group and a control group, providing a framework for evaluating the effects of the treatment. Another strength of this study lies in the application of a robust statistical model, the linear mixed model for repeated measures. This model is well-suited for analyzing longitudinal data with missing observations, which is particularly relevant in our study due to the follow-up nature and potential dropouts over time even when the sample size is small. Additionally, to minimize the risk of false positive (type I error) due to multiple comparisons, Dunnett's method was applied for adjustment.

However, it is also essential to acknowledge certain limitations in our study as well. Firstly, the sample size was relatively small in both cohorts, which may have restricted the generalizability of our findings to a broader population. Some analyses are also lacking, i.e., in the intervention study, the lack of bone biomarkers, such as PINP and CTX, limits the ability to fully assess bone turnover, including osteoblast and osteoclast activity, and while total serum alkaline phosphatase was measured, we did not differentiate between the bone and liver isoforms. Additionally, the randomized controlled trial's study

duration might not have been sufficiently long to capture the full extent of denosumab's long-term effects on bone outcomes as the 160-day follow-up occurs before denosumab bioavailability decreases, thus limiting conclusions about the lack of a subsequent increase in bone turnover. Furthermore, although, a linear mixed model was used to account for missing data, the small study cohorts (i.e., $n=12$) and the presence of some missing observations, especially on day 270, may still introduce some bias. Lastly, the study's focus on young infertile men limits the direct applicability of our findings to other populations, and we acknowledge the need for larger and more diverse study populations, longer follow-up periods, and consideration of additional outcome measures in future research.

Conclusions

In conclusion, our study explored the effects of denosumab on mineral homeostasis in a population not typically treated with the drug. It confirmed the initial drop in serum calcium, phosphate, and alkaline phosphatase, with a corresponding increase in PTH, followed by a return to baseline levels. These findings underscore the interplay between denosumab and bone mineral metabolism in young infertile men.

Abbreviations

BA	Bone area
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
CV	Coefficient of variation
CTX	Type I collagen cross-linked C-telopeptide
DXA	Dual-energy X-ray absorption
eGFR	Estimated glomerular filtration rate
LC–MS	Liquid chromatography–mass spectrometry
OPG	Osteoprotegerin
PINP	Procollagen type I N-terminal propeptide
PTH	Parathyroid hormone
RANK	Receptor activator of the nuclear factor κB
RANKL	Receptor activator of the nuclear factor κB ligand
RCT	Randomized controlled trial
SD	Standard deviation
$1,25(OH)_2D_3$	1,25-Dihydroxyvitamin D_3
25(OH)D	25-Hydroxyvitamin D

Supplementary Information

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Supplementary Material 1.

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Authors' contribution

M.B.J. designed the study and directed the analyses. S.K.Y. reviewed the literature, organized the data, and wrote the initial draft. In line with the mentioned authors, R.H., L.J.M., L.B.Á., I.M.B., A.J.1, and A.J.2. participated in the discussion and interpretation of the results and critically revised the manuscript for intellectual content. SKY is the guarantor of this work and takes responsibility for data integrity. All authors read and approved the final manuscript.

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Data availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The clinical trials in this study were approved by the Research Ethics Committee of Region Copenhagen (H-15001992 and H-16039285) and listed at ClinicalTrials.gov (NCT02422108 and NCT03030196). Written consent has been obtained from each patient or subject after a full explanation of the purpose and nature of all procedures used.

Consent for publication

Not applicable.

Competing interests

MBJ holds two patents on the use of RANKL inhibitors to treat male infertility and a spin out company (XY Therapeutics) has been generated based on this IP. All other authors state no conflicts of interest.

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