Increase in Osteocalcin Following Testosterone Therapy in Men With Type 2 Diabetes and Subnormal Free Testosterone

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Context: One-third of men with type 2 diabetes have subnormal free testosterone concentrations. We evaluated the following: (i) whether bone mineral density (BMD) and bone strength are affected by gonadal status in type 2 diabetes and (ii) the effect of testosterone replacement on markers of osteoblast and osteoclast activity.

Design: This is a **s**econdary analysis of a previously completed, randomized, placebo-controlled trial. Ninety-four men with type 2 diabetes were recruited; 44 had subnormal free testosterone concentrations. Men with subnormal free testosterone concentrations were randomized to receive intramuscular injections of testosterone or placebo every 2 weeks for 22 weeks. Dual energy X-ray absorptiometry scans were performed at baseline and at 23 weeks.

Results: Men with subnormal free testosterone had similar BMD compared with men with normal free testosterone. However, bone strength indices were lower in men with subnormal free testosterone. BMD was related to free estradiol concentrations ($r = 0.37$, $P = 0.004$ at hip), whereas bone strength was related to free testosterone concentrations ($r = 0.41$, $P < 0.001$). Testosterone replacement increased osteocalcin concentrations [mean change (95% CI), 3.52 (0.45, 6.59), $P = 0.008$]. C-Terminal telopeptide (CTx) concentrations also increased at 15 weeks but reverted to baseline following that. There were no changes in other bone turnover markers or BMD.

Conclusion: We conclude that testosterone replacement resulted in an increase in osteocalcin and a transient increase in CTx, indicating an increase in osteoblastic activity and transient increase in bone breakdown. Therefore, a major action of testosterone is to increase bone turnover in men with type 2 diabetes.

Osteoporosis is an established complication of the male hypogonadal state [1]. Subnormal testosterone concentrations lead to decreases in bone mineral density (BMD) in men at all ages, regardless of the cause of hypogonadism [2]. Osteopenia or osteoporosis is present in 40% of

men with Klinefelter syndrome [3]. Hypogonadism is common in elderly men, and testosterone concentrations are inversely related to BMD and fractures in these men [4]. Testosterone therapy in hypogonadal men induces an increase in BMD at the spine and hip [5, 6].

Obesity and type 2 diabetes have emerged as two of the common causes of subnormal testosterone concentrations in men. One-third of men with type 2 diabetes have subnormal free testosterone concentrations. The luteinizing hormone concentrations are not elevated in these men [7]. Men with type 2 diabetes have a higher risk of hip and nonvertebral fractures than nondiabetic men [8, 9]. Paradoxically, the BMD is higher by ~5% in men with type 2 diabetes compared with men without diabetes, possibly because they have a higher body weight and lean mass than nondiabetic men [10]. Estimates of bone strength, which can be calculated using variables measured by a dual energy X-ray absorptiometry (DEXA) scan, suggest that bone strength is lower in patients with type 2 diabetes. A low bone turnover state exists in type 2 diabetes, and this probably contributes to the high fracture risk [11–13]. It is not known if the high frequency of subnormal testosterone concentrations contributes to the higher fracture risk or low bone turnover in men with type 2 diabetes nor have the effects of testosterone replacement in men with type 2 diabetes and subnormal free testosterone on bone turnover and BMD been studied.

In a recent study conducted in men with type 2 diabetes and subnormal free testosterone concentrations, testosterone was shown to reduce adiposity, increase lean body mass, and increase insulin sensitivity. The study also demarcated the four molecular levels with which insulin signal transduction was interfered in these men and how this interference was reversed at all four sites by testosterone [14]. We have now investigated the effect of testosterone on the indices of osteoblast and osteoclast activity. We hypothesized that testosterone therapy will accomplish the following: (i) increase bone formation, as reflected by increase in osteocalcin, and (ii) decrease bone breakdown, as reflected by decreases in sclerostin, receptor activator of nuclear factor-κB (RANK), RANK ligand (RANKL), osteoprotegerin, and serum C-terminal telopeptides (CTx).

1. Patients and Methods

We recruited men with type 2 diabetes between the ages of 30 and 65 years, hemoglobin A1c (HbA1c) ≤ 8%, and stable diabetes regimen for 3 months in a randomized, placebo-controlled trial of testosterone replacement that was funded by the National Institutes of Health. Subnormal free testosterone was defined as concentration <5 ng/dL on two occasions along with low or normal luteinizing hormone concentrations. The details on study design, as well as the results on insulin sensitivity, inflammation, and body composition from that trial, have previously been published [14]. In brief, 50 men with normal free testosterone and 44 men with subnormal free testosterone concentrations participated in the primary trial. All subjects had been diagnosed with diabetes by their treating physician. All men with subnormal testosterone and 44 out of 50 men with normal testosterone were on antihyperglycemic therapy. Six men with normal testosterone were diet controlled. Men with subnormal free testosterone concentrations were randomized with a computerized random number generation program (Microsoft Office, Excel) to receive intramuscular injections of testosterone (therapy initiated with 200 mg) or placebo (saline 1 mL) every 2 weeks for 22 weeks. The dose of testosterone was adjusted to keep serum free testosterone concentrations in the midnormal range. Two men in the testosterone group and eight men in the placebo group dropped out. The predominant reason was lack of time to follow the study protocol in its entirety. Serum samples were collected at baseline, 15 weeks, and 23 weeks after the start of therapy. DEXA scans were performed at baseline and 1 week after the last injection (week 23). The results presented below are secondary analyses of the stored samples from the trial. As a result of the limited amount of material remaining for this secondary analysis, the number of samples tested in each assay varied depending on type and amount of sample needed per test (serum, plasma, or mRNA) and the order of testing. This limitation of sample availability was more prominent in the follow-up samples (week 23) as a result of

dropouts from the original study. The protocol was approved by the Human Research Board of the State University of New York at Buffalo, informed consent was signed by all subjects, and the trial was registered with clinicaltrials.gov (NCT01127659) [14].

A. Laboratory Assays

Testosterone and estradiol concentrations were measured by liquid chromatography–tandem mass spectrometry (Quest Diagnostics) [15]. A detailed description of the methodology has previously been published [15]. For testosterone, the sensitivity of the assay (limit of quantitation), set at a coefficient of variation (CV) of ≤20%, was 0.3 ng/dL. The intra-assay CV ranged from 7.6% to 10.8% and interassay CV ranged from 9.8% to 13.4% at testosterone concentrations between 10 and 1200 ng/dL. The CV of the assay for estradiol was 15% at an estradiol concentration of 1.5 ng/dL and 13% at 20 ng/dL. The limit of quantitation for estradiol in this assay was 0.2 ng/dL. Tracer equilibrium dialysis was used to separate the free testosterone and free estradiol (Nichols Institute, Chantilly, VA, and San Juan Capistrano, CA) [15, 16].

A-1. Bone turnover markers

In this manuscript, we have used the term "bone turnover" to indicate bone resorption and/or formation. ELISA was used to measure osteocalcin (Cat. #DSTCN0, R&D Systems, Minneapolis, MN; RRID:AB_2801529 [17]), sclerostin (Cat. #DSST00, R&D Systems; RRID: AB_2801530 [18]), RANKL, osteoprotegerin (Cat. #E-EL-H1341, Elabscience; RRID: AB_2801532 [19]), and CTx (Cat. #E-EL-H0873, Elabscience; RRID:AB_2801531 [20]).

A-2. Mononuclear cell isolation

Blood samples were collected in Na-EDTA and carefully layered on Lympholyte medium (Cedarlane Laboratories, Hornby, ON, Canada). Samples were centrifuged, and two bands separated out at the top of the red blood cell pellet. The mononuclear cell (MNC) band was harvested and washed twice with Hanks' balanced salt solution. This method provides yields >95% MNC preparation.

A-3. RANK and RANKL expression in MNC

mRNA expression of RANK and RANKL was measured in MNC by RT-PCR. Total RNA was isolated using the commercially available RNAqueous®-4PCR Kit (Ambion, Austin, TX). Real-time RT-PCR was performed using the Mx3000P QPCR System (Stratagene, La Jolla, CA), SYBR Green Master Mix (Qiagen, Valencia, CA), and gene-specific primers (Life Technologies, Gaithersburg, MD) for RANK and RANKL. All values were normalized to the expression of a group of housekeeping genes, including actin, ubiquitin C, and cyclophilin A.

B. DEXA Scan

Total and regional lean body mass, fat mass bone mineral content, and bone density were measured by DEXA (GE Lunar Prodigy) at baseline and week 23. Appendicular BMD (arm or leg) in the manuscript is the average of right and left limbs. The CV in the measurement of BMD, lean mass, and fat mass is 0.5%, 0.9%, and 1.2%, respectively [21, 22].

Estimates of structural properties of the hip were calculated using "Advanced Hip Analysis" software (GE Lunar). Femoral neck length was defined as distance along the neck axis from the center of the femoral head to the neck/shaft axis intersection. The CV for measurement of neck length is 0.6% on Lunar Prodigy [23]. Cross-sectional area (CSA) of the bone was estimated from mineral density. CSA of a bone refers to the surface of bone tissue after the subtraction of the area of voids, spaces, and marrow cavity that do not provide

substantial load support. Bone strength index (BSI) combines bone mineral and bone bio-mechanical properties to measure resistance to bending. It is calculated using age, height, weight, BMD, and femur geometry. The section modulus (Z) is another parameter of bending strength. Z is equal to the cross-sectional moment of inertia divided by the distance from the neutral axis to the subperiosteal surface of bone. A higher number on BSI and Z denote more strength. Finally, the buckling ratio (BR; the ratio of the outer radius to the cortical thickness) was calculated. Cortical instability may result when excessive cortical thinning is present. Higher BR is evidence of propensity to fracture.

C. Statistical Analysis

Group comparisons were performed by two-sided t tests, Mann-Whitney rank sum tests, one-way ANOVA, and χ^2 tests, as appropriate. Statistical analysis for change from baseline was carried out using paired t test. Non-normal data were logarithmically transformed to approximate normal distribution for application of parametric tests. Pearson correlation and multiple linear regression analyses between variables were performed using SPSS software (SPSS Inc, Chicago, IL). Data are presented as means ± SD for normally distributed data and median [25th, 75th percentile] for non-normal data. Pearson correlation coefficients are depicted as "r"; r^2 was calculated to demonstrate the variation in the dependent parameter explained by the independent variable. To perform multiple linear-regression analyses, BMD was chosen as the dependent variable, and independent variables of interest were inserted into the model to calculate the standardized coefficients (depicted as "β"). $P < 0.05$ was considered significant. The study end-points were not specified *a priori* with the design of the study. Hence, the results are hypothesis generating and not adjusted for multiple-point comparisons. No statistical assumptions were made to compute missing data as a result of dropouts or unavailability of samples.

C-1. Sample-size calculation

The primary endpoint of the study was to detect a difference in osteocalcin concentrations in subjects treated with testosterone for 23 weeks compared with placebo. The change from baseline to 23 weeks in serum osteocalcin concentrations was compared by t test between the testosterone and placebo arms. We estimated a change in osteocalcin of 50% in the testosterone arm compared with placebo. With the assumption that 15% of stored samples may not be evaluable, we estimated that 14 patients per treatment arm should provide adequate power ($\beta = 0.2$) to detect a significant difference ($\alpha = 0.05$) between the treatment arms, provided the SD of the residuals is not >40%.

2. Results

As previously reported [14], men with subnormal free testosterone concentrations had similar age but higher body mass index (BMI) than men with normal testosterone concentrations (Table 1). HbA1c was similar in the two groups ($7.0 \pm 1.1\%$ vs $7.1 \pm 1.1\%$, $P = 0.66$). Men with subnormal free testosterone concentrations had higher subcutaneous fat mass and lean mass than men with normal free testosterone concentrations. Measures of total and regional BMD were similar in the two groups. However, BMD was lower in men with subnormal free testosterone concentrations when expressed as a ratio of BMD to lean body mass. Four men with subnormal free testosterone and five men with normal free testosterone concentrations had osteopenia. No man in the study had osteoporosis.

BMI was strongly related to BMD at the hip ($r = 0.41$, $P < 0.001$) and leg ($r = 0.32$, $P = 0.002$) but not related to BMD at the arms ($r = 0.03$, $P = 0.76$) and spine ($r = 0.20$, $P = 0.06$). To assess the effect of lean mass and fat mass on BMD, we conducted multiple linear-regression analyses in a model with BMD as the dependent variable, whereas lean mass and fat mass were independent variables. Lean mass was a strong predictor of leg, hip, or total

Table 1. Body Composition, Bone Strength, and Bone Turnover Makers in Men With Subnormal and Normal Free Testosterone Concentrations

	Subnormal Free Testosterone	Normal Free Testosterone	P
Number of subjects	44	50	
Age, y	54.6 ± 7.9	51.5 ± 8.9	0.08
BMI, kg/m^2	39.8 ± 7.8	34.0 ± 6.4	<0.001
Total testosterone, ng/dL	252 ± 82	485 ± 183	<0.001
Free testosterone, ng/dL	4.4 ± 1.2	7.6 ± 2.2	<0.001
Total estradiol, pg/mL	29.6 ± 13.2	25.0 ± 9.9	0.11
Free estradiol, pg/mL	0.65 ± 0.32	0.60 ± 0.23	0.48
Body composition			
Total body subcutaneous fat mass, kg	46 ± 14	34 ± 12	<0.001
Total body lean mass, kg	71 ± 11	64 ± 9	0.002
Arm BMD, g/cm^2	1.10 ± 0.13	1.12 ± 0.13	0.56
Leg BMD, g/cm^2	1.50 ± 0.17	1.47 ± 0.12	0.33
Femoral neck BMD, g/cm^2	1.07 ± 0.17	1.07 ± 0.16	0.99
Hip BMD, g/cm^2	1.17 ± 0.18	1.14 ± 0.17	0.55
Spine BMD, g/cm^2	1.29 ± 0.18	1.25 ± 0.17	0.45
Total body BMD, g/cm^2	1.37 ± 0.12	1.32 ± 0.11	0.05
Hip BMD/total body lean mass	0.016 ± 0.004	0.018 ± 0.003	0.02
Spine BMD/total body lean mass	0.019 ± 0.004	0.020 ± 0.003	0.06
Total body BMD/total body lean mass	0.020 ± 0.003	0.021 ± 0.002	0.03
Hip structural parameters and estimates of bone strength			
CSA, mm^2	174 [163, 206]	178 [167, 206]	0.56
Femoral neck length, mm	54 ± 7	53 ± 9	0.39
Femoral neck diameter, mm	37 ± 3	37 ± 2	0.82
BSI	1.0 [0.8, 1.3]	1.3 [1.1, 1.6]	<0.001
Z, mm^3	839 [758, 986]	884 [754, 1019]	0.28
BR	3.6 [2.7, 5.0]	3.0 [2.5, 3.9]	0.08
Bone turnover markers			
Osteocalcin, ng/mL	2.02 [0.94, 3.74], n = 26	1.81 [0.94, 2.45], n = 25	0.78
Sclerostin, pg/mL	125 [97, 178], n = 26	130 [111, 169], n = 27	0.39
CTx, pg/mL	20.5 [4.0, 50.0], n = 22	6.0 [2.0, 20.5], n = 23	0.06
Osteoprotegerin, ng/mL	42.6 ± 23.2, n = 17	51.7 ± 31.2, n = 29	0.30
RANKL, pg/mL	60 [24, 809], n = 16	20 [12, 103], n = 23	0.20
RANKL expression in MNC, arbitrary units	0.26 [0.16, 0.34], n = 21	0.24 [0.16, 0.39], n = 22	0.98
RANK expression in MNC, arbitrary units	0.48 [0.30, 0.69], n = 21	0.57 [0.42, 0.79], n = 22	0.26

Some data have previously been reported [14]. Brackets indicate [25th, 75th percentile].

body BMD (P = 0.001 for all), whereas fat mass was not (P = 0.87, 0.62, and 0.27, respectively). Lean mass explained ~20% of the variation in BMD at these sites.

Free testosterone concentrations were not related to the hip (r = −0.11, P = 0.38) or spine BMD (r = −0.18, P = 0.08). In contrast, free estradiol concentrations were strongly related to both hip and spine BMD (Fig. 1). Total estradiol concentrations were also related to the hip (r = 0.28, P = 0.03) and spine (r = 0.25, P = 0.04) BMD. Total testosterone concentrations were inversely related to hip BMD (r = −0.37, P = 0.001) but not related to spine BMD (r = −0.18, P = 0.10). The relation of total testosterone with hip BMD was mediated via the association of both of these variables with obesity. Total testosterone was not related to hip BMD in the presence of sex hormone-binding globulin or BMI in multiple regression analyses (β = −0.02, P = 0.88).

To evaluate the contribution of lean mass and estradiol to BMD at various sites, we conducted multiple linear regression analyses with BMD as the dependent variable and lean mass, fat mass, free estradiol, and free testosterone as the independent variables. Lean mass and free estradiol were both independent predictors of BMD at the hip (β = 0.30 and 0.44, P = 0.002 and 0.01, respectively), as well as femoral neck, spine, leg, arm, and total body (data not shown). Estradiol concentrations and lean mass explained ~30% of the BMD at these sites.
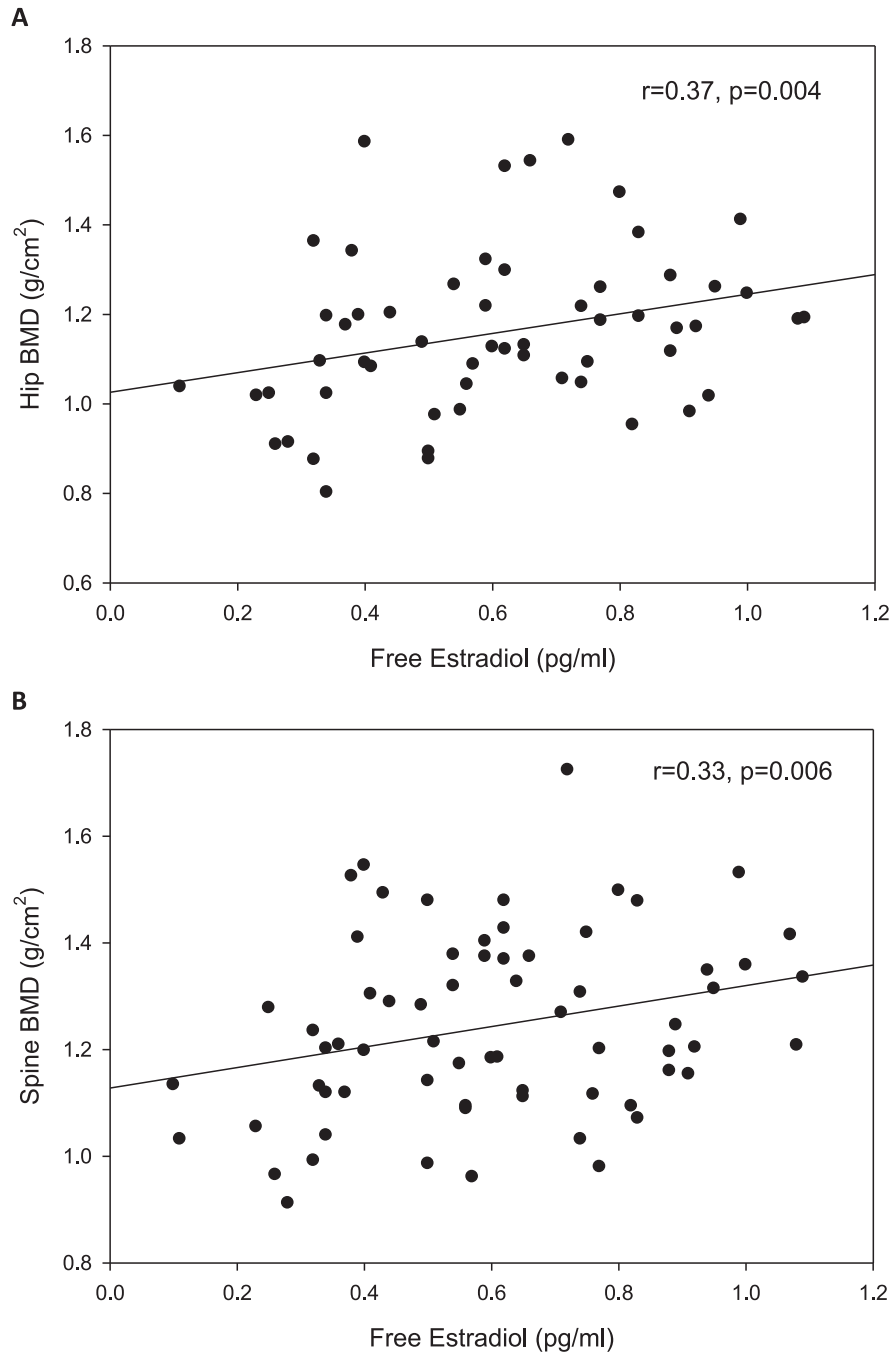
Figure 1. Relation of free estradiol with (A) hip and (B) spine BMD. Estradiol concentrations explained 14% of the variation in BMD at the hip ($r^2 = 0.14$) and 11% at the spine ($r^2 = 0.11$).

We then analyzed the relation of BMD with estradiol and lean mass in men with normal and subnormal free testosterone concentrations separately. BMD was the dependent variable, and lean mass, fat mass, free estradiol, and free testosterone were the independent variables in this model as well. The multiple linear analyses revealed that lean mass was a determinant of hip BMD in men with subnormal free testosterone concentrations ($\beta = 0.41$, $P = 0.01$), as well as men with normal free testosterone concentrations ($\beta = 0.51$, $P = 0.005$). However, free estradiol was a determinant of BMD in men with subnormal free testosterone ($\beta = 0.51$, $P = 0.002$) but not in those with normal free testosterone ($\beta = 0.15$, $P = 0.98$). The

strength of these significant associations was modest, explaining 20% to 25% of variation in BMD. Results were similar for femoral neck, spine, and total body BMD (data not shown).

A. BSIs

BSI was lower in men with subnormal free testosterone concentrations (Table 1). BSI was positively related to free testosterone but not to estradiol (Fig. 2). Z was also positively related
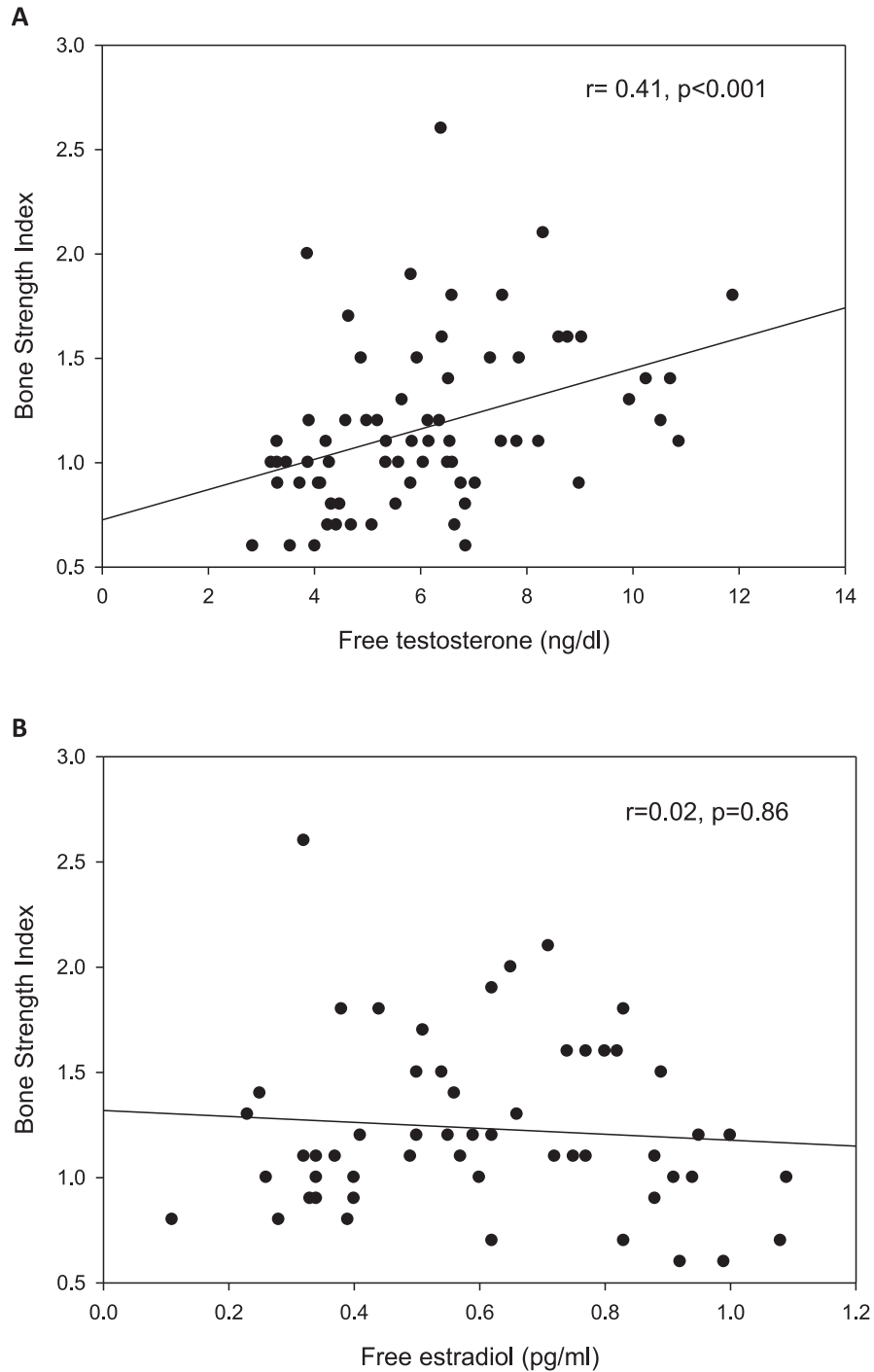

Figure 2. Relation of (A) free testosterone and (B) free estradiol with BSI. Testosterone explained 17% of the variation in bone strength ($r^2 = 0.17$).

to free testosterone concentrations (r = 0.46, P < 0.001) but not to estradiol (r = 0.03, P = 0.83). BR was not related to either free testosterone (r = −0.07, P = 0.58) or free estradiol concentrations (r = −0.19, P = 0.16). However, BR tended to be higher in men with subnormal free testosterone concentrations (Table 1). A ratio > 10 indicates the heightened chance of a precipitous loss of strength with local buckling. No subject in the study had BR > 10. However, a higher number of men with subnormal free testosterone had BR ≥ 5 compared with men with normal free testosterone concentrations (25% vs 8%, P = 0.05, by χ^2).

B. Bone Turnover Markers

There was no difference in bone formation or bone breakdown markers in men, with or without subnormal free testosterone concentrations (Table 1). None of the bone turnover markers were related to serum testosterone, estradiol, lean mass, fat mass, or BMD at any site (data not shown).

C. Effect of Testosterone Treatment in Men With Subnormal Free Testosterone Concentrations

Following testosterone treatment of 23 weeks, free testosterone and estradiol concentrations increased two- to threefold but did not change in the placebo group (Table 2) [14]. Changes in free testosterone and free estradiol concentrations were directly related (r = 0.56, P = 0.03). There was no change in HbA1c [14]. Total body subcutaneous fat mass decreased by ~3 kg, and lean mass increased by a similar amount after testosterone therapy (Table 2). There was no change in BMD at the hip, spine, or total body. BMD in arms or legs did not change either (data not shown). Hip CSA, femoral neck length or diameter, or BSIs did not change after testosterone or placebo therapy (data not shown). The BMD in the spine increased at 23 weeks in the placebo group. This is likely a chance finding that reflects type 2 error as a result of the multiple comparisons conducted in the study.

C-1. Bone turnover markers

There was a substantial increase in serum osteocalcin concentrations following testosterone therapy. This increase was evident at week 15 and persisted until the end of the study (Table 3). The change from baseline in osteocalcin at week 23 of the testosterone group was significantly different than the placebo group [mean change (95% CI), 3.52 [0.45, 6.59], P = 0.008]. There was also an increase in serum CTx concentrations after testosterone therapy at

Table 2. Changes in Testosterone, Estradiol, and Body Composition After Testosterone or Placebo Treatment

	Testosterone (n = 20)			Placebo (n = 14)		
	Baseline	23 Weeks	P	Baseline	23 Weeks	P
Weight, kg	123 ± 23	123 ± 24	0.69	124 ± 30	128 ± 31	0.22
Total testosterone, ng/dL	259 ± 85	561 ± 183	<0.001	239 ± 81	280 ± 132	0.08
Free testosterone, ng/dL	4.5 ± 1.3	13.8 ± 4.1	<0.001	4.2 ± 1.2	5.1 ± 1.7	0.07
Total estradiol, pg/mL	30.1 ± 17.2	62.6 ± 42.7	0.01	26.1 ± 8.3	26.4 ± 10.6	0.92
Free estradiol, pg/mL	0.66 ± 0.43	1.55 ± 1.10	0.02	0.57 ± 0.20	0.59 ± 0.22	0.96
SHBG, nM	27 ± 14	24 ± 10	0.06	26 ± 13	27 ± 13	0.82
Total body subcutaneous fat mass, kg	44.5 ± 13.7	42.1 ± 12.5	0.02	44.5 ± 15.0	45.4 ± 14.4	0.11
Total body lean mass, kg	70.6 ± 9.2	73.2 ± 10.7	0.001	69.1 ± 13.4	68.3 ± 13.0	0.41
Hip BMD, g/cm^2	1.15 ± 0.19	1.14 ± 0.19	0.61	1.15 ± 0.17	1.14 ± 0.15	0.20
Spine BMD, g/cm^2	1.27 ± 0.17	1.28 ± 0.15	0.57	1.25 ± 0.19	1.31 ± 0.21	0.04
Total body BMD, g/cm^2	1.36 ± 0.13	1.36 ± 0.13	0.85	1.35 ± 0.12	1.33 ± 0.13	0.45

Some data have previously been reported [14].
Abbreviation: SHBG, sex hormone-binding globulin.

Table 3. Bone Turnover Markers After Testosterone or Placebo Treatment

Bone Turnover Markers	Treatment Arm, n	Baseline	Week 15	Week 23
Osteocalcin, ng/mL	Testosterone 14	1.91 [0.74, 3.50]	4.52 [1.11, 8.11][a]	3.89 [2.19, 6.09][a]
	Placebo, 12	2.47 [1.51, 3.84]	3.09 [0.93, 4.23]	2.23 [1.64, 2.49]
Sclerostin, pg/mL	Testosterone, 16	102 [70,145]	104 [64, 117]	117 [88, 156]
	Placebo, 10	151 [125, 185]	127 [102, 183]	138 [112, 216]
CTx, pg/mL	Testosterone, 15	23.5 [4.5, 54.5]	73.5 [27.5, 94.5][a]	29.5 [19, 54.5]
	Placebo, 7	18.0 [5.5, 31]	24.5 [10.0, 63.0]	20.5 [9.5, 27.5]
Osteoprotegerin, ng/mL	Testosterone, 17	42.6 ± 23.2	49.5 ± 28.6	44.9 ± 22.5
	Placebo	Not done		
RANKL, pg/mL	Testosterone, 16	60 [24, 809]	55 [14, 839]	47 [15, 779]
	Placebo	Not done		
RANKL expression in MNC, arbitrary units	Testosterone, 15	0.26 [0.16, 0.36]	0.19 [0.14, 0.53]	0.20 [0.12, 0.39]
	Placebo, 6	0.26 [0.17, 0.33]	0.36 [0.14, 0.64]	0.29 [0.19, 0.37]
RANK expression in MNC, arbitrary units	Testosterone, 15	0.41 [0.29, 0.58]	0.35 [0.29, 0.50]	0.36 [0.24, 0.52]
	Placebo, 6	0.49 [0.35, 0.71]	0.52 [0.39, 1.11]	0.59 [0.37, 0.78]

The number of subjects used for each comparison is mentioned in the second column, except those indicated as Not done. Brackets indicate [25th, 75th percentile].

[a]$P < 0.05$ compared with baseline within the same treatment arm.

15 weeks, but it reverted to baseline by week 23. The change from baseline in CTx at week 23 was not different than placebo [mean change (95% CI), 4.4 [−17.4, 26.2], $P = 0.83$]. There was no change in serum sclerostin, osteoprotegerin, or RANKL concentrations. Expression of RANK or RANKL in MNC also did not change following testosterone treatment (Table 3).

The change in osteocalcin was not related to change in free testosterone (r = 0.26, $P = 0.39$) or estradiol (r = 0.56, $P = 0.15$) concentrations.

3. Discussion

Our data show clearly that testosterone replacement induced a twofold increase in plasma concentrations of osteocalcin, a secretory product of the osteoblast and an indicator of osteoblastic activity in the bone. This increase was observed at 15 weeks and was maintained at 23 weeks at the end of the study. Contrary to our hypothesis, there was also a transient increase in plasma concentrations of CTx, an indicator of increased bone breakdown. The nature of bone remodeling necessitates that an effect on either bone formation or bone breakdown is reciprocated by a compensatory change in the other. It thus appears that testosterone replacement in men with type 2 diabetes and subnormal free testosterone concentrations enhances bone remodeling. This may be of benefit in the context of a low bone turnover state that exists in type 2 diabetes [24]. Thus, testosterone induces osteoblastic activity with a concomitant increase in bone turnover necessary to allow new bone formation.

This observation of an increase in osteocalcin after testosterone therapy is consistent with that from some previous small studies in nondiabetic hypogonadal men. Wang *et al.* [25, 26] found an increase in markers of bone formation (serum osteocalcin, procollagen, and bone-specific alkaline phosphatase concentrations) after transdermal or sublingual testosterone application in hypogonadal men. This was accompanied by a decrease in a marker of bone breakdown, the urinary *N*-telopeptide/creatinine ratio. An increase in osteocalcin was also observed after intramuscular testosterone replacement for 3 months in a small study of eight patients but not seen in another study [27, 28]. None of these studies were randomized, placebo-controlled trials.

Testosterone replacement in men with subnormal testosterone concentrations leads to a proportional rise in estradiol concentrations, as noted by the direct relation between change in free testosterone and free estradiol concentrations after treatment in our study population. Hence, it is difficult to delineate the role of estradiol and testosterone on bone markers

separately in our study. It has been well documented that BMD in men is related more closely with serum estradiol concentrations than with serum testosterone [29, 30]. Consistent with results obtained in other populations, we found that serum estradiol concentrations and BMD are positively related in men with subnormal free testosterone concentrations and type 2 diabetes as well. However, it is likely that there exists a continuum in the effect of both testosterone and estrogen on bone. In young men (aged 18 to 20 years), estradiol is positively related to volumetric BMD but negatively related to cortical thickness in the distal tibia and leg [31]. Free testosterone was positively related to the cortical CSA but not related to volumetric BMD. Epidemiological studies have shown that elderly men with a combination of both low testosterone and low estrogen are more likely to have lower BMD and higher fracture rates than those with either low testosterone or low estrogen [4, 32].

Although BMD is predictive of fracture risk, it is not a direct measure of bone quality or resistance to failure. Other aspects of skeletal geometry are also important in the determination of the fracture risk. We found that men with subnormal free testosterone concentrations had lower estimated bone strength (bending resistance) than men with normal free testosterone concentrations. Contrary to BMD, bone strength was related to testosterone but not to estradiol concentrations. There are limited data on bone strength in hypogonadal men. One study found that men with Klinefelter syndrome had lower estimated failure load, bone stiffness, and cortical thickness at the tibia (based on data obtained by high-resolution peripheral quantitative computed tomography) compared with age- and weight-matched eugonadal men [33]. A cross-sectional survey of middle-aged men showed that Z was positively associated with estradiol concentrations but not with testosterone [34]. Testosterone treatment in elderly men, for 1 year, increases estimated bone strength [5]. We did not observe any changes in BMD or bone strength in our study, likely because the treatment duration of 6 months was not long enough to detect changes on DEXA scans.

Induction of hypogonadism with androgen-deprivation therapy in men with prostate cancer causes a decline in BMD by 3% to 4% within the first year of therapy [35]. This is accompanied by an increase in bone breakdown and formation markers (urine N-terminal telopeptide, serum procollagen type 1 N-terminal propeptide, bone-specific alkaline phosphatase, and osteocalcin). However, there is little to no decline in bone density beyond the first year [36]. Change in bone turnover markers is most rapid in the first 6 months after androgen-deprivation therapy. Bone turnover stabilizes, and markers return to baseline after 12 months of androgen-deprivation therapy [35]. Likewise, menopausal bone loss and bone turnover are most rapid in the first few years following menopause [37]. In our study, we found that a cross-sectional comparison of men with normal or subnormal testosterone concentrations did not reveal any differences in bone turnover markers, suggesting the achievement of a "steady state." However, men with subnormal free testosterone concentrations had lower BMD than expected from their body weight and lean mass.

It is believed that the predominant role of estradiol is via osteoclast suppression, leading to a decrease in bone breakdown [38]. Treatment of hypogonadal men with aromatase inhibitors increases serum testosterone concentrations but decreases BMD [39]. Finkelstein *et al.* [40] found an increase in bone breakdown (measured by serum CTx) after induction of hypogonadism in healthy men with a long-acting gonadotropin-releasing hormone agonist. These men with experimental hypogonadism were then replaced with either testosterone alone or a testosterone + aromatase inhibitor for 20 weeks. The increase in CTx was prevented by testosterone replacement but not if an aromatase inhibitor was also given. This indicates that the increase in estradiol was responsible for the change in CTx. In another study, the treatment of obese hypogonadal patients with testosterone, after a period of a very low-calorie diet, prevented the rise in CTx that often accompanies weight loss [41]. Whether the severe dietary restriction had an independent and lasting effect on CTx requires clarification. However, we found that testosterone therapy in men with type 2 diabetes leads to a transient increase in bone breakdown, despite the increase in estradiol. This is possibly a response to the increase in osteoblast activity. The increase in osteocalcin and CTx, observed after testosterone treatment, is comparable with the increases in these markers after

teriparatide, the most well-known anabolic treatment of bone. Teriparatide treatment usually leads to two- to threefold increases in serum osteocalcin and CTx concentrations within a few weeks of starting therapy [42, 43]. These changes precede the increase in BMD [44]. It is likely that a longer duration of testosterone treatment will also increase BMD in men with type 2 diabetes, but that remains to be determined. The increase in osteocalcin following testosterone is also similar to the increases observed after fluoride treatment [45].

In contrast to the increases in osteocalcin and CTx concentrations following testosterone therapy, sclerostin concentrations were not altered. Plasma osteoprotegerin also did not change significantly nor did RANK or RANKL expression transform in the MNCs. Plasma concentration and the expression of RANKL fell, but the change was not substantial, as a result of the scatter of the data. Prior studies have also not shown an effect of testosterone replacement on sclerostin concentrations in men [41, 46]. However, the use of an aromatase inhibitor increases serum sclerostin and CTx concentrations in men [46, 47].

Our study has many limitations. The study duration is too short—and is likely underpowered—to evaluate changes in BMD or bone strength parameters. The baseline comparisons of bone turnover markers with sex hormones are also hampered by the relatively small number of men and the large variation in the bone turnover markers. The study end-points were not prespecified. Hence, our results need to be confirmed in larger studies of longer duration. It should also be specified that BSIs are currently not validated for clinical use, and their precision needs to be derived in future studies.

In conclusion, testosterone replacement resulted in an increase in plasma concentrations of osteocalcin and CTx, indicating an increase in osteoblastic activity and some increase in bone turnover. Therefore, a major action of testosterone in men with type 2 diabetes is to increase bone turnover.

Acknowledgments

Additional Information

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