

Title

Higher serum undercarboxylated osteocalcin and other bone turnover markers are associated with reduced diabetes risk and lower estradiol concentrations in older men.

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The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Abstract

Context

In mice undercarboxylated osteocalcin (ucOC) modulates insulin secretion and sensitivity, and increases testosterone (T) secretion from Leydig cells, but human data are lacking. We hypothesised that ucOC is associated with diabetes risk and modulates **sex hormone concentrations** in older men, distinct from other bone turnover markers.

Participants

Community-dwelling men aged 70-89 years resident in Perth, Western Australia.

Main outcome measures

Serum **total osteocalcin** (TOC), N-terminal propeptide of type I collagen (P1NP) and collagen type I C-terminal cross-linked telopeptide (CTX) were measured by immunoassay, and ucOC by hydroxyapatite binding. Plasma total T, dihydrotestosterone (DHT) and estradiol (E2) were assayed by mass spectrometry.

Results

Excluding men with osteoporosis, on bisphosphonates, glucocorticoids or warfarin, and conditions affecting sex hormones 2,966 men were included. In multivariate analyses, higher ucOC was associated with reduced diabetes risk (odds ratio [OR] per 1 SD increase=0.55, $p<0.001$). Similar results were seen for TOC (OR=0.60, $p<0.001$), P1NP (OR=0.64, $p<0.001$) and CTX (OR=0.60, $p<0.001$) but not ucOC/TOC. **When all four markers were included in the fully-adjusted model, higher ucOC (OR=0.56, $p<0.001$) and CTX (OR=0.76, $p=0.008$) remained associated with reduced diabetes risk.** E2 was inversely associated with ucOC (coefficient -0.04, $p=0.031$), TOC (-0.05, $p=0.001$) and CTX (-0.04, $p=0.016$); and positively with ucOC/TOC (0.05, $p=0.002$). DHT was inversely associated with ucOC/TOC (-0.04, $p=0.040$). T was not associated with bone turnover.

Conclusions

Higher bone remodelling rates are associated with reduced diabetes risk in older men. Higher ucOC is both a marker of bone remodelling and an independent predictor of reduced diabetes risk. E2 is inversely associated with bone turnover markers. We found no evidence ucOC modulates circulating T in older men.

Introduction

Osteocalcin, an osteoblast secreted protein which constitutes a major component of bone matrix, functions as an endocrine regulator of glucose metabolism in mice [1]. Mice with a targeted deletion of the osteocalcin gene (*Ocn*^{-/-}) develop glucose intolerance and obesity in later life, with reduced pancreatic beta cell mass and insulin content. In the circulation total osteocalcin (TOC), a marker of bone turnover, comprises both gamma-carboxylated osteocalcin and undercarboxylated osteocalcin (ucOC) lacking gamma-carboxylation at one or more sites [2]. Gamma-carboxylation increases binding *in vitro* to hydroxyapatite thus osteocalcin in bone is predominantly carboxylated, while ucOC comprises a large proportion of osteocalcin in the circulation and is considered to be the principal fraction regulating glucose metabolism. Consistent with this, administration of ucOC *in vitro* and in wild type mice increased insulin secretion and sensitivity identifying it as the metabolically active form [3,4]. The receptor for ucOC is a G-protein coupled receptor GPRC6A, expressed in murine Leydig cells [5]. In mice, ucOC regulated production of testosterone (T) in a parallel pathway to luteinising hormone (LH) illuminating a complex relationship between bone turnover and androgen secretion [5].

Translation of these findings from the experimental context to humans has been problematic. While several epidemiological studies have associated lower levels of TOC with adiposity, metabolic syndrome or diabetes [6-10], it is not known whether these observations might

reflect differences in underlying bone turnover rather than a specific influence of osteocalcin. Studies which have reported ucOC data in relation to measures of glucose metabolism showed contrasting results or no clear differentiation between associations of carboxylated osteocalcin vs ucOC with outcomes [11-15]. This is due to the small size of previous studies and the use of immunoassays to measure ucOC without any precipitation step to remove carboxylated osteocalcin, which have been shown to overestimate ucOC [2]. However, Levinger et al using a euglycemic-hyperinsulinaemic clamp, recently showed that ucOC was associated with whole body insulin sensitivity both at rest and post-exercise in obese men [16].

Data examining whether ucOC regulates T levels in men are even more limited. In a study of 69 men with Type 2 diabetes, Kanazawa et al reported a positive association of ucOC with calculated free T and a negative association of ucOC with LH [17]. However, free T was measured using an invalid radioimmunoassay, the results of which correspond poorly to the reference method of equilibrium dialysis [18]. In addition, the relationship of ucOC to the T-derived sex steroids dihydrotestosterone (DHT) and estradiol (E2), and to sex hormone-binding globulin (SHBG) which also modulates bone loss and fracture risk in older men [19-22] remains unclear.

In the present study we tested the hypothesis that ucOC is 1. independently associated with diabetes risk, and 2. modulates circulating T levels in older men. To overcome limitations of previous studies, we examined the associations of T, DHT, E2, SHBG and LH with TOC, ucOC and the ratio of ucOC/TOC, and compared these with N-terminal propeptide of type I collagen (P1NP) and collagen type I C-terminal cross-linked telopeptide (CTX) as distinct

markers of bone formation and resorption respectively [23], in a large population-based cohort of older men.

Participants and methods

Study population

The Health In Men Study (HIMS) is a cohort study of community-dwelling older men from Perth, Western Australia, which has been described previously [24]. Briefly, men aged 65 years or more were randomly selected from the electoral roll (voting being compulsory for Australian citizens) and invited to participate in the study, from which 12,203 men completed a questionnaire and attended for physical examination in Wave 1 (W1, 1996-1999). 4,248 men attended for re-assessment and venesection in Wave 2 (W2, 2001-2004). **Approximately 95% of the men were** of Caucasian ethnic origin. The University of Western Australia Human Research Ethics Committee approved the study, and all men gave written informed consent.

Assessment of medical comorbidities

Medical data collected at W2 were utilised to identify men with a history of prostate cancer, osteoporosis or bone fracture and Paget's disease. Medications data were analysed to identify men receiving androgens or anti-androgen therapy, bisphosphonates or glucocorticoids. The list of medications included then available oral and parenteral bisphosphonates (alendronate, risedronate and zoledronic acid) and the range of glucocorticoid preparations (cortisone, hydrocortisone, dexamethasone and prednisolone). As gamma-carboxylation is a vitamin K-dependent process, we also identified men who were receiving warfarin. Men were considered to have hypertension if they reported this diagnosis at W1 or W2, or used anti-hypertensive medication or had blood pressure $\geq 140/90$ mmHg at W2. Dyslipidemia was

defined as having fasting HDL <0.9 mmol/L, LDL \geq 3.4 mmol/L, triglycerides \geq 1.8 mmol/L or total cholesterol \geq 5.5 mmol/L, or receiving lipid-lowering therapy at W2. Men diagnosed with diabetes, reporting use of glucose-lowering medication, or with fasting or non-fasting glucose at W2 of \geq 7 mmol/L or \geq 11.1 mmol/L respectively, were considered to have (predominantly Type 2) diabetes. Further assessment of morbidity was performed via the Western Australian Data Linkage System (WADLS) which provides electronic linkage to records from death, hospital, and cancer registries and captures admissions to all public and private hospitals in Western Australia [25]. We used the Charlson score to determine the presence of significant medical comorbidity [26]. Medical diagnoses are weighted for severity and summed to provide a weighted index of medical comorbidity. Data were included from 1990 to the time of blood sampling, providing a measure of recent comorbidity.

Laboratory assays

Blood samples were collected between 0800h and 1030h at W2. Aliquots of plasma and serum were prepared immediately following phlebotomy and stored at -80°C until assayed. Serum TOC, procollagen type 1 N-propeptide (P1NP) and collagen type 1 C-terminal cross-linked telopeptide (CTX) were measured by electrochemiluminescence immunoassay using a Modular E170 analyser (Roche Diagnostics, Australia). Coefficient of variation (CV) was 3.7% and 2.9% at 18 and 89 ug/L TOC; 4.0% and 5.7% at 28 and 191 ug/L P1NP; and 4.1% and 3.8% at 0.31 and 0.71 ug/L CTX. Serum samples were incubated with hydroxyapatite (5 mg/mL), mixed and centrifuged to separate out carboxylated osteocalcin **as previously described** [2]. The ucOC in the supernatant was measured using the same assay as for TOC and was reported as a concentration and as fraction of the total. For a reference osteocalcin standard with expected fractional hydroxyapatite binding of 0.80, kindly supplied by

Professor Caren Gundberg (Yale School of Medicine, New Haven, CT), mean fractional hydroxyapatite-bound osteocalcin was 0.77 and between-run imprecision was 6.0%. Plasma total T, DHT and E2 were quantified using liquid chromatography-tandem mass spectrometry (LC-MS) without derivatization using atmospheric pressure photo-ionisation in positive mode for androgens and negative mode for estrogens, from 200 μ L samples as previously reported [27]. Precision profiles displayed CV <6% for T (>0.4 nmol/L), <13% for DHT (>0.7 nmol/L) and <8% for E2 (>25 pmol/L). LH and SHBG had been previously determined by chemiluminescent immunoassay on an Immulite 2000 analyser (DPC-Biomediq, Australia) with CV of <7% for both. Free T was calculated using empirical formula which provides closer concordance with free T measured by equilibrium dialysis compared with calculations based on equilibrium binding equations [28]. Insulin resistance was estimated using homeostasis model assessment (HOMA-IR), as previously described [29]. Vitamin D was measured using a chemiluminescent immunoassay, **as previously reported** [30].

Statistical analysis

The statistical package Stata version 12.1 (StataCorp, College Station, Texas, USA) was used. Baseline descriptive data were shown as mean and standard deviations (SD), or percentages (%). Comparisons of means were performed using two sample t tests with equal variances, which are robust for parametric and modestly skewed distributions with sufficiently large sample sizes [31]. Linear regression analyses were used to examine the associations of each bone turnover marker and the ratio of ucOC/TOC with diabetes, insulin resistance, and with sex hormones. Models were adjusted for age, smoking, body mass index (BMI), waist:hip ratio (WHR), hypertension, dyslipidemia, creatinine, vitamin D and medical comorbidity (Charlson index). Trimmed analyses were performed excluding the lowest and the highest 1% of values to ensure the analyses were not biased by low or high outliers. The

fully adjusted models for DHT and E2 were further explored by incorporation of SHBG. A two tailed p value of <0.05 was considered significant.

Results

Baseline characteristics of the study population

Of the 4,248 men who participated at W2, 4,233 had sex hormone measurements, and 4,010 had measurements of bone turnover markers. 3,992 men had both sex hormones and bone turnover assessed. From these men, we excluded 71 receiving testosterone or anti-androgen therapy, 420 with prostate cancer or orchidectomy and 23 men with Paget's disease of bone. We excluded a further 277 men with a history of osteoporosis, fracture or bisphosphonate use, and finally 53 men using glucocorticoids and 182 using warfarin (see Supplementary Figure SF1). This left 2,966 men who were included in the analysis. Baseline characteristics of men included and excluded from the analysis are shown (Table 1). Men who were excluded from the analysis were older, more likely to have smoked, had more medical comorbidity, and had higher ucOC, ucOC/TOC, P1NP and CTX levels.

SUPPLEMENTARY FIGURE S1

TABLE 1

Associations of ucOC and other bone turnover markers with prevalent diabetes

There were 445 men with diabetes (15.0%). Men with diabetes had lower levels of TOC, ucOC, P1NP and CTX, and a higher ratio of ucOC/TOC compared to men without diabetes (Table 2). In analyses adjusting for age, smoking, BMI, waist:hip ratio, hypertension, dyslipidemia, creatinine, vitamin D and medical comorbidity, a one SD increase in ucOC was associated with an odds ratio (OR) of 0.55 for having diabetes ($p < 0.001$) (Table 3). There

were comparable results with TOC (OR=0.60, $p<0.001$), P1NP (OR=0.64, $p<0.001$) and CTX (OR=0.60, $p<0.001$). These results were not altered by exclusion of men treated with insulin (Supplementary Tables S1 and S2). **The results remained consistent when men with eGFR <60 ml/min were excluded (Supplementary Tables S3 and S4). When TOC, ucOC, P1NP and CTX were included in the multivariate model simultaneously, higher ucOC (OR=0.56, $p<0.001$) and CTX (OR=0.76, $p=0.008$) remained associated with reduced diabetes risk (Table 3).**

TABLES 2 & 3

SUPPLEMENTARY TABLES S1-S4

Associations of ucOC and other bone turnover markers with insulin resistance

Associations of ucOC, TOC, ucOC/TOC, P1NP and CTX with insulin resistance assessed using HOMA-IR were analysed (Supplementary Table S5). In unadjusted analyses, TOC, ucOC and CTX were inversely associated with HOMA-IR, while ucOC/TOC was positively associated. However, these associations were not significant in the fully-adjusted model.

SUPPLEMENTARY TABLE S5

Associations of ucOC and other bone turnover markers with sex hormones

In the fully adjusted analysis, total T was not associated with any bone turnover marker, nor with ucOC/TOC (Table 4). However DHT was inversely associated with ucOC/TOC. E2 was inversely associated with TOC, ucOC and CTX, and positively with ucOC/TOC. SHBG and LH were positively associated with TOC, ucOC, P1NP and CTX, and **inversely** associated with ucOC/TOC.

TABLE 4

Bone turnover and sex hormones: trimmed analyses

The adjusted analyses were repeated after excluding men with hormone levels in the lowest and highest 1% of values, to ensure that the results were not biased by low or high outliers. Associations of LH with bone turnover markers and ucOC/TOC were no longer significant (Table 5). DHT remained **inversely**, and E2 positively associated with ucOC/TOC. E2 remained **inversely** associated with TOC, and SHBG positively associated with TOC, ucOC, P1NP and CTX.

TABLE 5

Combined models with T and SHBG, DHT and SHBG, and E2 and SHBG

The fully adjusted trimmed analyses were repeated, with inclusion of SHBG in the models containing T, DHT and E2 (Table 6). In the combined model, T was not associated with any bone turnover marker, nor with ucOC/TOC. DHT was **inversely** associated with TOC and ucOC. E2 remained **inversely** associated with TOC, ucOC and CTX, and positively associated with ucOC/TOC.

TABLE 6

Discussion

In this large cross-sectional analysis of older men, we showed that ucOC and other markers of bone turnover are associated with lower risk of predominantly Type 2 diabetes. This

association of higher bone remodelling with reduced diabetes risk was **not accounted for by** age, BMI, or other risk factors or comorbidities. **While higher** ucOC may reflect rates of bone remodelling, **it remains independently associated with** lower risk of having diabetes. E2, but not T, was inversely associated with bone turnover. There was no evidence of a specific association of ucOC with T; however ucOC was inversely associated with circulating DHT, the more potent androgenic metabolite of T.

These results extend previous cohort studies **in older men where TOC concentrations have been lower in the presence of diabetes, and inversely associated with BMI and fat mass [6]; and inversely associated with fasting insulin, plasma glucose and HOMA IR [8,9].** However, these studies did not measure ucOC. We found that while older men with diabetes had lower ucOC concentrations compared with non-diabetic men, comparable associations were present for TOC, and for P1NP and CTX.

Previous studies of ucOC have been smaller in size with results differing between studies. **Both** ucOC and carboxylated osteocalcin (cOC) were associated inversely with fasting and 2-hour post-challenge glucose concentrations, and HOMA-IR in a study of 199 men aged a mean of 47 years [11]. **In** a study of 348 non-diabetic men and women aged on average 68 years, ucOC was not associated with HOMA-IR at baseline or after 3 years [12]. **In** a prospective, nested case-control study of 153 cases with newly diagnosed diabetes and 306 matched controls both ucOC and cOC were lower in cases compared with controls at baseline, and cOC not ucOC was inversely associated with HOMA-IR in non-diabetic controls [15]. However, differences in assays for ucOC may have affected these findings.

Although these studies did not support a role for ucOC as a modulator of glucose metabolism as proposed by Lee et al [1], other studies supported a specific role for ucOC in diabetes risk. In 289 adults with Type 2 diabetes, ucOC correlated negatively with HbA1c and fasting plasma glucose in men but not in post-menopausal women [13]. In 79 older men (aged 55-80 years), increases in ucOC over a 2 year follow-up were associated with decreases in HOMA-IR [14]. Both these studies used immunoassays for ucOC. Levinger et al using a hydroxyapatite-binding assay showed that ucOC was associated with whole body insulin sensitivity both at rest and post-exercise in obese men [16]. However, in a study of 129 adults with Type 2 diabetes, ucOC did not correlate with insulin resistance assessed using a euglycemic hyperinsulinemic clamp [32]. In a Japanese study of 1,597 men aged 65 years and above [33], both TOC and ucOC, and the ratio of ucOC/TOC were lower in men with diabetes, but the association of ucOC with diabetes remained after adjustment for TOC in that study.

By contrast, our results from a larger cohort of older men showed that ucOC (measured using a hydroxyapatite-binding step) and higher bone remodelling rates in general were associated with lower risk of diabetes, almost entirely Type 2. Furthermore, in non-diabetic older men TOC, ucOC and CTX were associated inversely with HOMA-IR in unadjusted analyses but not after adjustment for covariates. **An association of lower ucOC but not TOC, P1NP or CTX with diabetes would have supported the Karsenty hypothesis. However, finding that higher ucOC, TOC, P1NP and CTX are all associated with lower diabetes risk suggested that ucOC may behave as a marker of bone remodelling without exhibiting a specific association with glucose metabolism. The combined model indicates that ucOC and CTX are robust predictors which contribute independently of the other bone turnover markers. While the difference between mean ucOC concentrations in the groups of men with and without**

diabetes small ($\sim 2 \mu\text{g/L}$), its significance as a biomarker is appreciable with a 1 SD increase in ucOC ($\sim 5 \mu\text{g/L}$) corresponding to a 45% reduction in the odds of having diabetes.

Interestingly, recent work by De Toni et al showed ucOC increased vitamin D production from murine Leydig cells [34]. In our study the association of ucOC with diabetes risk was independent of vitamin D concentrations.

Oury et al described a mechanism by which ucOC acting via the G-protein coupled receptor GPRC6A expressed in testes regulated production of T in a parallel pathway to LH in mice [5]. However, the relevance of this mechanism to humans had been unclear. Kanazawa et al reported a study of 69 men with Type 2 diabetes in which ucOC and ucOC/TOC correlated positively with free T concentrations [17]. UcOC and ucOC/TOC correlated inversely with LH, and neither TOC nor urinary N-telopeptide concentrations were associated [17]. In that study ucOC was measured using immunoassay without a hydroxyapatite-binding step, and free T was measured by direct radioimmunoassay which does not correspond to the reference method making those results difficult to interpret. However, other studies have shown correlations of ucOC/TOC with total T in overweight middle-aged men and age-matched controls [34], and of TOC with total T and more favourable metabolic parameters in predominantly middle-aged men [35].

In a study of 159 young adult men attending fertility clinics for assessment with their partners, cOC was separated from ucOC by hydroxyapatite binding, and total T measured by immunoassay [36]. In that study, after adjustment for age, and for BMI or waist-hip ratio, neither TOC, cOC or ucOC was associated with total T. In our large cohort study of older men with sex hormones assayed using LC-MS, there was no association of total or calculated free T with ucOC or any other bone turnover marker. Higher SHBG was associated with

TOC, ucOC, P1NP and CTX. In fully-adjusted models including SHBG, DHT was associated inversely with TOC and ucOC, and E2 was associated inversely with TOC, ucOC and CTX, and positively with ucOC/TOC. Therefore although we found no evidence that ucOC modulates circulating T in older men, its association with the more potent circulating androgen DHT indicates that the relationship of bone remodelling with overall androgen status needs to be further clarified. The association of DHT with TOC and ucOC requires further exploration, as this was specific for osteocalcin and was not seen with P1NP or CTX. While lower circulating T has been identified as a risk predictor for bone loss and fracture in older men [19,20], our results with E2 are consistent with previous reports identifying E2 as being the major sex hormone acting on bone in man and a determinant of osteoporosis and fracture risk in ageing men [21,22,37-39].

Strengths of our study include the size of the cohort which provides ample statistical power to detect associations of ucOC and other bone turnover markers with outcomes of interest, robust measurement of ucOC using hydroxyapatite-binding, inclusion of P1NP and CTX for comparison, and accurate measurement of T, DHT and E2 using LC-MS. In our regression analyses we adjusted systematically for potential confounders, and we performed a trimmed analysis to ensure results were not biased by high or low outliers. [The WADLS links individual men to state-wide and national databases covering hospital admissions and related morbidity as well as mortality \[25\]. As outcomes for the entire state of Western Australia are captured and as few men of this age emigrate, the resulting dataset is comprehensive with minimal loss to cross-boundary flows.](#) We acknowledge several limitations of our study, including its observational and cross-sectional nature, which limits our ability to infer causation. [The definition of diabetes encompassing elevated glucose concentrations differs from clinical definitions which require symptomatic hyperglycemia \[40\].](#) We were not able to

adjust for vitamin K intake which may have influenced ucOC concentrations. Men participating in HIMS were drawn from a larger group seen earlier hence a “healthy survivor” effect may be present. We did not have serial blood samples for repeat assays of either bone turnover markers or hormones. Nevertheless, men were venesected early in the morning which would reduce confounding from circadian variation and a single sample provides a reasonable estimate of hormone levels [41]. Men in HIMS were almost entirely of Caucasian ethnicity therefore our results may not apply to younger men, men from different ethnic backgrounds or to women. **The exclusion of men with prostate cancer or osteoporosis, as well as those receiving hormonal therapy or bisphosphonates, enhances the internal validity of our results but limits their wider generalisability.**

Older men are the expanding demographic group with the highest prevalence of Type 2 diabetes [42] and also the greatest risk of osteoporosis and its related morbidity [37]. **Higher bone remodelling rates in older men are considered markers for osteoporosis risk, yet may be associated with metabolically favourable outcomes.** It is conceivable that **while** higher bone remodelling rates result in increased concentrations of all bone turnover markers including ucOC, ucOC **also** exerts metabolically favourable effects. In older men ucOC behaves as a marker of bone remodelling and **of reduced diabetes risk**, while a lower E2 concentration is strongly associated with increased bone remodelling. Gamma-carboxylation of osteocalcin is a vitamin K-dependent phenomenon, higher TOC has been associated with reduced progression of abdominal aortic calcification [43]. Additional studies would be warranted to determine whether ucOC is a biomarker for other health outcomes distinct from TOC, P1NP or CTX.

In conclusion, higher bone remodelling rates are associated with reduced diabetes risk in older men. While higher ucOC was associated with reduced diabetes risk, higher TOC, P1NP and CTX were similarly associated. When all four markers were combined in the same model, higher ucOC remained associated, indicating its role as a marker of bone remodelling and an independent predictor of reduced diabetes risk. Older men with diabetes have an increased risk of fracture compared to non-diabetic men, despite relatively preserved bone mineral density [44]. The association of diabetes with reduced bone remodelling rates illuminates another point of difference with non-diabetic older men, which should be considered when investigating bone metabolism. E2, and to a lesser extent DHT, were inversely associated with bone remodelling rate. While we found no evidence ucOC modulates circulating T in men, its association with circulating DHT and therefore net androgen status require further investigation.

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Table 1 Characteristics of the study population, stratified according to whether men were included in or excluded from the analysis. Data are shown as n (%) for categorical variables, and mean \pm SD for continuous variables. OC=osteocalcin, P1NP=N-terminal propeptide of type I collagen, CTX=collagen type I C-terminal cross-linked telopeptide.

Variable	Men included in analysis N=2,966	Men excluded from analysis N=1,026	P value
Age 70-74 yrs	1,183 (39.9)	313 (30.5)	<0.001
75-79 yrs	1,271 (42.9)	463 (45.1)	
80-84 yrs	434 (14.6)	193 (18.8)	
85+	78 (2.6)	57 (5.6)	
Smoker - Never	1,032 (34.8)	315 (30.7)	0.04
Past	1,786 (60.2)	663 (64.7)	
Current	148 (5.0)	47 (4.6)	
BMI \geq 25 kg/m ²	1,922 (65.0)	683 (66.7)	0.33
WHR \geq 0.90	2,512 (84.9)	886 (86.5)	0.204
Hypertension	2,294 (77.3)	781 (76.1)	0.422
Dyslipidemia	2,159 (72.8)	715 (69.7)	0.056
Diabetes	445(15.0)	166(16.2)	0.367
Charlson index	0.75 \pm 1.48	1.59 \pm 1.88	<0.001
Creatinine, μ mol/L	93.2 \pm 32.0	95.0 \pm 31.1	0.118
Vitamin D, nmol/L	68.4 \pm 23.4	68.2 \pm 23.3	0.812
Total OC, μ g/L	20.8 \pm 12.6	21.4 \pm 13.2	0.204
Undercarboxylated OC, μ g/L	11.0 \pm 5.0	12.3 \pm 6.5	<0.001
Undercarboxylated/total OC	0.55 \pm 0.09	0.60 \pm 0.14	<0.001
P1NP, μ g/L	42.1 \pm 25.9	49.7 \pm 57.8	<0.001
CTX, μ g/L	0.31 \pm 0.17	0.34 \pm 0.22	<0.001

Table 2 Bone turnover markers in men without and with diabetes who were included in the analysis (N=2,966). Men reporting use of glucose-lowering medication, or with fasting or non-fasting glucose at W2 of ≥ 7 mmol/L or ≥ 11.1 mmol/L respectively, were considered to have diabetes. OC=osteocalcin, P1NP=N-terminal propeptide of type I collagen, CTX=collagen type I C-terminal cross-linked telopeptide.

variable	No diabetes N=2,521	Diabetes N=445	P value
Total OC, $\mu\text{g/L}$	21.21 \pm 10.85	18.56 \pm 19.47	<0.001
Undercarboxylated OC, $\mu\text{g/L}$	11.22 \pm 4.73	9.58 \pm 6.29	<0.001
Undercarboxylated/total OC	0.54 \pm 0.09	0.56 \pm 0.10	0.005
P1NP, $\mu\text{g/L}$	42.96 \pm 25.09	37.45 \pm 29.61	<0.001
CTX, $\mu\text{g/L}$	0.32 \pm 0.17	0.26 \pm 0.18	<0.001

Table 3 Odds ratio of prevalent diabetes according to a 1 SD increase in ucOC and other bone turnover markers in 2,966 older men. Men diagnosed with diabetes, reporting use of glucose-lowering medication, or with fasting glucose of ≥ 7 mmol/L or non-fasting ≥ 11.1 mmol/L respectively, were considered to have diabetes. **Individual models: each bone turnover marker and the ratio of ucOC/TOC was analysed separately, combined model: TOC, ucOC, P1NP and CTX were included in the model simultaneously. The ratio of ucOC/TOC was excluded from the combined model.** Adjusted: adjustment for age, smoking, body mass index, waist:hip ratio, hypertension, dyslipidemia, creatinine, vitamin D and medical comorbidity (Charlson index).

variable	Individual models				Combined model	
	Unadjusted		Adjusted		Adjusted	
	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Total OC	0.66 (0.55,0.79)	<0.001	0.60 (0.50,0.72)	<0.001	1.18 (0.96, 1.45)	0.106
ucOC	0.58 (0.50,0.68)	<0.001	0.55 (0.47,0.64)	<0.001	0.56 (0.44, 0.72)	<0.001
ucOC/total OC	1.16 (1.05,1.28)	0.005	1.11 (0.99,1.24)	0.062	N/A	N/A
P1NP	0.72 (0.62,0.84)	<0.001	0.64 (0.54,0.76)	<0.001	1.05 (0.87, 1.28)	0.587
CTX	0.64 (0.56,0.73)	<0.001	0.60 (0.52,0.69)	<0.001	0.76 (0.62, 0.93)	0.008

Table 4 Associations of T, DHT, E2, SHBG and LH with total OC, ucOC, the ratio of ucOC/TOC, P1NP and CTX, adjusted for age, smoking, body mass index, waist hip ratio, hypertension, dyslipidemia, creatinine, vitamin D, and for medical comorbidity (Charlson index) in 2,966 community-dwelling older men. Data are shown as standardised regression coefficients with corresponding p values.

variable	Total OC		UcOC		ucOC/total OC		P1NP		CTX	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
T	-0.005	0.772	-0.002	0.928	-0.029	0.121	0.009	0.651	0.008	0.678
Free T	-0.027	0.121	-0.025	0.177	-0.012	0.523	-0.013	0.487	-0.016	0.397
DHT	-0.009	0.577	-0.014	0.400	-0.038	0.040	-0.001	0.949	-0.009	0.609
E2	-0.052	0.001	-0.037	0.031	0.055	0.002	-0.009	0.630	-0.043	0.016
SHBG	0.058	0.001	0.072	<0.001	-0.051	0.008	0.056	0.004	0.069	<0.001
LH	0.057	0.001	0.051	0.004	-0.055	0.003	0.037	0.043	0.061	0.001

Table 5 Associations of T, DHT, E2, SHBG and LH with total OC, ucOC, the ratio of ucOC/TOC, P1NP and CTX, adjusted for age, smoking, BMI, WHR, hypertension, dyslipidemia, creatinine, vitamin D, and for medical comorbidity (Charlson index), excluding men with hormone levels in the lowest or highest 1% of values in 2,643 community-dwelling older men.

variable	Total OC		UcOC		ucOC/total OC		P1NP		CTX	
	Coefficient	p-value	Coefficient	p-value	Coefficient	variable	Coefficient	p-value	Coefficient	p-value
T	0.015	0.454	0.023	0.289	-0.036	0.13	0.011	0.642	0.036	0.119
Free T	-0.004	0.851	0.002	0.946	-0.024	0.318	-0.008	0.725	0.016	0.495
DHT	-0.010	0.608	-0.019	0.374	-0.049	0.035	0.001	0.972	-0.001	0.969
E2	-0.051	0.005	-0.030	0.115	0.066	0.002	-0.018	0.388	-0.040	0.055
SHBG	0.055	0.007	0.066	0.002	-0.046	0.051	0.053	0.022	0.073	0.002
LH	0.042	0.072	0.025	0.316	-0.044	0.108	0.031	0.247	0.040	0.141

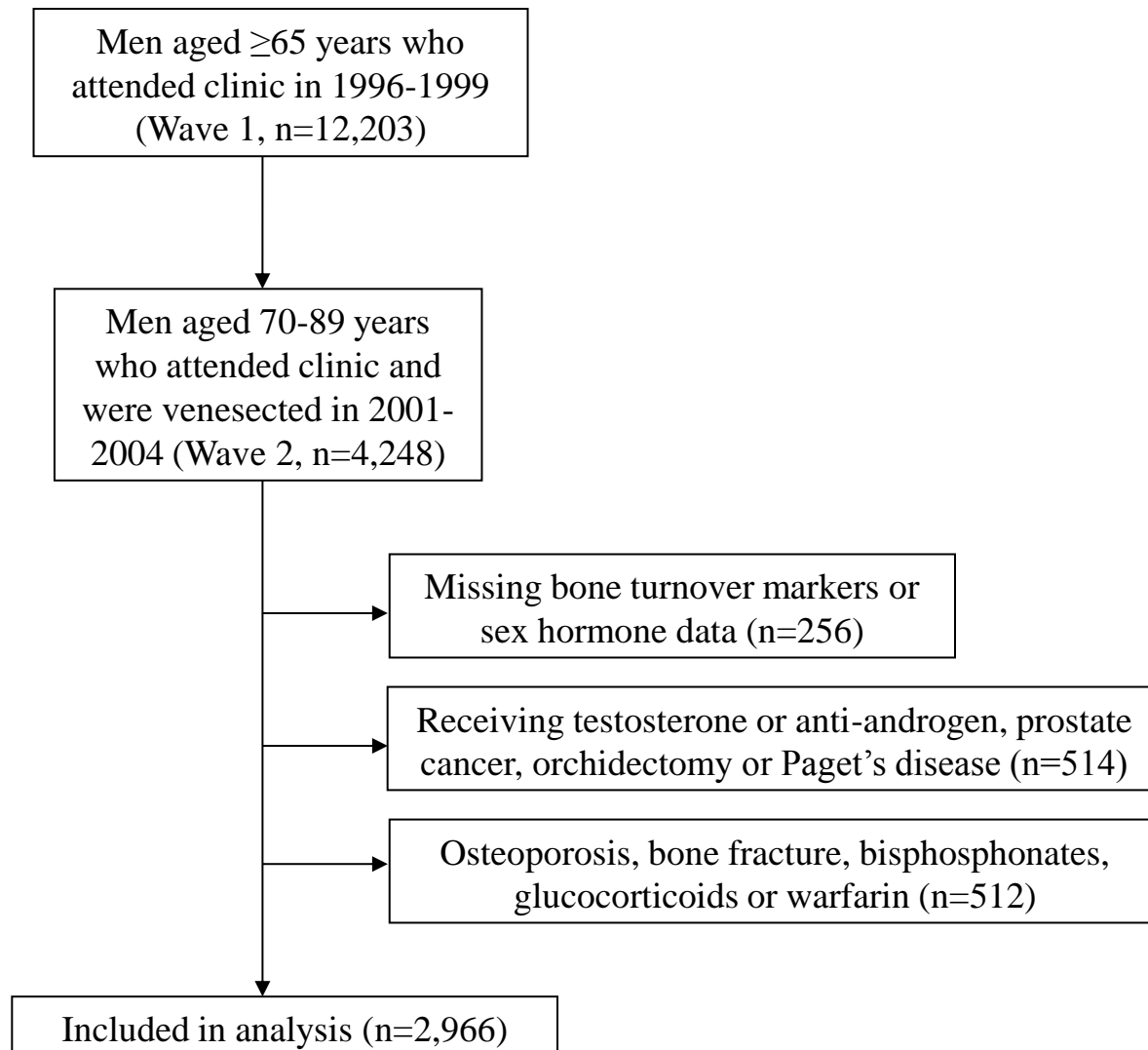
Table 6 Multivariable analysis of associations between the T and its metabolites DHT and E2, with total OC, ucOC, the ratio of ucOC/total OC, P1NP and CTX in 2,643 community-dwelling older men, with both T and SHBG included in the model (A), DHT and SHBG included in the model (B), and E2 and SHBG included in the model (C). Analyses were adjusted for age, smoking, BMI, WHR, hypertension, dyslipidemia, creatinine, vitamin D, and for medical comorbidity and excluded men with hormone levels in the lowest or highest 1% of values.

A	Total OC		UcOC		ucOC/total OC		P1NP		CTX	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
variable										
T	-0.037	0.173	-0.036	0.206	-0.009	0.763	-0.043	0.166	-0.020	0.520
SHBG	0.080	0.003	0.091	0.002	-0.040	0.203	0.082	0.009	0.086	0.006

B	Total OC		UcOC		ucOC/total OC		P1NP		CTX	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
variable										
DHT	-0.047	0.042	-0.064	0.008	-0.035	0.180	-0.031	0.233	-0.045	0.085
SHBG	0.078	0.001	0.098	<0.001	-0.029	0.278	0.068	0.010	0.094	<0.001

C	Total OC		UcOC		ucOC/total OC		P1NP		CTX	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
variable										
E2	-0.065	0.001	-0.045	0.022	0.078	<0.001	-0.029	0.164	-0.056	0.008
SHBG	0.071	0.001	0.077	0.001	-0.065	0.007	0.061	0.011	0.086	<0.001

Supplementary Figure SF1



Supplemental Material

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