Progressive Temporal Change in Serum SHBG, but not in Serum Testosterone or Estradiol, is Associated with Bone Loss and Incident Fractures in Older Men:

The Concord Health and Ageing in Men Project

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ABSTRACT

This study aimed to examine progressive temporal relationships between changes in major reproductive hormones across three waves of a cohort study of older men and (a) changes in bone mineral density (BMD) and (b) incident fractures (any, hip or non-vertebral) over an average of 6-years follow-up. The CHAMP cohort of men aged 70 years and older were assessed at baseline (2005-07, n=1705), 2-year (n=1367) and 5-year follow-up (n=958). Serum testosterone (T), dihydrotestosterone (DHT), estradiol (E2), and estrone (E1) (by liquid chromatography-tandem mass spectrometry (LC-MS/MS)), and of SHBG, LH and FSH (by immunoassay) were measured at all time-points, while free testosterone (cFT) was calculated using a well validated formula. Hip BMD was measured by dual X-ray absorptiometry (DXA) at all three time-points, and fracture data were verified radiographically. Statistical modeling was done using general estimating equations (GEE). For total hip BMD, univariable analyses revealed inverse associations with temporal changes in serum SHBG, FSH and LH and positive associations for serum E1 and cFT across the three time-points. In models adjusted for multiple covariables, serum SHBG (β=-0.029), FSH (β=-0.065), LH (β=-0.049), E1 (β=0.019) and cFT (β=0.033) remained significantly associated with hip BMD. However for femoral neck BMD, only FSH (β=-0.048) and LH (β=-0.036) remained associated in multivariable-adjusted models. Temporal
change in serum SHBG, but not T, E2 or other hormonal variables, was significantly associated with any, non-vertebral or hip fracture incidence in univariable analyses. In multivariable-adjusted models, temporal increase in serum SHBG over time remained associated with any ($\beta=0.060$) and hip fracture ($\beta=0.041$) but not non-vertebral fracture incidence. These data indicate that a progressive increase in circulating SHBG over time predicts bone loss and fracture risk in older men. Further studies are warranted to further characterize changes in circulating SHBG as a mechanism and/or biomarker of bone health during male ageing.

Key words: reproductive hormone; SHBG; bone mineral density; fracture; aging
While it is well known that there is a firm relationship between reproductive hormones and bone health in women, these relationships remain less clear in men. To date, all previous studies in men have used only single baseline hormone measurements as a predictor of subsequent bone loss and fracture risk. Such studies, which may not distinguish between prevalent genetic or other background factors and prevailing hormonal variables, have provided contradictory findings with some, but not all studies showing low baseline serum T or E2 or high serum SHBG levels predicting deterioration in bone health. The majority of previous studies also measured serum T and E2 by immunoassay and reported associations mostly for derived “free” or “bioavailable” fractions of T or E2 using calculations of uncertain validity and interpretation. The few studies that have measured baseline serum T and E2 by mass spectrometry have also provided contradictory findings in predicting deterioration in bone health of older men based on single time-point hormone measurement. Our previous study reported that high baseline serum SHBG levels, but not low baseline serum T and E2, measured by mass spectrometry, was predictive of bone health. However, the progressive temporal relationship between ongoing changes in prevailing circulating hormone levels over time, and bone loss and incident fracture
risk has not been reported. Such investigation may provide more insight into the
dynamics of these contemporaneous relationships over time.

The objectives of this study were to examine temporal associations between
ongoing dynamic changes in circulating reproductive hormones levels integrated with
changes in hip bone mineral density (BMD) and incident fracture risk using general
estimating equations (GEE) over three cohort study waves spanning an average of 6-
years follow-up in older men.

MATERIALS AND METHODS

Study Subjects

The Concord Health and Ageing in Men Project (CHAMP) is a longitudinal,
observational study of the epidemiology of male aging conducted among men living
within three local government areas (Burwood, Canada Bay, and Strathfield)
surrounding Concord Hospital in Sydney, New South Wales, Australia.\textsuperscript{(13)} Men were
selected from the New South Wales electoral roll; enrollment is compulsory in
Australia. Potential subjects were community-dwelling men aged at least 70 years,
with no other inclusion or exclusion criteria. A total of 1705 subjects were enrolled in
the CHAMP study. The study design has been reported in detail elsewhere.\textsuperscript{(13)}
Baseline measurements were conducted between January 2005 and June 2007.
Data were collected using self-reported questionnaires, interviewer-administered questionnaires, and a wide range of clinical assessments. Follow-up assessments of were conducted between January 2007 and October 2009 for 2-year follow-up (n=1367), and August 2010 and July 2013 for the 5-year follow-up (n=958), with identical measurements in each waves as at baseline.

**Hormone Measurement**

Participants had an early morning fasting blood sample taken at all three time-points, with serum stored at -80°C until assay. Measurements of serum T, DHT, E2 and E1 were by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as originally described. Changes to improve assay performance by substituting ultrapressure for high pressure liquid chromatography with corresponding changes in extraction methodology were validated according to FDA criteria (see supplementary methods in (15)). The steroid measurements were calibrated against certified reference materials for T and DHT (National Measurement Institute, North Ryde, Australia), E2 (European Commission’s Institute for Reference Materials and Measurements) and E1 (Cerilliant Corporation). The assays had between-run coefficients of variation (CV) at three levels (low, medium, high) of quality control (QC) specimens of 1.9-4.5%, 3.8-7.6%, 2.9-13.6% and 5.7-8.7%, respectively, over 224 runs including all samples from
the three waves of this study. Overlapping QC samples were routinely run at the start, middle and end of every run with each new QC control run multiple times for calibration before use. There was no evidence of assay drift over the three waves of the present study in QC plots (see supplementary figure in (16)). Steroid profiles were measured in separate batches for the baseline, 2-year and 5-year follow-up samples with all samples from one study period run together. The change to ultrapressure liquid chromatography was introduced between 2 and 5 year follow-up samplings after rigorous confirmation that it did not change accuracy or precision for any QC samples. The steroid assays had limits of quantification (defined by FDA/EMEA as lowest detectable measurement with CV<20%) of 0.025 ng/ml (T), 0.10 ng/ml (DHT), 5 pg/ml (E2) and 3 pg/ml (E1). Serum LH, FSH and SHBG were measured by automated immunoassays (Roche Diagnostics Australia, Dee Why, Australia) subject to ongoing external QC program calibration with between-assay CV for 3 levels of QC specimens in each run of 2.1-2.2% for LH, 2.7-3.0 for FSH, and 2.0-2.8% (two QC levels only) SHBG. cFT levels were computed using an empirical formula validated in two separate studies comprising more than 6000 blood samples. At baseline, serum 25 hydroxy vitamin D (25D) and 1,25 dihydroxy vitamin D (1,25D) levels were measured by radioimmunoassays (RIA) using single-batch reagents (DiaSorin Inc., Stillwater, MN) and serum levels of intact PTH were determined by a
two-site chemiluminescent ELISA on an Immulite 1000 analyser (Diagnostic Products, Los Angeles, CA), as described previously.\(^{(18)}\)

156 **Outcome Measurement**

Bone mineral density at the total hip and femoral neck was measured by dual X-ray absorptiometry (DXA) using a Hologic Discovery-W scanner (Hologic Inc., Bedford, MA, USA). The same DXA scanner was used for all scans at the three time-points. The coefficient of variation (CV) for scans duplicated on 30 men from the study cohort were 1.6% for the total hip. The quality control scans were conducted daily using the Hologic whole body phantom and indicated no shifts or drifts. Neither spinal nor whole body BMD were included in the analyses because age-related osteoarthritis creates difficulty in interpreting spine BMD in older men.

Following the baseline assessment, men were contacted by telephone every four months to ascertain any incident fractures. Phone calls were made up to January 2014. If a fracture was reported, radiology reports were obtained either from the participant, or from hospital medical records and radiology practices. Additional manual searching for fractures were conducted for the men’s medical records within our health district. Only fractures confirmed by radiographic reports were included in the present analysis. Pathological fractures and fractures of hands, fingers, feet, toes and
the skull were excluded. Only the first incident fractures that met the inclusion criteria were included, regardless of trauma level or any additional subsequent fractures reported.\textsuperscript{(19,20)} Time to censorship was either date of death, date of official withdrawal from the study or date of the last telephone contact. The date of first fracture was the date on the radiology report.

\textbf{Potential Confounder Measurement}

Tobacco usage status (current, ex- or never smoker) was self-reported. At clinic assessment, BMI was calculated from height (measured by a Harpendenstadiometer) and weight on regularly calibrated scales, and waist circumference was measured. A comorbidity score was calculated as the sum of the number of conditions reported from 19 disorders listed in the questionnaire.\textsuperscript{(13)} Physical activity was measured using the Physical Activity Scale for the Elderly (PASE).\textsuperscript{(21)} Body fat percentage was measured using dual X-ray absorptiometry. Baseline serum 25D and 1,25D and PTH were included in the confounder sub-analysis. Weight loss was defined, using the modified Frailty Phenotype criteria, as current weight lower by 15\% or more than self-reported heaviest weight (or than weight at 25 years old, if missing data on heaviest weight).\textsuperscript{(22,23)}
Statistical Analysis

Descriptive baseline characteristics based on any, non-vertebral and hip fractures were generated for the analytic sample. Men that were on androgen or anti-androgen treatments (n=20) were excluded from analysis. The temporal associations between changes in reproductive hormones, and changes in BMD across baseline, 2-year and 5-year follow-up and incident fracture risk over an average of 6-year follow-up were assessed by GEE with exchangeable working correlation and robust variance estimator. GEE method is known to be robust and more efficient when treating missing data in longitudinal data. GEE longitudinal analysis included a term for the study follow-up period (baseline, 2 year and 5 year follow-up) which served as a covariate vector to adjust for between study periods (eg in assay or other methodology). Hormones were fitted as continuous variables with results expressed in terms of a 1-SD increase in hormone levels. Our models included hormones and covariates data at all three time-points. Models were fitted using SPSS software version 20 (IBM Corp., Armonk, NY, USA) and SAS software 9.3 (SAS Institute Inc., Cary, NC, USA).

RESULTS

The descriptive characteristic of study participants at baseline, 2-year and 5-year
follow-up are shown in Table 1. The baseline characteristics of the study sample and their stratification by fracture type (all fractures, n=168; non-vertebral fractures, n=137; hip fractures, n=43) are shown in Table 2. There were statistically significant difference between men with or without any fracture in age (78.1 vs 76.8 years), number of comorbidities (2.9 vs 2.5), SHBG (53.7 vs 49.7 nmol/L), hip BMD (0.89 vs 0.94 g/cm²) and femoral neck BMD (0.73 vs 0.77 g/cm²). Likewise in men with or without hip fractures, there were significant difference in age (80.7 vs 76.8 years), number of comorbidities (3.2 vs 2.5), SHBG (56.5 vs 50.0 nmol/L), hip BMD (0.84 vs 0.94 g/cm²) and femoral neck BMD (0.68 vs 0.76 g/cm²).

The temporal association between changes in reproductive hormones and changes in total hip and femoral neck BMD across baseline, 2-years and 5-years of follow-up are shown in Table 3.

For total hip BMD, changes in serum SHBG (β=-0.029), FSH (β=-0.065), LH (β=-0.049), E1 (β=0.019) and cFT (β=0.033), but not serum DHT and E2 were statistically significantly associated with changes in hip BMD in both univariable and multivariable-adjusted models. Change in serum T was significantly associated with changes in total hip BMD only in multivariable-adjusted models (β=0.035) but not in the univariable model.

For femoral neck BMD, progressive temporal changes in serum SHBG, FSH, LH
and cFT were associated with changes in femoral neck BMD over time in univariable models. However, only FSH ($\beta=-0.048$) and LH ($\beta=-0.036$) remained statistically significantly (inversely) associated with changes in femoral neck BMD in the multivariable model.

For fracture risks, the temporal association between changes in reproductive hormones and fracture risks across baseline, 2-years and 5-years of follow-up are shown in Table 4. Temporal changes in serum SHBG, FSH, LH and cFT were associated with any fractures in univariable analyses, but only the increase in serum SHBG ($\beta=0.060$) over time remained significantly associated with the incidence of any fractures in multivariable-adjusted model. The increase in serum SHBG over time was also associated with the incidence of hip fracture ($\beta=0.041$), but not with that of non-vertebral fracture in multivariable-adjusted models. No other hormones measured in the present study were associated with either any, hip or non-vertebral fracture in multivariable-adjusted models.

Due to the strong dependency of serum SHBG on obesity, we performed additional GEE sub-analyses to examine whether the link between SHBG and BMD loss or fracture was mediated by adiposity. Adjusting for waist circumference, body fat percentage or unintentional weight loss in replacement of BMI did not attenuate the observed associations between SHBG and bone loss or fractures (data not shown).
We further performed GEE sub-analysis by adjusting for baseline levels of both serum 25D and 1,25D, and serum PTH levels. The observed associations in our main analyses remained similar after adjusting for both vitamin D metabolites and PTH (data not shown). Similarly, when we excluded men on bisphosphonate, corticosteroid and thyroid medications, the findings remained very similar to the main analyses findings (data not shown).

**DISCUSSION**

This study examines the temporal relationship between changes in major reproductive hormones with concurrent changes in hip BMD and fracture risk in older men across three time-points spanning 6-years continuous follow-up. Our analyses revealed increases in serum SHBG, LH and FSH, and decreases in serum E1 and cFT, but not T, DHT and E2, over time were significantly associated with a decrease in hip BMD. However, only the temporal increase in circulating SHBG levels was predictive of fracture risk. Our findings suggest that changes in serum SHBG levels over time may be an important biomarker of bone health in older men and that this function may not be mediated solely or to any great extent by obesity.

The novel finding of this study was to reveal consistent associations between a temporal increase in serum SHBG levels over time with bone loss and fracture risks.
This finding extends to our previous cross-sectional observations, which suggested that high serum SHBG levels at a single time-point (baseline) were associated with bone health in older men.\(^{(12)}\) Furthermore, recent results from the analysis of the combined MrOS Sweden and MrOS Hong Kong cohorts (n=4324), and MrOS USA cohort (n=1463) demonstrated that baseline serum SHBG is an independent predictor of incident clinical and radiographic vertebral fractures in older men.\(^{(9,11)}\) Together, there is strong evidence suggesting change in circulating SHBG in men may have an important and previously underestimated significance for bone health including maintenance of bone density and fracture risk in older men. However, predictive effects from a single time-point may reflect the impact of background genetic or other fixed risk factors rather than prevailing dynamic effects of hormonal variables. Higher baseline serum SHBG has been reported to be associated in some, but not all, studies with BMD loss and incident fracture risk.\(^{(2)}\) It is widely believed that SHBG effects on bone density and fractures in men may be indirect via modulating androgen effects on bone through influencing passage of androgens (T, DHT) and/or estrogens (E2, E1) from the circulation to bone cells. Yet, changes in serum T and cFT in the present study had similar associations as did serum SHBG with hip BMD over time, but unlike serum SHBG, neither were significantly associated with fracture risk. Furthermore, although SHBG is implicated in the unproven and controversial free
hormone hypothesis which asserts inter alia that SHBG bound T is a biologically inert buffer, there is contrary evidence that SHBG-bound T can participate in signal transduction via a SHBG membrane receptor \(^{(26)}\) or via cellular uptake of SHBG-bound biologically active androgens and estrogens via the megalin mediated endocytotic receptor \(^{(27)}\). Furthermore, although higher blood SHBG does reduce metabolic clearance rate of testosterone \(^{(28)}\) and estradiol \(^{(29)}\) in non-human primates, and thereby prolongs their transit time in the circulation, it is unclear how this mechanism relates to the present findings on bone structure and function. Thus, the mechanism by which SHBG modulates fracture risk remains unclear and other undefined mechanisms and/or unmeasured confounders such as insulin-like growth factor-1 (IGF-1) levels may be relevant \(^{(30,31)}\). It is also possible that SHBG has as yet undefined independent effects on bone health in older men by directly influencing skeletal tissues.

This study has revealed no dynamic temporal associations between progressive decline in serum T and E2 levels with changes in bone health over time in older men. This is consistent with our previous study in which low baseline serum T and E2 did not predict significant bone loss or incident fracture risks \(^{(12)}\). Hence, based on the CHAMP cohort, there is little evidence supporting a direct measurable effect of serum T and/or E2 on bone density and fractures in older men. Previous studies in
men have found associations between low baseline serum T and E2 with loss of BMD.\textsuperscript{(2)} However, most of those studies have reported associations using derived “free” or “bioavailable” fractions of immunoassay measured T or E2 using calculations of uncertain validity.\textsuperscript{(3,5)} Previous studies examining the associations between reproductive hormones and fracture risk in men have been inconsistent. Therefore, it remains possible that low serum T and E2 in older men may have an effect on bone density and fracture risk through other factors such as frailty, muscle mass and/or strength. In that context, decreases in serum T and E2 are reported to be associated with frailty, muscle mass and strength which may subsequently lead to falls and fractures.\textsuperscript{(32,33)}

Our analyses revealed that decreasing circulating E1, an estrogen of low intrinsic potency but capable of conversion to E2 by 17-beta steroid dehydrogenase, over time was associated with greater hip bone loss over time. Serum E1 is usually considered to have no clinical significance in men due to its minimal intrinsic estrogenic bioactivity.\textsuperscript{(34,35)} Hence, the potential functions or biomarker role of circulating E1 in men have not been widely studied in relation to health and diseases.\textsuperscript{(34,35)} Our novel dynamic temporal finding is consistent with previous cross-sectional snapshot studies of older men showing serum E1 positively correlated with total hip BMD\textsuperscript{(36)} and total body BMD\textsuperscript{(37)}. Further investigation is warranted to better understand serum E1 as a
Another major finding of this study was the inverse association observed between temporal changes in FSH and LH and change in both hip and femoral neck BMD. This indicated that men with increasing FSH and LH over time were more likely to have greater bone loss at the hip or femoral neck. The temporal finding from this study is consistent with our previous study which have reported high baseline FSH and LH was statistically significantly associated with hip bone loss. One possible explanation has been FSH and/or LH may have direct effect on bone metabolism, independent of other sex hormones, and the two gonadotropins are highly correlated so that a genuine biological relationship with one may be reflected indirectly in the other. This was proposed for FSH in female mice, but refuted for both FSH and LH in studies showing the effects of FSH and LH on bone are indirect and mediated via gonadal steroid secretion. The most plausible explanation may be that subtle changes due to gradually declining exposure to circulating T over time are more sensitively reflected in men’s serum FSH and LH via the negative feedback mechanism of an intact hypothalamic-pituitary-testicular axis.

This study had a number of major strengths. One was the consistent analysis by the GEE analytical framework of the dynamic temporal relationships between changes in reproductive hormones and bone health over three follow-up time-points.
of continuous follow-up. Another was the use of LC-MS/MS, the gold standard for
steroid assays providing multi-analyte steroid profiling as well as the use of a well
validated formula for cFT.\(^{(5,43)}\) Finally, a further strength is that CHAMP includes a
large and representative group of older Australian men, as demonstrated by the similar
socio-demographic and health characteristics in CHAMP men compared to men in the
nationally representative MATeS study.\(^{(44)}\)

Our study has several limitations. We recorded 20% loss to follow-up from
baseline to 2-year and a further 30% loss from 2-years to 5-years. However, loss to
follow up in cohort studies of older people is inevitable because of the high mortality
rate, which accounted for over a third of the loss in our cohort over 5-years. Diurnal
and seasonal variations in hormone concentrations could potentially have influenced
our results; however, single morning fasting blood samples were obtained to minimize
possible variations due to timing of sample collection\(^{(45)}\) and seasonal variation
appears negligible.\(^{(46)}\) Furthermore, we have not measured insulin sensitivity which
may influence circulating SHBG as well as representing a potentially modifiable risk
factor. The measurements for serum vitamin D metabolites (25D and 1,25D) and PTH
were only available at baseline, hence these possible major regulatory factors were
not adjusted for in the main analysis but only in the sub-analysis.

This is the first study to investigate and report the ongoing dynamic temporal
relationship between changes in circulating SHBG levels with concurrent changes in BMD and incident fracture risk in older men. Men with increasing serum SHBG levels (but not changes in circulating reproductive hormones) over time were more likely to have significant hip bone loss and incident fracture risks at any site or hip. Further longitudinal studies examining temporal changes in circulating SHBG and other major reproductive hormones are warranted to confirm and explain these new findings.

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514 variation in serum sex hormone levels in middle-aged to older men in the
TABLE 1. Characteristics of study participants at baseline, 2-year and 5-year follow-up

<table>
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<th></th>
<th>Baseline Mean (SD) or N (%)</th>
<th>2-year Mean (SD) or N (%)</th>
<th>5-year Mean (SD) or N (%)</th>
<th>Loss to follow-up at 2-year N=332</th>
<th>Loss to follow-up at 5-year N=735</th>
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<td>Age (years)</td>
<td>76.9 (5.5)</td>
<td>79.5 (5.3)</td>
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<td>PASE</td>
<td>124.4 (62.1)</td>
<td>119.7 (59.7)</td>
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<td>100.4 (63.2)</td>
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<td>Current smoker</td>
<td>101 (6%)</td>
<td>52 (4%)</td>
<td>40 (4%)</td>
<td>31 (9%)</td>
<td>49 (7%)</td>
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<td>No. of Comorbidty</td>
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<td>2.5 (1.7)</td>
<td>2.5 (1.6)</td>
<td>2.9 (63.2)</td>
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<td>T (ng/ml)</td>
<td>4.3 (1.9)</td>
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<td>3.4 (1.8)</td>
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<td>DHT (ng/ml)</td>
<td>0.4 (0.2)</td>
<td>0.4 (0.2)</td>
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<td>SHBG (nmol/L)</td>
<td>50.1 (20.7)</td>
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<td>E2 (pg/ml)</td>
<td>25.3 (12.4)</td>
<td>24.2 (9.8)</td>
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<td>25.3 (11.4)</td>
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<td>E1 (pg/ml)</td>
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<td>cFT (pg/ml)</td>
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<td>BMD (g/cm³)</td>
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<td>Hip</td>
<td>0.94 (0.1)</td>
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### TABLE 2. Characteristics of study participants stratified by fracture type (any fracture, n=168; hip fracture, n=43; non-vertebral fracture, n=137)

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<th>Non-Vertebral Fracture</th>
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<td>Age (years)</td>
<td>Yes (n=168)</td>
<td>No (n=1491)</td>
<td>p-value</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>78.1 (5.7)</td>
<td>76.8 (5.5)</td>
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<td>PASE</td>
<td>27.6 (3.9)</td>
<td>27.8 (4.1)</td>
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<td>Waist circumference (cm)</td>
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<td>125.4 (61.7)</td>
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<td>Current smoker</td>
<td>8 (5%)</td>
<td>93 (6%)</td>
<td>0.9</td>
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<tr>
<td>No. of Comorbidity</td>
<td>2.9 (1.8)</td>
<td>2.5 (1.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>T (ng/ml)</td>
<td>4.2 (2.0)</td>
<td>4.3 (1.8)</td>
<td>0.8</td>
</tr>
<tr>
<td>DHT (ng/ml)</td>
<td>0.4 (0.2)</td>
<td>0.4 (0.3)</td>
<td>0.4</td>
</tr>
<tr>
<td>cFT (pg/ml)</td>
<td>58.3 (26.9)</td>
<td>59.7 (21.9)</td>
<td>0.4</td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>53.7 (23.2)</td>
<td>49.7 (20.4)</td>
<td>0.02</td>
</tr>
<tr>
<td>E2 (pg/ml)</td>
<td>24.7 (10.6)</td>
<td>25.4 (12.6)</td>
<td>0.5</td>
</tr>
<tr>
<td>E1 (pg/ml)</td>
<td>39.8 (18.2)</td>
<td>40.5 (16.1)</td>
<td>0.6</td>
</tr>
<tr>
<td>FSH (IU/L)</td>
<td>16.6 (17.7)</td>
<td>14.5 (14.5)</td>
<td>0.08</td>
</tr>
<tr>
<td>LH (IU/L)</td>
<td>10.6 (10.4)</td>
<td>9.5 (8.6)</td>
<td>0.1</td>
</tr>
<tr>
<td>BMD (g/cm²)</td>
<td>Total Hip</td>
<td>0.89 (0.17)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Femoral Neck</td>
<td>0.73 (0.16)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
**TABLE 3. Longitudinal association between temporal change in reproductive hormones* across baseline, 2-year and 5-year follow-up with change in total hip and femoral neck BMD across the three time-points**

<table>
<thead>
<tr>
<th></th>
<th>Total Hip BMD</th>
<th>Femoral Neck BMD</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate†</td>
<td>Univariate</td>
<td>Multivariate†</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>p-value</td>
<td>β</td>
<td>p-value</td>
</tr>
<tr>
<td><strong>T</strong></td>
<td>0.016</td>
<td>0.07</td>
<td><strong>0.035</strong></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>DHT</strong></td>
<td>0.006</td>
<td>0.3</td>
<td>0.015</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>cFT</strong></td>
<td>0.006</td>
<td>0.3</td>
<td><strong>0.033</strong></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>SHBG</strong></td>
<td>-0.081</td>
<td>&lt;0.001</td>
<td>-0.027</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>E2</strong></td>
<td>0.011</td>
<td>0.06</td>
<td>0.008</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>E1</strong></td>
<td>0.018</td>
<td>0.02</td>
<td><strong>0.019</strong></td>
<td>0.009</td>
</tr>
<tr>
<td><strong>FSH</strong></td>
<td>-0.094</td>
<td>&lt;0.001</td>
<td>-0.065</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>LH</strong></td>
<td>-0.078</td>
<td>&lt;0.001</td>
<td>-0.049</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*For per one-SD increase in reproductive hormone levels as estimated by general estimating equations (GEE).
†Multivariate model was adjusted for age, BMI, smoking status, physical activity and number of comorbidities.
TABLE 4. Longitudinal association between temporal change in reproductive hormones* across baseline, 2-year and 5-year follow-up with fractures risk over an average of 6-year follow-up

<table>
<thead>
<tr>
<th></th>
<th>Any Fracture</th>
<th>Hip Fracture</th>
<th>Non-Vertebral Fracture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate†</td>
<td>Univariate</td>
</tr>
<tr>
<td>T</td>
<td>-0.037 0.08</td>
<td>-0.017 0.4</td>
<td>-0.024 0.3</td>
</tr>
<tr>
<td>DHT</td>
<td>-0.003 0.9</td>
<td>-0.0003 0.9</td>
<td>-0.020 0.5</td>
</tr>
<tr>
<td>cFT</td>
<td><strong>-0.043 0.04</strong></td>
<td>-0.019 0.4</td>
<td>-0.025 0.2</td>
</tr>
<tr>
<td>SHBG</td>
<td><strong>0.092 0.004</strong></td>
<td><strong>0.060 0.04</strong></td>
<td><strong>0.072 0.02</strong></td>
</tr>
<tr>
<td>E2</td>
<td>-0.014 0.08</td>
<td>-0.008 0.3</td>
<td>-0.014 0.06</td>
</tr>
<tr>
<td>E1</td>
<td>-0.033 0.08</td>
<td>-0.019 0.2</td>
<td>-0.024 0.2</td>
</tr>
<tr>
<td>FSH</td>
<td><strong>0.079 0.04</strong></td>
<td>0.048 0.2</td>
<td>0.072 0.1</td>
</tr>
<tr>
<td>LH</td>
<td><strong>0.078 0.04</strong></td>
<td>0.043 0.2</td>
<td>0.061 0.2</td>
</tr>
</tbody>
</table>

*For per one-SD increase in reproductive hormone levels as estimated by general estimating equations (GEE).
†Multivariate model was adjusted for age, BMI, smoking status, physical activity and number of comorbidities