Associations between Sex Steroids and the Development of Metabolic Syndrome: a Longitudinal Study in European Men

Postprint after peer review

Leen Antonio, MD[1,2,3], Frederic C.W. Wu, MD[4,5], Terence W. O'Neill, MD[6,7], Stephen R. Pye, PhD[6], Emma L. Carter, PhD[4], Joseph D. Finn, B.Sc.[4], Martin K. Rutter, MD[4,8,9], Michaël R. Laurent, MD[2,10], Ilpo T. Huhtaniemi, MD, PhD[11], Thang S. Han, MD, PhD[12], Michael E. J. Lean, MD[13], Brian G Keevil, MSc[14], Neil Pendleton, MD[15], Giulia Rastrelli, MD, PhD[16,17], Gianni Forti, MD[16], Gyorgy Bartfai, MD[18], Felipe F. Casanueva, MD, PhD[19], Krzysztof Kula, MD, PhD[20], Margus Punab, MD, PhD[21], Aleksander Giwercman, MD, PhD[22], Frank Claessens, PhD[2], Brigitte Decallonne, MD, PhD[1,3], Dirk Vanderschueren, MD, PhD[1,3] and the EMAS Study Group†.

Affiliations:

1. Department of Clinical and Experimental Medicine, KU Leuven, Laboratory of Clinical and Experimental Endocrinology, Leuven, Belgium
2. Department of Cellular and Molecular Medicine, KU Leuven, Laboratory of Molecular Endocrinology, Leuven, Belgium
3. Department of Endocrinology, University Hospitals Leuven, Leuven, Belgium
4. Andrology Research Unit, Endocrinology and Diabetes Research Group, Institute of Human Development, Faculty of Medical and Human Sciences, The University of Manchester, Manchester, UK
5. Manchester Royal Infirmary, Central Manchester University Hospitals NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester M13 9WL
6. Arthritis Research UK Centre of Epidemiology, The University of Manchester, Manchester Academic Health Science Centre, Manchester, UK
7. NIHR Manchester Musculoskeletal Biomedical Research Unit, Central Manchester NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester, UK.
8. The Endocrinology and Diabetes Research Group, Institute of Human Development, Faculty of Medical and Human Sciences, University of Manchester, Manchester Academic Health Science Centre, UK

9. Manchester Diabetes Centre, Central Manchester University Hospitals NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester M13 9WL, UK
10. Department of Clinical and Experimental Medicine, KULeuven, Laboratory of Gerontology and Geriatrics, Leuven, Belgium.
11. Department of Surgery and Cancer, Imperial College London, Hammersmith Campus, London, UK
12. Department of Endocrinology, Ashford and St Peter's NHS Foundation Trust Hospital, Chertsey, Surrey, KT16 0PZ, UK
13. Department of Human Nutrition, University of Glasgow, Glasgow, UK
14. Department of Clinical Biochemistry, University Hospital of South Manchester, Manchester, UK
15. School of Community Based Medicine, The University of Manchester, Salford Royal NHS Trust, Salford, UK
16. Endocrinology Unit, Department Of Experimental Clinical And Biochemical Sciences, University of Florence, Florence, Italy
17. Department Of Sexual Medicine And Andrology Unit, Department Of Experimental, Clinical And Biochemical Sciences, University Of Florence, Italy
18. Department of Obstetrics, Gynaecology and Andrology, Albert Szent-György Medical University, Szeged, Hungary
19. Department of Medicine, Santiago de Compostela University, Complejo Hospitalario Universitario de Santiago (CHUS); CIBER de Fisiopatología Obesidad y Nutricion (CB06/03), Instituto Salud Carlos III; Santiago de Compostela, Spain
20. Department of Andrology and Reproductive Endocrinology, Medical University of Łódź, Łódź, Poland
21. Andrology Unit, United Laboratories of Tartu University Clinics, Tartu, Estonia
22. Reproductive Medicine Centre, Skåne University Hospital, University of Lund, Sweden

†The EMAS Study Group: Florence (Gianni Forti, Luisa Petrone, Giovanni Corona); Leuven (Dirk Vanderschueren, Herman Borghs, Leen Antonio); Łódź (Krzysztof Kula, Jolanta Slowikowska-Hilczer, Renata Walczak-Jedrzejowska); London (Ilpo Huhtaniemi); Malmö (Aleksander Giwercman); Manchester (Frederick Wu, Alan Silman, Terence O'Neill, Joseph Finn, Philip Steer, Stephen Pye, Martin Rutter); Santiago (Felipe Casanueva, Ana I Castro); Szeged (Gyorgy Bartfai, Imre Földesi, Imre Fejes); Tartu (Margus Punab, Paul Korrovitz)

Abbreviated title: Sex Steroids and Metabolic Syndrome Risk

Keywords: Metabolic syndrome, testosterone, estradiol, E2/T ratio, prospective study

Word count: 3649

Number of figures: 1

Number of tables: 3

Corresponding author and reprints:

Leen Antonio

Clinical and Experimental Endocrinology - Laboratory of Molecular Endocrinology

University of Leuven

Campus GHB O/N 1

Herestraat 49 box 902

3000 Leuven

Belgium

Email: leen.antonio@med.kuleuven.be

Grants/fellowships:

The European Male Ageing Study is funded by the Commission of the European Communities Fifth Framework Programme 'Quality of Life and Management of Living Resources' Grant QLK6-CT-2001-00258 and supported by Arthritis Research UK.

This work was supported by grant #G085413N from the Research Foundation Flanders (FWO) and by a research grant from the Academische Stichting Leuven.

Disclosure statement:

Part of this research was presented at the annual meeting of the Endocrine Society 2014, Chicago, Illinois. Abstract # 12458.

LA: received the 'Ipsen poster award in Endocrinology' for the abovementioned abstract and poster presentation.

TWON, SRP, ELC, JDF, MKR, ITH, TSH, MEJL, NP, GR, GF, GB, FFC, KK, MP, AG, FC: nothing to disclose

FCCW: consultant for Besins Healthcare and Repros Inc., research funding from Besins Healthcare and Bayer Schering

MRL: Fellow of the Research Foundation Flanders (FWO), consultant for Novartis. MRL has received lecture fees from Flanders' Agricultural Marketing Board (VLAM).

BGK: consultant for Thermo Scientific

BD: Senior clinical investigator of the Research Foundation Flanders (FWO)

DV: Senior clinical investigator of the University Hospitals Leuven

Abstract

Context: Low testosterone (T) has been associated with incident metabolic syndrome (MetS), but it remains unclear if this association is independent of sex hormone binding globulin (SHBG). Estradiol (E2) may also be associated with MetS, but few studies have investigated this.

Objective: To study the association between baseline sex steroids and the development of incident MetS and to investigate the influence of SHBG, BMI and insulin resistance on this risk.

Methods: 3369 community-dwelling men aged 40-79 years were recruited for participation in EMAS. MetS was defined by the updated NCEP ATP III criteria. Testosterone and E2 levels were measured by liquid and gas chromatography/mass spectrometry respectively. Logistic regression was used to assess the association between sex steroids and incident MetS.

Results: 1651 men without MetS at baseline were identified. During follow-up 289 men developed incident MetS, while 1362 men did not develop MetS. Men with lower baseline total T levels were at higher risk for developing MetS (Odds ratio (OR)=1.72, $p<0.001$), even after adjustment for SHBG (OR=1.43, $p=0.001$), BMI (OR=1.44, $p<0.001$) or HOMA-IR (OR=1.64, $p<0.001$). E2 was not associated with development of MetS (OR=1.04; $p=0.56$). However, a lower E2/T ratio was associated with a lower risk of incident MetS (OR=0.38; $p<0.001$), even after adjustment for SHBG (OR=0.48; $p<0.001$), BMI (OR=0.60; $p=0.001$) or HOMA-IR (OR=0.41; $p<0.001$).

Conclusions: In men, lower T levels, but not E2, are linked with an increased risk of developing MetS, independent of SHBG, BMI or insulin resistance. A lower E2/T ratio may be protective against developing MetS.

Introduction

Metabolic syndrome (MetS) describes a cluster of features including abdominal obesity, dyslipidemia, hypertension and insulin resistance that are associated with an increased risk of developing type 2 diabetes, cardiovascular disease and death (1,2). Moreover, these risks are higher than those associated with individual components of the syndrome (2). Up to one-quarter to one-third of the adult population in Europe and the United States can be diagnosed with MetS (1-3), making it an important public health target for disease prevention.

Both low total testosterone (T) and low sex hormone binding globulin (SHBG) have been associated with an increased risk of MetS in men (4-9). However, serum concentrations of total and free T are strongly linked to SHBG, especially in men with obesity (10). Whether the risk of MetS associated with low T is independent of SHBG or vice versa, remains unclear.

Furthermore, T is converted to estradiol (E2) by the aromatase enzyme, which is highly expressed in adipose tissue (11) . A recent experimental study showed that lowering E2 levels in healthy males increased body fat, independent of T (12). Although both T and E2 are associated with variations in body composition in men (12,13), the potential impact of E2 and the extent of aromatisation on the risk for incident MetS has not been investigated prospectively.

Using data from the European Male Aging Study (EMAS), a prospective study of aging in European men, we studied the association between baseline sex steroids (T and E2) and the risk of developing MetS at follow-up and investigated if this association was independent of SHBG, body mass index (BMI), insulin resistance and body fat measurements. We also assessed whether sex steroids were associated with change in individual MetS components.

Methods

Subjects and study design

The prospective study design of EMAS has been described previously (14). From 2003 to 2005, 3369 men aged 40-79 years were recruited from population registers in eight European centres: Manchester, United Kingdom; Leuven, Belgium; Malmö, Sweden; Tartu, Estonia; Lodz, Poland; Szeged, Hungary;

Florence, Italy and Santiago de Compostela, Spain. After a median follow-up time of 4.3 years (range 2.95-5.7 years), 2736 men participated in phase 2. From the original cohort, 193 men had died and 440 were lost to follow-up. 150 men were excluded because of known pituitary or testicular disease or current drug use of medications that could affect pituitary or testicular function or sex steroid clearance (e.g. GnRH agonists, testosterone, anticonvulsants). Ethical approval for the study was acquired in accordance with local institutional requirements at each centre. All subjects gave written informed consent.

Assessments

At both phases, participants completed a postal questionnaire that included information about general health (response set: excellent, very good, good, fair or poor), smoking history (current, past or non-smoker) and frequency of alcohol consumption in the previous month (none, less than once a week, 1-2, 3-4, 5-6 or 7 days per week) (14). Current prescription and non-prescription medication use was recorded.

Height, weight and waist circumference were measured in a standing position. Body weight was measured to the nearest 0.1 kg using an electronic scale (SECA UK Ltd, Birmingham, UK) and height to the nearest 1 mm using a stadiometer (Leicester Height Measure, SECA UK Ltd). Waist circumference was measured using anthropometric tape, and the median of three measurements was used as the recorded value. Body mass index (BMI) was calculated as body weight (kilograms) divided by the square of height (meters). Body fat percentage was calculated by the Siri equation, based on a subject's average density (body mass divided by body volume) (15). Seated blood pressure (Omron 500I, Omron Healthcare (UK) Ltd, Milton Keynes, UK) was recorded after a 5 min rest period. Physical function was assessed via gait speed in a timed 50-feet walk (16).

Laboratory measurements

At both phases, a single fasting morning (before 10.00 h) venous blood sample was obtained from each subject.

Total T was measured by liquid chromatography-tandem mass spectrometry as described previously (17). The lower limit of quantification (LOQ) was 0.25 nmol/l. The coefficients of variation were less than 10% within runs and between runs. Measurement of total E2 was carried out by gas chromatography-tandem mass spectrometry as described previously (18). The LOQ for E2 was 7.34 pmol/l. The coefficients of variation were less than 5% within runs and between runs. The E2/T ratio, a measure of aromatisation, was calculated by dividing total E2 concentration in nmol/L by total T concentration in nmol/L. SHBG was measured by the Modular E170 platform electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany). Free T and E2 levels were calculated from total hormone levels, SHBG, and individual albumin concentrations by the Vermeulen formula (19).

Albumin, glucose, cholesterol and triglyceride measurements were assessed at the local health care facility. Insulin was assayed using quimioluminiscence (University of Santiago de Compostela). Insulin resistance was calculated using the homeostasis model assessment of insulin resistance (HOMA-IR) (20).

Dual energy X-ray absorptiometry

In the Leuven and Manchester cohort, body composition at baseline was assessed by dual energy X-ray absorptiometry (DXA) (QDR 4500A Discovery scanner, Hologic Inc, Bedford, MA, USA), as described elsewhere (21). Percentage body fat and trunk fat was calculated by dividing body fat mass or trunk fat mass by total body mass, multiplied by 100.

Definition of the metabolic syndrome and its components

MetS was defined according to the updated NCEP ATPIII criteria (22). Subjects were classified with MetS when three or more of the following criteria were present: waist circumference ≥ 102 cm, triglyceride level ≥ 1.7 mmol/l (150 mg/dl), HDL cholesterol levels < 1.03 mmol/l (40 mg/dl), blood pressure ≥ 130/85 mmHg and a fasting glucose level ≥ 5.6 mmol/l (100 mg/dl).

Subjects on antihypertensive drugs or antidiabetic drugs were classified positive for the blood pressure or glucose criterion respectively. Subjects without MetS at baseline, but who developed MetS during

follow-up, were classified as 'Incident MetS'. Subjects without MetS at either time point were classified as 'No MetS'.

Subjects with missing data were excluded if MetS could not be determined with certainty. For instance, a subject with four positive MetS criteria and one missing was classified as having MetS. However, in a subject with two positive, two negative and one missing MetS criterion, the MetS status could not be determined with certainty, and these subjects were excluded from the analysis.

Statistical analysis

Descriptive statistics were used to characterise subjects at baseline. Smoking status was categorised as current versus never and ex-smokers (referent). Alcohol intake was stratified as less than four days per week (referent) versus five or more days per week. General health was defined as poor/fair versus good/very good/excellent (referent). To assess associations with decreasing sex steroid and SHBG levels, these variables were multiplied by -1 and converted to standardised z-scores. Baseline total T, free T and SHBG were also categorized into quintiles, with the middle quintile as referent.

Logistic regression analysis was used to determine associations between baseline sex steroids (predictor) and incident MetS (outcome). Results were expressed as standardised odds ratios (OR) with 95% confidence intervals (CI). The analysis was performed unadjusted, with adjustments for age, study centre, smoking status, alcohol intake, physical activity and general health, and with additional adjustments for SHBG or total T as indicated. As both insulin sensitivity and obesity can influence T and SHBG levels, we subsequently adjusted for HOMA-IR, BMI and % body fat. We further explored relationships between baseline sex steroids and individual components of MetS at follow-up, using linear regression with adjustments for age, centre, alcohol intake, smoking status, physical activity, general health and the baseline value of the individual component. To meet linear regression assumptions, glucose and triglyceride levels were log transformed. Results were expressed as standardised β-coefficients and 95% CI. $P<0.05$ was considered statistically significant. All analyses were performed by using STATA version 13 (Stata corp. College station, TX, USA).

Results

Subject characteristics

In EMAS, MetS status at baseline and follow-up could be assessed in 2376 men. Of these men, 725 men with MetS at baseline were excluded from the analysis. 1651 men did not have MetS at baseline. Of these, 289 (17.5%) developed MetS during the follow-up period. Baseline characteristics of the 1651 men without baseline MetS are presented in Table 1. Their mean (SD) age was 58.5 (10.7) and mean BMI was 26.3 kg/m² (3.3). 21.8% of the study subjects used antihypertensive drugs, 8.8% were on statins and 2.0% were treated for diabetes.

Men with incident MetS had a higher weight, BMI, calculated body fat % and HOMA-IR. They had a lower baseline self-reported health, compared to the men that did not develop MetS (Table 1). Men with incident MetS had lower levels of total and free T, a higher E2/T ratio and lower SHBG. Baseline E2 levels were not significantly different between both groups. Already at baseline, subjects with incident MetS had a higher waist circumference, systolic and diastolic blood pressure, fasting triglyceride and glucose levels and lower HDL levels, compared to subject without MetS. Compared to men who did not develop MetS, those with incident MetS were more often prescribed antihypertensive drugs, statins and antidiabetic drugs at baseline (Table 1).

Association between baseline sex steroids and incident MetS

Logistic regression analysis showed that lower total T and free T were associated with an increased risk of incident MetS (OR=1.64 (CI 1.41-1.90) and OR=1.31 (CI 1.14-1.51) respectively). This association persisted after adjustment for age, centre and lifestyle factors (alcohol intake, current smoking status, physical activity and general health) (OR=1.72 (CI 1.48-2.01) for total T and OR=1.36 (CI 1.17-1.59) for free T) and for total T after further adjustment for SHBG (OR=1.43 (CI 1.16-1.76)). Total E2 levels were not significantly associated with the risk of developing MetS (OR=1.04 (CI 0.91-1.19)). Adding SHBG to the model had no effect on this risk (OR=0.90 (CI 0.78-1.04)). A lower E2/T ratio was associated with a decreased risk for incident MetS, independent of age, centre and lifestyle factors and SHBG (OR=0.48 (CI 0.35-0.64)). SHBG itself was also independently associated with

incident MetS (OR=1.78 (CI 1.48-2.13)). However, after adjustment for total T levels, the association between SHBG and incident MetS was attenuated, but remained significant (OR=1.33 (CI 1.05-1.68)) (Figure 1).

Further analysis after categorising sex steroid levels into quintiles, with the middle quintile as referent, showed no evidence of a threshold effect (data not shown). Adding an interaction term for age (below or above 60) or for BMI (below or above 30) had no significant effects, indicating that the sex-steroid associated MetS risk does not vary in different age or BMI groups (data not shown).

Influence of BMI, body fat and insulin resistance on relation of sex steroids and incident MetS

- Insulin resistance

After further adjustment for HOMA-IR, total T, free T, E2/T ratio and SHBG remained strongly associated with incident MetS (OR=1.64 (CI 1.40-1.91), OR=1.29 (CI 1.11-1.51), OR=0.41 (CI 0.31-0.54), OR=1.75 (CI 1.45-2.10)). Further the association between total E2 and incident MetS became borderline significant (OR=1.37 (CI 1.00-1.88), p=0.049)) (Table 2).

- BMI and percentage body fat

After further adjustment for BMI, the associations between lower total T, free T, E2/T ratio, SHBG and incident MetS remained significant (OR=1.44 (CI 1.23-1.69), OR=1.24 (CI 1.06-1.45) and OR=0.60 (CI 0.44-0.81), OR=1.49 (CI 1.23-1.79) respectively). After adjustment for BMI, total E2 was not associated with incident MetS (OR=1.15 (CI 0.99-1.33)). Adjusting for calculated body fat percentage yielded similar results (Table 2).

In the Manchester and Leuven cohorts, baseline DXA data are available in 713 men. In this subgroup, MetS status could be determined in in 595 men at both study phases. Of these men, 402 (67.6%) had no MetS at either time point. 59 (9.9%) men developed MetS during the study period.

In this subgroup, lower total T, free T and SHBG were also associated with an increased risk for incident MetS after adjustment for age, centre and lifestyle factors (OR=2.28 (CI 1.60-3.25), OR=1.90 (CI 1.34-2.69) and OR=1.90 (CI 1.28-2.81) respectively), and a lower E2/T ratio showed an inverse

association (OR=0.46 (CI 0.27-0.76)). Total E2 was not associated with MetS (OR=1.22 (CI 0.91-1.64)).

For total and free T and SHBG, the association with incident MetS remained after further adjusting for % body fat (OR=2.25 (CI 1.52-3.31) for total T, OR=1.91 (CI 1.29-2.81) for free T and OR=1.87 (CI 1.23-2.85) for SHBG) or % trunk fat (OR=2.15 (CI 1.45-3.18) for total T, OR=1.85 (CI 1.25-2.74) for free T and OR=1.78 (CI 1.17-2.71) for SHBG). The E2/T ratio was no longer significantly associated with MetS after further adjustment for body fat or trunk fat measurements (OR=0.63 (CI 0.35-1.14) and OR=0.68 (CI 0.37-1.24)). Total E2 was weakly associated with incident MetS when % body fat or % trunk fat was added to the model (OR=1.49 (CI 1.06-2.10) and OR=1.50 (CI 1.06-2.12)).

Association between baseline sex steroids and SHBG and MetS components at follow-up

In the unadjusted model, lower total T, free T and SHBG levels at baseline were associated with a higher waist circumference at follow-up (β= 3.91 (CI 3.47-4.35), β=3.32 (CI 2.86-3.79), β=2.45 (CI 1.97-2.94)) and E2/T ratio was associated with a lower waist circumference (β=-8.01 (CI -8.76—7.26). However, these associations disappeared after adjustments for age, centre, lifestyle factors and baseline waist circumference. Similar results were seen for systolic and diastolic blood pressure, except for the multivariable adjusted association of baseline SHBG and systolic blood pressure at follow-up (β=1.35 (CI 0.52- 2.17)).

After adjustment for age, centre and lifestyle factors and baseline values of the components, lower total and free T levels were associated with a higher triglyceride level, lower HDL and higher glucose levels (β=0.06 (CI 0.04- 0.08), β=-0.04 (CI -0.05- -0.03) and β=0.01 (CI 0.01 - 0.02) for total T and β=0.04 (CI 0.02- 0.06), β=-0.02 (CI -0.03- -0.01) and β=0.01 (CI 0.0001 - 0.01) for free T). Similar associations were seen for SHBG. A lower E2/T ratio was associated with lower triglyceride levels, higher HDL levels and lower glucose levels (β=-0.06 (CI -0.09- -0.03) β=0.05 (CI 0.03-0.07) β=-0.03 (CI -0.04- -0.01)). Lower total E2 levels were only associated with higher triglyceride levels (β=0.02 (CI 0.004 - 0.04)) (Table 3).

340 ***Discussion***

341 In this prospective study of middle-aged and elderly men, lower baseline serum T was prospectively
342 associated with an increased risk for incident MetS. Moreover, the association between low T and
343 incident MetS persisted after adjustments for SHBG, HOMA-IR, BMI and calculated body fat. In the
344 Leuven-Manchester subcohort, this association was also independent of DXA-measured body fat and
345 trunk fat. Total E2 levels were not associated with the development of MetS. A lower E2/T ratio,
346 reflecting lower aromatisation of T into E2, was associated with a reduced risk of developing MetS.
347 This association was also independent of SHBG, HOMA-IR and BMI, but not of body fat measured
348 by DXA. Lower baseline total and free T and SHBG levels were associated with higher triglyceride
349 and glucose levels and lower HDL levels at follow-up. A lower E2/T ratio was associated with lower
350 triglyceride and glucose levels and higher HDL levels. Total E2 was only associated with a higher
351 triglyceride level.
352
353 Similar to the present results, other longitudinal studies such as the Baltimore Longitudinal Study of
354 Aging (4), the Kuopio Ischemic Heart Disease Risk Factor Study (5) and the Massachusetts Male
355 Ageing Study (7) showed an increased risk of MetS in men with lower levels of total T or SHBG at
356 baseline. In contrast, the Framingham Heart Study, the Study of Health in Pomerania and the Concord
357 Health and Ageing in Men Project, found that only SHBG and not total T was independently
358 associated with incident MetS (8,9,23). Discrepancies between our findings and other longitudinal
359 studies could in part be related to differences in the populations studied and the methods used to
360 measure serum T levels.
361 Our MetS component data are consistent with other cross-sectional and longitudinal studies,
362 suggesting that lower baseline total T levels were associated with a less favorable lipid profile (24) and
363 higher glucose levels (25).
364
365 Previous analysis of baseline EMAS cross-sectional data had revealed that obesity was strongly
366 associated with low T and low or inappropriately normal LH levels, reflecting dysfunction at the
367 hypothalamic-pituitary-testicular axis (HPT-axis) and secondary hypogonadism (26). Obesity was also

associated with a reduced circulating SHBG concentration. Both low SHBG and HPT-axis dysfunction in obese men may therefore account for the low total T, and both can be induced by higher levels of proinflammatory cytokines and insulin resistance, associated with adiposity (26,27). However, adjusting for SHBG and different measures of fat mass as well as insulin resistance did not affect the association between low T and MetS. This suggests that factors directly associated with low T may be important in driving the progression to metabolic syndrome in men, independent of SHBG, insulin resistance and obesity.

However, it remains unclear if low T is a biomarker of an unfavourable metabolic state or a mediating factor in the development of MetS. Androgen deprivation therapy in prostate cancer patients results in a higher prevalence of MetS and a higher cardiovascular mortality (28). On the other hand, weight reduction in obese men increases T levels (29). Testosterone replacement therapy in men with MetS may improve several MetS components, such as insulin sensitivity, waist circumference and LDL cholesterol (30). The association between low T and MetS may therefore be bidirectional.

In contrast to the abundance of data investigating the link between T and MetS, there are few prospective data investigating the association between E2 and MetS. In men, circulating E2 levels are in the picomolar range and chromatography/mass spectrometry methods are therefore needed for accurate measurement of serum E2 (31). Around 60% of circulating E2 in men is produced by aromatisation of T (11,32). Estrogens play an essential role in male physiology. They are not only important for bone maintenance, but they also have metabolic effects on carbohydrate and lipid metabolism and fat distribution, not only in humans, but also in rodents. Estrogen receptor α disruption, both in the presence or absence of androgen receptor, increases fat mass in male mice (33,34). An absolute lack of E2, such as in men with congenital aromatase or estrogen receptor alpha deficiency and in aromatase knockout mice, has also been associated with the development of several MetS components such as truncal obesity, lipid disorders and insulin resistance (35). Moreover, a recent study showed that experimentally-induced short-term estrogen deficiency resulted in an increase in body fat in men (12). In our study, we found no association between baseline E2 levels and incident MetS. Our results are in line with a cross-sectional study in middle-aged and elderly men (36)

and a recent longitudinal study in elderly men(23). Only in the latter study E2 was measured by liquid chromatography-tandem mass spectrometry. In other recent cross-sectional studies, both higher (37) and lower (38) E2 levels, measured by radioimmunoassay (which may be unreliable), have been associated with MetS in men.

The activity of the aromatase enzyme can be upregulated by multiple factors, such as inflammatory adipocytokines, insulin and free fatty acids. This results in increased intracellular E2 levels that can activate the estrogen receptor (27,39). Circulating E2 levels may not reflect the local actions of E2 in target tissues. The E2/T ratio may therefore be a better indicator of aromatisation than a single measurement of circulating E2. A positive correlation between the E2/T ratio and BMI as well as different measures of body fat has been reported, but these associations were not independent of visceral adipose tissue (40). More recently, cross-sectional data from the Boston Area Community Health/Bone survey also showed a positive association between the E2/T ratio and body composition, measured anthropometrically and by DXA (13). In a recent short-term intervention study, administration of an aromatase inhibitor with T replacement to GnRH analog-treated men, thereby lowering E2/T ratio, resulted in an increase of body fat (12). Interestingly, in our study, a lower E2/T ratio, reflecting lower aromatisation of T into E2, was strongly associated with a reduced risk for incident MetS. However, this association was not independent of DXA-measured body fat, indicating that changes in body composition may modify the association between the estrogen-androgen balance and MetS. This may account for the discrepancy between our findings and those from short-term aromatase inhibition. Moreover, administration of an aromatase inhibitor to young men results in virtually undetectable, non-physiologic E2 levels, which are clearly different from the E2 levels observed in our study population of middle-aged and older men.

Our study has several strengths. It is a large, population based study and standardised methods in design and analysis were used. As recommended by the Endocrine Society (41), serum T and E2 measurements were done by respectively liquid or gas chromatography-tandem mass spectrometry, giving more accurate results as compared to other population studies that have used immunoassays (31,42). Furthermore, the prospective design allows insights into the temporal nature of the

associations. By adjusting our findings for a range of putative confounding factors, including SHBG, insulin resistance and body composition, these results add insights into the specific effects of sex hormones independent of adiposity.

There are some limitations which need to be considered. Our results were based on an analysis of responders to both baseline and follow up phases and in whom data on MetS were available. Therefore caution is needed in interpreting data on incidence of MetS. Any response or loss to follow-up bias is though unlikely to influence our findings as these were based on an internal comparison of responders. Finally, our data were based on analysis of a relatively healthy proportion of European men. Extrapolating these data to other populations should be done with care.

In conclusion, low T but not E2 levels in men may be regarded as a biomarker or risk predictor for MetS, independently of SHBG, insulin resistance and body composition. A lower E2/T ratio may be protective against developing MetS. The importance of aromatase activity in MetS requires further investigation. These findings may have implications for the assessment of cardiometabolic risks in older and obese men.

Author contributions:

- LA analysed and interpreted the data and wrote the manuscript
- FCCW: designed and led the European Male Ageing Study, contributed to the interpretation of data and preparation of the manuscript
- TWON, DV: concept and design of the study, collection and interpretation of data, preparation of the manuscript
- MKR, MRL, BD and FC assisted with interpretation of data and preparation of the manuscript
- SRP and JDF collected data, contributed to the statistical analysis and interpretation of data
- ELC contributed to the statistical analysis and interpretation of data
- GF, GB, FFC, KK, MP, AG collected data
- All authors reviewed and edited the manuscript

DV is the guarantor of this work and has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Acknowledgments and funding

We thank the men who participated and the research and nursing staff in the eight centres.

We thank Dr. K. Antonio, Research Centre Insurance, Faculty of Economics and Business, KULeuven, Belgium for statistical advice.

Part of this research was presented at the annual meeting of the Endocrine Society 2014, Chicago, Illinois. Abstract # 12458.

The European Male Ageing Study is funded by the Commission of the European Communities Fifth Framework Programme 'Quality of Life and Management of Living Resources' Grant QLK6-CT-2001-00258 and supported by Arthritis Research UK.

This work was supported by a grant from the Fund for Scientific Research Flanders (FWO-Vlaanderen grant #G085413N) and by a research grant from the Academische Stichting Leuven.

References

1. Cornier MA, Dabelea D, Hernandez TL, Lindstrom RC, Steig AJ, Stob NR, Van Pelt RE, Wang H, Eckel RH. The metabolic syndrome. *Endocrine reviews* 2008;29:777-822.

2. Gami AS, Witt BJ, Howard DE, Erwin PJ, Gami LA, Somers VK, Montori VM. Metabolic syndrome and risk of incident cardiovascular events and death: a systematic review and meta-analysis of longitudinal studies. *Journal of the American College of Cardiology* 2007;49:403-414.

3. Scuteri A, Laurent S, Cucca F, Cockcroft J, Cunha PG, Manas LR, Raso FU, Muiesan ML, Ryliskyte L, Rietzschel E, Strait J, Vlachopoulos C, Volzke H, Lakatta EG, Nilsson PM. Metabolic syndrome across Europe: different clusters of risk factors. *European journal of preventive cardiology* 2014.

4. Rodriguez A, Muller DC, Metter EJ, Maggio M, Harman SM, Blackman MR, Andres R. Aging, androgens, and the metabolic syndrome in a longitudinal study of aging. *The Journal of clinical endocrinology and metabolism* 2007;92:3568-3572.

5. Laaksonen DE, Niskanen L, Punnonen K, Nyyssonen K, Tuomainen TP, Valkonen VP, Salonen R, Salonen JT. Testosterone and sex hormone-binding globulin predict the metabolic syndrome and diabetes in middle-aged men. *Diabetes care* 2004;27:1036-1041.

6. Li C, Ford ES, Li B, Giles WH, Liu S. Association of testosterone and sex hormone-binding globulin with metabolic syndrome and insulin resistance in men. *Diabetes care* 2010;33:1618-1624.

7. Kupelian V, Page ST, Araujo AB, Travison TG, Bremner WJ, McKinlay JB. Low sex hormone-binding globulin, total testosterone, and symptomatic androgen deficiency are associated with development of the metabolic syndrome in nonobese men. *The Journal of clinical endocrinology and metabolism* 2006;91:843-850.

8. Bhasin S, Jasjua GK, Pencina M, D'Agostino R, Sr., Coviello AD, Vasan RS, Travison TG. Sex hormone-binding globulin, but not testosterone, is associated prospectively and independently with incident metabolic syndrome in men: the framingham heart study. *Diabetes care* 2011;34:2464-2470.

9. Haring R, Volzke H, Spielhagen C, Nauck M, Wallaschofski H. The role of sex hormone-binding globulin and testosterone in the risk of incident metabolic syndrome. *European journal of preventive cardiology* 2013;20:1061-1068.

10. de Ronde W, van der Schouw YT, Pierik FH, Pols HA, Muller M, Grobbee DE, Gooren LJ, Weber RF, de Jong FH. Serum levels of sex hormone-binding globulin (SHBG) are not associated with lower levels of non-SHBG-bound testosterone in male newborns and healthy adult men. *Clinical endocrinology* 2005;62:498-503.

11. Kaufman JM, Vermeulen A. The decline of androgen levels in elderly men and its clinical and therapeutic implications. *Endocrine reviews* 2005;26:833-876.

12. Finkelstein JS, Lee H, Burnett-Bowie SA, Pallais JC, Yu EW, Borges LF, Jones BF, Barry CV, Wulczyn KE, Thomas BJ, Leder BZ. Gonadal steroids and body composition, strength, and sexual function in men. *The New England journal of medicine* 2013;369:1011-1022.

13. Gates MA, Mekary RA, Chiu GR, Ding EL, Wittert GA, Araujo AB. Sex steroid hormone levels and body composition in men. *The Journal of clinical endocrinology and metabolism* 2013;98:2442-2450.

14. Lee DM, O'Neill TW, Pye SR, Silman AJ, Finn JD, Pendleton N, Tajar A, Bartfai G, Casanueva F, Forti G, Giwercman A, Huhtaniemi IT, Kula K, Punab M, Boonen S, Vanderschueren D, Wu FC. The European Male Ageing Study (EMAS): design, methods and recruitment. *International journal of andrology* 2009;32:11-24.

15. Siri WE. Body composition from fluid spaces and density: Analysis of methods. In: Brozek J, Henzchel A, eds. Techniques for Measuring Body Composition. Washington: National Academy of Sciences; 1961:224–244.

16. Reuben DB, Siu AL. An objective measure of physical function of elderly outpatients. The Physical Performance Test. *Journal of the American Geriatrics Society* 1990;38:1105-1112.

17. Gallagher LM, Owen LJ, Keevil BG. Simultaneous determination of androstenedione and testosterone in human serum by liquid chromatography-tandem mass spectrometry. *Annals of clinical biochemistry* 2007;44:48-56.

18. Labrie F, Belanger A, Belanger P, Berube R, Martel C, Cusan L, Gomez J, Candas B, Castiel I, Chaussade V, Deloche C, Leclaire J. Androgen glucuronides, instead of testosterone, as the new markers of androgenic activity in women. *The Journal of steroid biochemistry and molecular biology* 2006;99:182-188.

19. Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. *The Journal of clinical endocrinology and metabolism* 1999;84:3666-3672.

20. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985;28:412-419.

21. Verschueren S, Gielen E, O'Neill TW, Pye SR, Adams JE, Ward KA, Wu FC, Szulc P, Laurent M, Claessens F, Vanderschueren D, Boonen S. Sarcopenia and its relationship with bone mineral density in middle-aged and elderly European men. *Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA* 2013;24:87-98.

22. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WP, Loria CM, Smith SC, Jr. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation* 2009;120:1640-1645.

23. Hsu B, Cumming RG, Naganathan V, Blyth FM, Le Couteur DG, Seibel MJ, Waite LM, Handelsman DJ. Associations between Circulating Reproductive Hormones and SHBG and Prevalent and Incident Metabolic Syndrome in Community-Dwelling Older Men: The Concord Health and Ageing in Men Project. *The Journal of clinical endocrinology and metabolism* 2014:jc20142464.

24. Monroe AK, Dobs AS. The effect of androgens on lipids. *Current opinion in endocrinology, diabetes, and obesity* 2013;20:132-139.

25. Stellato RK, Feldman HA, Hamdy O, Horton ES, McKinlay JB. Testosterone, sex hormone-binding globulin, and the development of type 2 diabetes in middle-aged men: prospective results from the Massachusetts male aging study. *Diabetes care* 2000;23:490-494.

26. Wu FC, Tajar A, Pye SR, Silman AJ, Finn JD, O'Neill TW, Bartfai G, Casanueva F, Forti G, Giwercman A, Huhtaniemi IT, Kula K, Punab M, Boonen S, Vanderschueren D. Hypothalamic-pituitary-testicular axis disruptions in older men are differentially linked to age and modifiable risk factors: the European Male Aging Study. *The Journal of clinical endocrinology and metabolism* 2008;93:2737-2745.

27. Gregor MF, Hotamisligil GS. Inflammatory mechanisms in obesity. *Annual review of immunology* 2011;29:415-445.

28. Shahani S, Braga-Basaria M, Basaria S. Androgen deprivation therapy in prostate cancer and metabolic risk for atherosclerosis. *The Journal of clinical endocrinology and metabolism* 2008;93:2042-2049.

29. Corona G, Rastrelli G, Monami M, Saad F, Luconi M, Lucchese M, Facchiano E, Sforza A, Forti G, Mannucci E, Maggi M. Body weight loss reverts obesity-associated hypogonadotropic hypogonadism: a systematic review and meta-analysis. *European journal of endocrinology / European Federation of Endocrine Societies* 2013;168:829-843.

30. Wang C, Jackson G, Jones TH, Matsumoto AM, Nehra A, Perelman MA, Swerdloff RS, Traish A, Zitzmann M, Cunningham G. Low testosterone associated with obesity and the metabolic syndrome contributes to sexual dysfunction and cardiovascular disease risk in men with type 2 diabetes. *Diabetes care* 2011;34:1669-1675.

31. Huhtaniemi IT, Tajar A, Lee DM, O'Neill TW, Finn JD, Bartfai G, Boonen S, Casanueva FF, Giwercman A, Han TS, Kula K, Labrie F, Lean ME, Pendleton N, Punab M, Silman AJ, Vanderschueren D, Forti G, Wu FC, Group E. Comparison of serum testosterone and estradiol measurements in 3174 European men using platform immunoassay and mass spectrometry; relevance for the diagnostics in aging men. *European journal of endocrinology / European Federation of Endocrine Societies* 2012;166:983-991.

587 32. de Ronde W, Pols HA, van Leeuwen JP, de Jong FH. The importance of oestrogens in males.
588 *Clinical endocrinology* 2003;58:529-542.
589 33. Callewaert F, Venken K, Ophoff J, De Gendt K, Torcasio A, van Lenthe GH, Van Oosterwyck H,
590 Boonen S, Bouillon R, Verhoeven G, Vanderschueren D. Differential regulation of bone and body
591 composition in male mice with combined inactivation of androgen and estrogen receptor-alpha.
592 *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*
593 2009;23:232-240.
594 34. Vanderschueren D, Laurent MR, Claessens F, Gielen E, Lagerquist MK, Vandenput L, Borjesson
595 AE, Ohlsson C. Sex steroid actions in male bone. *Endocrine reviews* 2014;35:906-960.
596 35. Jones ME, McInnes KJ, Boon WC, Simpson ER. Estrogen and adiposity--utilizing models of
597 aromatase deficiency to explore the relationship. *The Journal of steroid biochemistry and molecular*
598 *biology* 2007;106:3-7.
599 36. Muller M, Grobbee DE, den Tonkelaar I, Lamberts SW, van der Schouw YT. Endogenous sex
600 hormones and metabolic syndrome in aging men. *The Journal of clinical endocrinology and*
601 *metabolism* 2005;90:2618-2623.
602 37. Maggio M, Lauretani F, Ceda GP, Bandinelli S, Basaria S, Paolisso G, Giumelli C, Luci M, Najjar
603 SS, Metter EJ, Valenti G, Guralnik J, Ferrucci L. Estradiol and metabolic syndrome in older italian
604 men: The InCHIANTI Study. *Journal of andrology* 2010;31:155-162.
605 38. Cheng KH, Huang SP, Huang CN, Lee YC, Chu CS, Chang CF, Lai WT, Liu CC. The impact of
606 estradiol and 1,25(OH)2D3 on metabolic syndrome in middle-aged Taiwanese males. *PloS one*
607 2013;8:e60295.
608 39. Williams G. Aromatase up-regulation, insulin and raised intracellular oestrogens in men, induce
609 adiposity, metabolic syndrome and prostate disease, via aberrant ER-alpha and GPER signalling.
610 *Molecular and cellular endocrinology* 2012;351:269-278.
611 40. Phillips GB, Jing T, Heymsfield SB. Relationships in men of sex hormones, insulin, adiposity, and
612 risk factors for myocardial infarction. *Metabolism: clinical and experimental* 2003;52:784-790.

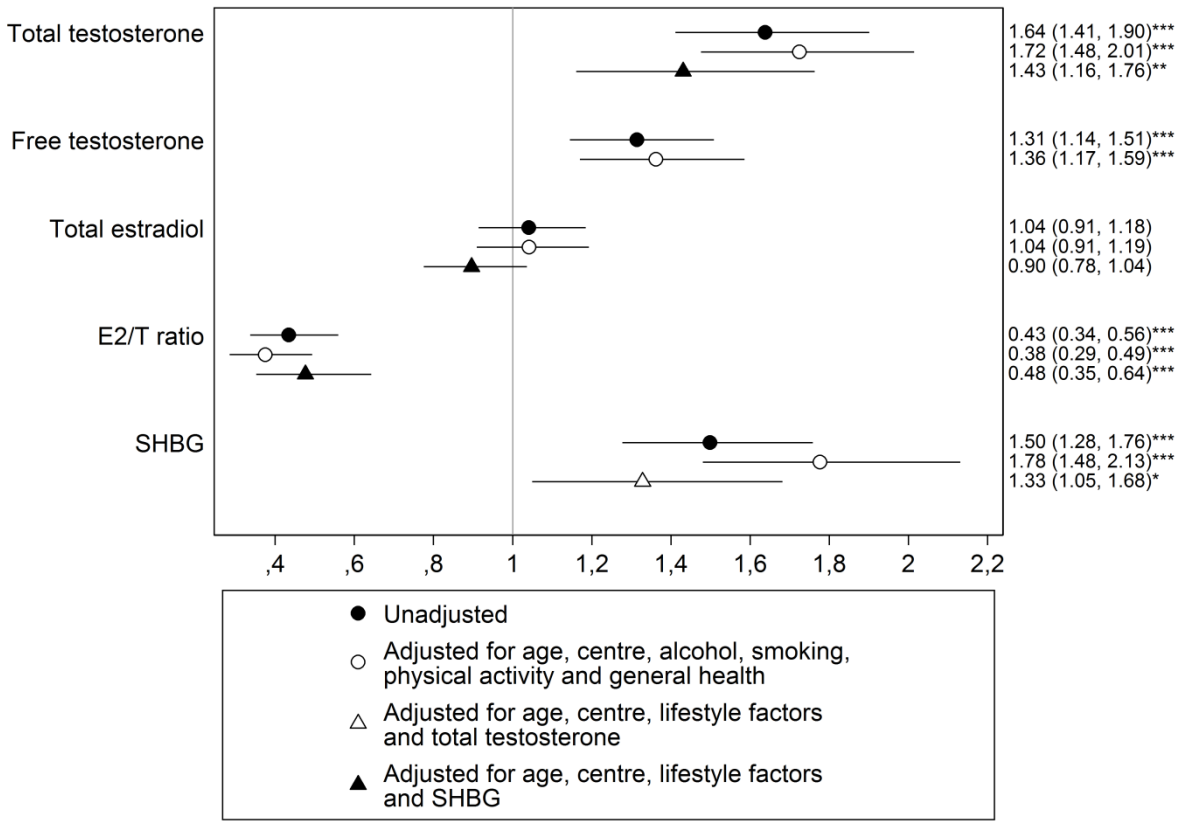
41. Rosner W, Auchus RJ, Azziz R, Sluss PM, Raff H. Position statement: Utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement. *The Journal of clinical endocrinology and metabolism* 2007;92:405-413.

42. Ohlsson C, Nilsson ME, Tivesten A, Ryberg H, Mellstrom D, Karlsson MK, Ljunggren O, Labrie F, Orwoll ES, Lee DM, Pye SR, O'Neill TW, Finn JD, Adams JE, Ward KA, Boonen S, Bartfai G, Casanueva FF, Forti G, Giwercman A, Han TS, Huhtaniemi IT, Kula K, Lean ME, Pendleton N, Punab M, Vanderschueren D, Wu FC, Vandenput L. Comparisons of immunoassay and mass spectrometry measurements of serum estradiol levels and their influence on clinical association studies in men. *The Journal of clinical endocrinology and metabolism* 2013;98:E1097-1102.

640 ***Figures***

641 Figure 1:

642



643

644 Associations between decreasing baseline sex steroids and SHBG levels and the development of

645 metabolic syndrome

646 Data are reported as standardised odds ratios with 95% confidence intervals for the risk of developing

647 metabolic syndrome associated with lower baseline sex steroids or SHBG (per 1 SD decrease).

648 Black circles represent the unadjusted model. White circles represent the multivariable adjusted

649 model, with adjustments for age, centre, alcohol intake, smoking, physical activity and general health.

650 Additional adjustments were made for total testosterone (white triangles) or SHBG (black triangles).

651 *p<0.05; ** p<0.01; *** p<0.001

652

653

654

655

656 **Table 1: Baseline characteristics of study subjects**

	No metabolic syndrome at baseline (n=1651)		No MetS at follow-up (n = 1362, 82.5%)		Incident MetS at follow-up (n=289, 17.5%)		
Physiological measures	Mean	SD	Mean	SD	Mean	SD	p-value
Age (yr)	58.5	10.7	58.5	10.9	58.6	10.1	0.85
Height (cm)	174.1	7.1	174.1	7.2	174.4	6.7	0.43
Weight (kg)	79.9	11.2	78.5	10.6	86.2	11.4	<0.001
BMI (kg/m^2)	26.3	3.3	25.9	3.1	28.3	3.3	<0.001
Calculated body fat (%)	26.6	4.9	26.1	4.8	28.6	4.7	<0.001
HOMA-IR	2.2	2.5	2.0	1.6	3.1	4.6	<0.001
Alcohol (five or more days/wk) (%)	24.3		25.2		19.7		0.047
Current smoker (%)	20.6		19.7		24.7		0.055
Physical activity (time to walk) (m/s)	13.1	2.4	13.1	2.3	13.4	2.6	0.07
General health (fair or poor) (%)	25.4		23.4		34.4		<0.001
Hormones							
Total T (nmol/L)	18.2	6.1	18.7	6.1	16.1	5.5	<0.001
Free T (pmol/L)	315.6	87.0	319.5	87.7	297.3	81.3	<0.001
Total E2 (pmol/L)	74.5	25.1	74.7	25.3	73.7	24.2	0.55
Free E2 (pmol/L)	1.26	0.43	1.26	0.44	1.30	0.42	0.11
E2/T ratio	0.0043	0.0015	0.0042	0.0014	0.0048	0.0016	<0.001
SHBG (nmol/L)	45.0	19.4	46.1	19.8	39.8	16.8	<0.001
MetS Components							
Waist circumference (cm)	94.4	9.0	93.1	8.6	100.5	8.3	<0.001
HDL cholesterol (mmol/L)	1.49	0.37	1.52	0.35	1.36	0.42	<0.001
Systolic BP (mmHg)	143.0	20.3	142.1	20.1	146.9	21.0	<0.001
Diastolic BP (mmHg)	85.8	11.7	85.4	11.4	87.9	12.7	0.001
Fasting triglycerides (mmol/L)	1.24	0.68	1.19	0.65	1.46	0.79	<0.001
Fasting glucose (mmol/L)	5.29	0.83	5.25	0.79	5.48	0.95	<0.001
Number of MetS components	1.30	0.68	1.23	0.68	1.67	0.55	<0.001
Medication use							
Using antihypertensive drugs (%)	21.8		19.8		31.1		<0.001
Using statins (%)	8.8		7.9		13.2		0.004
Using antidiabetic drugs (%)	2.0		1.5		4.2		0.004

Table 2: Impact of insulin resistance, BMI and body fat on the association between lower baseline sex steroids and SHBG and incident Metabolic syndrome

	Total T	Free T	E2/T ratio	Total E2	SHBG
Entire study sample					
Adjusted for age, centre and lifestyle factors	1.72 (1.48, 2.01)***	1.36 (1.17, 1.59)***	0.38 (0.29, 0.49)***	1.04 (0.91, 1.19)	1.78 (1.48, 2.13)***
+ HOMA-IR	1.64 (1.40, 1.91)***	1.29 (1.11, 1.51)**	0.41 (0.31, 0.54)***	1.37 (1.00, 1.88)*	1.75 (1.45, 2.10)***
+ BMI	1.44 (1.23, 1.69)***	1.24 (1.06, 1.45)**	0.60 (0.44, 0.81)**	1.15 (0.99, 1.33)	1.49 (1.23, 1.79)***
+ Calculated % body fat	1.52 (1.30, 1.79)***	1.26 (1.08, 1.47)**	0.49 (0.37, 0.66)***	1.08 (0.94, 1.24)	1.57 (1.31, 1.89)***
Subgroup analysis in Manchester and Leuven cohorts					
Adjusted for age, centre and lifestyle factors	2.28 (1.60, 3.25)***	1.90 (1.34, 2.69)***	0.46 (0.27, 0.76)**	1.22 (0.91, 1.64)	1.90 (1.28, 2.81)**
+ % Body fat DXA	2.25 (1.52, 3.31)***	1.91 (1.29, 2.81)**	0.63 (0.35, 1.14)	1.49 (1.06, 2.10)*	1.87 (1.23, 2.85)**
+ % Trunk fat DXA	2.15 (1.45, 3.18)***	1.85 (1.25, 2.74)**	0.68 (0.37, 1.24)	1.50 (1.06, 2.12)*	1.78 (1.17, 2.71)**

Data are reported as standardised odds ratios with 95% confidence intervals for the risk of developing metabolic syndrome per standard deviation decrease in baseline sex steroids or SHBG. Lifestyle factors: alcohol, current smoking status, physical activity and general health.
In the complete study sample, insulin resistance (HOMA-IR), BMI or calculated body fat percentage were included in the model.
In the Manchester and Leuven cohort, DXA measurements of percentage body fat or percentage trunk fat were included in the model.
*p<0.05; ** p<0.01; *** p<0.001.

Table 3: Associations between a decrease in baseline sex steroids and SHBG and MetS components at follow up

Baseline hormones	Follow-up Metabolic syndrome variables					
	Waist circumference	Log triglycerides	HDL	Log glucose	SBP	DBP
Total T						
Model 1	3.91 (3.47, 4.35)***	0.12 (0.10, 0.14)***	-0.09 (-0.10, -0.07)***	0.03 (0.02, 0.04)***	1.75 (0.91, 2.58)***	0.73 (0.24, 1.21)**
Model 2	0.08 (-0.15, 0.31)	0.06 (0.04, 0.08)***	-0.04 (-0.05, -0.03)***	0.01 (0.01, 0.02)***	0.40 (-0.34, 1.13)	-0.05 (-0.48, 0.38)
Free T						
Model 1	3.32 (2.86, 3.79)***	0.06 (0.04, 0.08)***	-0.04 (-0.05, -0.02)***	0.03 (0.02, 0.03)***	1.77 (0.91, 2.62)***	-0.53 (-1.02, -0.03)*
Model 2	0.19 (-0.05, 0.44)	0.04 (0.02, 0.06)***	-0.02 (-0.03, -0.01)**	0.01 (0.0001, 0.01)*	-0.44 (-1.24, 0.36)	-0.31 (-0.78, 0.15)
E2/T ratio						
Model 1	-8.01 (-8.76, -7.26)***	-0.16 (-0.19, -0.12)***	0.13 (0.10, 0.15)***	-0.06 (-0.07, -0.04)***	-4.13 (-5.57, -2.69)**	-1.13 (-1.97, -0.29)**
Model 2	-0.33 (-0.11, 0.76)	-0.06 (-0.09, -0.03)**	0.05 (0.03, 0.07)***	-0.03 (-0.04, -0.01)***	0.17 (-1.14, 1.48)	0.64 (-0.12, 1.41)
Total E2						
Model 1	-0.45 (-0.93, 0.03)	0.06 (0.01, 0.06)**	-0.02 (-0.03, 0.00)	0.0003 (-0.008, 0.009)	-1.20 (-2.06, -0.35)**	-0.14 (-0.64, 0.35)
Model 2	0.21 (-0.02, 0.43)	0.02 (0.004, 0.04)*	-0.01 (-0.02, 0.01)	0.001 (-0.01, 0.01)	-0.16 (-0.91, 0.59)	-0.06 (-0.51, 0.38)
SHBG						
Model 1	2.45 (1.97, 2.94)***	0.13 (0.11, 0.15)***	-0.09 (-0.11, -0.07)***	0.02 (0.01, 0.03)***	0.84 (-0.03, 1.72)	1.81 (1.31, 2.32)***
Model 2	-0.08 (-0.34, 0.17)	0.06 (0.04, 0.08)***	-0.05 (-0.06 -0.03)***	0.02 (0.01, 0.02)***	1.35 (0.52, 2.17)**	0.32 (-0.16, 0.81)

Data are reported as β coefficients with 95% confidence interval per standard deviation decrease in baseline sex steroids and SHBG. *p<0.05; ** p<0.01; *** p<0.001
Model 1: unadjusted. Model 2: adjusted for age, centre, alcohol, current smoking status, physical activity, general health, and baseline value of component
To meet linear regression assumptions, follow-up triglyceride and glucose levels were log-transformed. A total of 27 outliers with baseline waist circumference <40 cm, baseline SHBG >190 nmol/L, baseline E2/T ratio >0.03, follow-up glucose level >12 mmol/L and follow-up triglyceride levels >14 mmol/L were excluded from the analysis.
Abbreviations: HDL: high density lipoprotein, SBP: systolic blood pressure, DBP: diastolic blood pressure, T: testosterone, E2: estradiol, SHBG: sex hormone binding globulin.