



UNIVERSITÀ  
DEGLI STUDI  
FIRENZE

## FLORE

# Repository istituzionale dell'Università degli Studi di Firenze

### **Associations between sex steroids and the development of metabolic syndrome: A longitudinal study in European men**

Questa è la versione Preprint (Submitted version) della seguente pubblicazione:

*Original Citation:*

Associations between sex steroids and the development of metabolic syndrome: A longitudinal study in European men / Antonio, Leen; Wu, Frederick C. W.; O'Neill, Terence W.; Pye, Stephen R.; Carter, Emma L.; Finn, Joseph D.; Rutter, Martin K.; Laurent, Michaël R.; Huhtaniemi, Ilpo T.; Han, Thang S.; Lean, Michael E. J.; Keevil, Brian G.; Pendleton, Neil; Rastrelli, Giulia; Forti, Gianni; Bartfai, Gyorgy; Casanueva,

*Availability:*

The webpage <https://hdl.handle.net/2158/1058697> of the repository was last updated on 2016-11-06T21:24:21Z

*Published version:*

DOI: 10.1210/jc.2014-4184

*Terms of use:*

Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (<https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf>)

*Publisher copyright claim:*

La data sopra indicata si riferisce all'ultimo aggiornamento della scheda del Repository FloRe - The above-mentioned date refers to the last update of the record in the Institutional Repository FloRe

(Article begins on next page)



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

53

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

60

61

62 **Abbreviated title:** Sex Steroids and Metabolic Syndrome Risk

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

64 **Word count:** 3649

65 **Number of figures:** 1

66 **Number of tables:** 3

67

68 **Corresponding author and reprints:**

69 Leen Antonio

70 Clinical and Experimental Endocrinology - Laboratory of Molecular Endocrinology

71 University of Leuven

72 Campus GHB O/N 1

73 Herestraat 49 box 902

74 3000 Leuven

75 Belgium

76 Email: leen.antonio@med.kuleuven.be

77

78 **Grants/fellowships:**

79 The European Male Ageing Study is funded by the Commission of the European Communities Fifth  
80 Framework Programme 'Quality of Life and Management of Living Resources' Grant QLK6-CT-  
81 2001-00258 and supported by Arthritis Research UK.

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

84

85 **Disclosure statement:**

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

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

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

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

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

96 BGK: consultant for Thermo Scientific

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

98 DV: Senior clinical investigator of the University Hospitals Leuven

99

100

101

102

103

104

105

106

107

108

109

110

111

112

113

114

115

116

117

118 **Abstract**

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

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

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

128 **Results:** 1651 men without MetS at baseline were identified. During follow-up 289 men developed  
129 incident MetS, while 1362 men did not develop MetS. Men with lower baseline total T levels were at  
130 higher risk for developing MetS (Odds ratio (OR)=1.72,  $p<0.001$ ), even after adjustment for SHBG  
131 (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  
132 associated with development of MetS (OR=1.04;  $p=0.56$ ). However, a lower E2/T ratio was associated  
133 with a lower risk of incident MetS (OR=0.38;  $p<0.001$ ), even after adjustment for SHBG (OR=0.48;  
134  $p<0.001$ ), BMI (OR=0.60;  $p=0.001$ ) or HOMA-IR (OR=0.41;  $p<0.001$ ).

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

138

139

140

141

142

143

144

145

146 ***Introduction***

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

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

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

162  
163 Using data from the European Male Aging Study (EMAS), a prospective study of aging in European  
164 men, we studied the association between baseline sex steroids (T and E2) and the risk of developing  
165 MetS at follow-up and investigated if this association was independent of SHBG, body mass index  
166 (BMI), insulin resistance and body fat measurements. We also assessed whether sex steroids were  
167 associated with change in individual MetS components.

168

169 ***Methods***

170 *Subjects and study design*

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

174 Florence, Italy and Santiago de Compostela, Spain. After a median follow-up time of 4.3 years (range  
175 2.95-5.7 years), 2736 men participated in phase 2. From the original cohort, 193 men had died and 440  
176 were lost to follow-up. 150 men were excluded because of known pituitary or testicular disease or  
177 current drug use of medications that could affect pituitary or testicular function or sex steroid  
178 clearance (e.g. GnRH agonists, testosterone, anticonvulsants). Ethical approval for the study was  
179 acquired in accordance with local institutional requirements at each centre. All subjects gave written  
180 informed consent.

181

### 182 *Assessments*

183 At both phases, participants completed a postal questionnaire that included information about general  
184 health (response set: excellent, very good, good, fair or poor), smoking history (current, past or non-  
185 smoker) and frequency of alcohol consumption in the previous month (none, less than once a week, 1-  
186 2, 3-4, 5-6 or 7 days per week) (14). Current prescription and non-prescription medication use was  
187 recorded.

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

197

### 198 *Laboratory measurements*

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

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

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

215

#### 216 *Dual energy X-ray absorptiometry*

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

221

#### 222 *Definition of the metabolic syndrome and its components*

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

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

229 follow-up, were classified as ‘Incident MetS’. Subjects without MetS at either time point were  
230 classified as ‘No MetS’.

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

235

### 236 *Statistical analysis*

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

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

255

256

257 **Results**

258 *Subject characteristics*

259 In EMAS, MetS status at baseline and follow-up could be assessed in 2376 men. Of these men, 725  
260 men with MetS at baseline were excluded from the analysis. 1651 men did not have MetS at baseline.  
261 Of these, 289 (17.5%) developed MetS during the follow-up period. Baseline characteristics of the  
262 1651 men without baseline MetS are presented in Table 1. Their mean (SD) age was 58.5 (10.7) and  
263 mean BMI was 26.3 kg/m<sup>2</sup> (3.3). 21.8% of the study subjects used antihypertensive drugs, 8.8% were  
264 on statins and 2.0% were treated for diabetes.

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

273

274 *Association between baseline sex steroids and incident MetS*

275 Logistic regression analysis showed that lower total T and free T were associated with an increased  
276 risk of incident MetS (OR=1.64 (CI 1.41-1.90) and OR=1.31 (CI 1.14-1.51) respectively). This  
277 association persisted after adjustment for age, centre and lifestyle factors (alcohol intake, current  
278 smoking status, physical activity and general health) (OR=1.72 (CI 1.48-2.01) for total T and OR=1.36  
279 (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)).  
280 Total E2 levels were not significantly associated with the risk of developing MetS (OR=1.04 (CI 0.91-  
281 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  
282 ratio was associated with a decreased risk for incident MetS, independent of age, centre and lifestyle  
283 factors and SHBG (OR=0.48 (CI 0.35-0.64)). SHBG itself was also independently associated with

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

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

291

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

#### 293 - Insulin resistance

294 After further adjustment for HOMA-IR, total T, free T, E2/T ratio and SHBG remained strongly  
295 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-  
296 0.54), OR=1.75 (CI 1.45-2.10)). Further the association between total E2 and incident MetS became  
297 borderline significant (OR=1.37 (CI 1.00-1.88), p=0.049)) (Table 2).

298

#### 299 - BMI and percentage body fat

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

305

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

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

312 association (OR=0.46 (CI 0.27-0.76)). Total E2 was not associated with MetS (OR=1.22 (CI 0.91-  
313 1.64)).

314 For total and free T and SHBG, the association with incident MetS remained after further adjusting for  
315 % 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  
316 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  
317 free T and OR=1.78 (CI 1.17-2.71) for SHBG). The E2/T ratio was no longer significantly associated  
318 with MetS after further adjustment for body fat or trunk fat measurements (OR=0.63 (CI 0.35-1.14)  
319 and OR=0.68 (CI 0.37-1.24)). Total E2 was weakly associated with incident MetS when % body fat or  
320 % trunk fat was added to the model (OR=1.49 (CI 1.06-2.10) and OR=1.50 (CI 1.06-2.12)).

321

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

323 In the unadjusted model, lower total T, free T and SHBG levels at baseline were associated with a  
324 higher waist circumference at follow-up ( $\beta= 3.91$  (CI 3.47-4.35),  $\beta=3.32$  (CI 2.86-3.79),  $\beta=2.45$  (CI  
325 1.97-2.94)) and E2/T ratio was associated with a lower waist circumference ( $\beta=-8.01$  (CI -8.76—  
326 7.26)). However, these associations disappeared after adjustments for age, centre, lifestyle factors and  
327 baseline waist circumference. Similar results were seen for systolic and diastolic blood pressure,  
328 except for the multivariable adjusted association of baseline SHBG and systolic blood pressure at  
329 follow-up ( $\beta=1.35$  (CI 0.52- 2.17)).

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

338

339

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

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

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

381

382 In contrast to the abundance of data investigating the link between T and MetS, there are few  
383 prospective data investigating the association between E2 and MetS. In men, circulating E2 levels are  
384 in the picomolar range and chromatography/mass spectrometry methods are therefore needed for  
385 accurate measurement of serum E2 (31). Around 60% of circulating E2 in men is produced by  
386 aromatisation of T (11,32). Estrogens play an essential role in male physiology. They are not only  
387 important for bone maintenance, but they also have metabolic effects on carbohydrate and lipid  
388 metabolism and fat distribution, not only in humans, but also in rodents. Estrogen receptor  $\alpha$   
389 disruption, both in the presence or absence of androgen receptor, increases fat mass in male mice  
390 (33,34). An absolute lack of E2, such as in men with congenital aromatase or estrogen receptor alpha  
391 deficiency and in aromatase knockout mice, has also been associated with the development of several  
392 MetS components such as truncal obesity, lipid disorders and insulin resistance (35). Moreover, a  
393 recent study showed that experimentally-induced short-term estrogen deficiency resulted in an  
394 increase in body fat in men (12). In our study, we found no association between baseline E2 levels and  
395 incident MetS. Our results are in line with a cross-sectional study in middle-aged and elderly men (36)

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

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

418

419 Our study has several strengths. It is a large, population based study and standardised methods in  
420 design and analysis were used. As recommended by the Endocrine Society (41), serum T and E2  
421 measurements were done by respectively liquid or gas chromatography-tandem mass spectrometry,  
422 giving more accurate results as compared to other population studies that have used immunoassays  
423 (31,42). Furthermore, the prospective design allows insights into the temporal nature of the

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

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

433

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

439

440

441

442

443

444

445

446

447

448

449

450

451

452 ***Author contributions:***

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

463

464 DV is the guarantor of this work and has full access to all the data in the study and takes responsibility

465 for the integrity of the data and the accuracy of the data analysis.

466

467 ***Acknowledgments and funding***

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

469 We thank Dr. K. Antonio, Research Centre Insurance, Faculty of Economics and Business,

470 KULeuven, Belgium for statistical advice.

471 Part of this research was presented at the annual meeting of the Endocrine Society 2014, Chicago,

472 Illinois. Abstract # 12458.

473

474 The European Male Ageing Study is funded by the Commission of the European Communities Fifth

475 Framework Programme 'Quality of Life and Management of Living Resources' Grant QLK6-CT-

476 2001-00258 and supported by Arthritis Research UK.

477 This work was supported by a grant from the Fund for Scientific Research Flanders (FWO-Vlaanderen

478 grant #G085413N) and by a research grant from the Academische Stichting Leuven.

479

480 **References**

481

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

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

487 3. Scuteri A, Laurent S, Cucca F, Cockcroft J, Cunha PG, Manas LR, Raso FU, Muiesan ML,  
488 Ryliskyte L, Rietzschel E, Strait J, Vlachopoulos C, Volzke H, Lakatta EG, Nilsson PM. Metabolic  
489 syndrome across Europe: different clusters of risk factors. *European journal of preventive cardiology*  
490 2014.

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

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

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

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

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

- 506 9. Haring R, Volzke H, Spielhagen C, Nauck M, Wallaschofski H. The role of sex hormone-binding  
507 globulin and testosterone in the risk of incident metabolic syndrome. *European journal of preventive*  
508 *cardiology* 2013;20:1061-1068.
- 509 10. de Ronde W, van der Schouw YT, Pierik FH, Pols HA, Muller M, Grobbee DE, Gooren LJ, Weber  
510 RF, de Jong FH. Serum levels of sex hormone-binding globulin (SHBG) are not associated with lower  
511 levels of non-SHBG-bound testosterone in male newborns and healthy adult men. *Clinical*  
512 *endocrinology* 2005;62:498-503.
- 513 11. Kaufman JM, Vermeulen A. The decline of androgen levels in elderly men and its clinical and  
514 therapeutic implications. *Endocrine reviews* 2005;26:833-876.
- 515 12. Finkelstein JS, Lee H, Burnett-Bowie SA, Pallais JC, Yu EW, Borges LF, Jones BF, Barry CV,  
516 Wulczyn KE, Thomas BJ, Leder BZ. Gonadal steroids and body composition, strength, and sexual  
517 function in men. *The New England journal of medicine* 2013;369:1011-1022.
- 518 13. Gates MA, Mekary RA, Chiu GR, Ding EL, Wittert GA, Araujo AB. Sex steroid hormone levels  
519 and body composition in men. *The Journal of clinical endocrinology and metabolism* 2013;98:2442-  
520 2450.
- 521 14. Lee DM, O'Neill TW, Pye SR, Silman AJ, Finn JD, Pendleton N, Tajar A, Bartfai G, Casanueva F,  
522 Forti G, Giwercman A, Huhtaniemi IT, Kula K, Punab M, Boonen S, Vanderschueren D, Wu FC. The  
523 European Male Ageing Study (EMAS): design, methods and recruitment. *International journal of*  
524 *andrology* 2009;32:11-24.
- 525 15. Siri WE. Body composition from fluid spaces and density: Analysis of methods. In: Brozek J,  
526 Henzchel A, eds. *Techniques for Measuring Body Composition*. Washington: National Academy of  
527 Sciences; 1961:224–244.
- 528 16. Reuben DB, Siu AL. An objective measure of physical function of elderly outpatients. The  
529 Physical Performance Test. *Journal of the American Geriatrics Society* 1990;38:1105-1112.
- 530 17. Gallagher LM, Owen LJ, Keevil BG. Simultaneous determination of androstenedione and  
531 testosterone in human serum by liquid chromatography-tandem mass spectrometry. *Annals of clinical*  
532 *biochemistry* 2007;44:48-56.

- 533 18. Labrie F, Belanger A, Belanger P, Berube R, Martel C, Cusan L, Gomez J, Candas B, Castiel I,  
534 Chaussade V, Deloche C, Leclaire J. Androgen glucuronides, instead of testosterone, as the new  
535 markers of androgenic activity in women. *The Journal of steroid biochemistry and molecular biology*  
536 2006;99:182-188.
- 537 19. Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the  
538 estimation of free testosterone in serum. *The Journal of clinical endocrinology and metabolism*  
539 1999;84:3666-3672.
- 540 20. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model  
541 assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin  
542 concentrations in man. *Diabetologia* 1985;28:412-419.
- 543 21. Verschueren S, Gielen E, O'Neill TW, Pye SR, Adams JE, Ward KA, Wu FC, Szulc P, Laurent M,  
544 Claessens F, Vanderschueren D, Boonen S. Sarcopenia and its relationship with bone mineral density  
545 in middle-aged and elderly European men. *Osteoporosis international : a journal established as result*  
546 *of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis*  
547 *Foundation of the USA* 2013;24:87-98.
- 548 22. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WP,  
549 Loria CM, Smith SC, Jr. Harmonizing the metabolic syndrome: a joint interim statement of the  
550 International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung,  
551 and Blood Institute; American Heart Association; World Heart Federation; International  
552 Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation*  
553 2009;120:1640-1645.
- 554 23. Hsu B, Cumming RG, Naganathan V, Blyth FM, Le Couteur DG, Seibel MJ, Waite LM,  
555 Handelsman DJ. Associations between Circulating Reproductive Hormones and SHBG and Prevalent  
556 and Incident Metabolic Syndrome in Community-Dwelling Older Men: The Concord Health and  
557 Ageing in Men Project. *The Journal of clinical endocrinology and metabolism* 2014;jc20142464.
- 558 24. Monroe AK, Dobs AS. The effect of androgens on lipids. *Current opinion in endocrinology,*  
559 *diabetes, and obesity* 2013;20:132-139.

- 560 25. Stellato RK, Feldman HA, Hamdy O, Horton ES, McKinlay JB. Testosterone, sex hormone-  
561 binding globulin, and the development of type 2 diabetes in middle-aged men: prospective results  
562 from the Massachusetts male aging study. *Diabetes care* 2000;23:490-494.
- 563 26. Wu FC, Tajar A, Pye SR, Silman AJ, Finn JD, O'Neill TW, Bartfai G, Casanueva F, Forti G,  
564 Giwercman A, Huhtaniemi IT, Kula K, Punab M, Boonen S, Vanderschueren D. Hypothalamic-  
565 pituitary-testicular axis disruptions in older men are differentially linked to age and modifiable risk  
566 factors: the European Male Aging Study. *The Journal of clinical endocrinology and metabolism*  
567 2008;93:2737-2745.
- 568 27. Gregor MF, Hotamisligil GS. Inflammatory mechanisms in obesity. *Annual review of immunology*  
569 2011;29:415-445.
- 570 28. Shahani S, Braga-Basaria M, Basaria S. Androgen deprivation therapy in prostate cancer and  
571 metabolic risk for atherosclerosis. *The Journal of clinical endocrinology and metabolism*  
572 2008;93:2042-2049.
- 573 29. Corona G, Rastrelli G, Monami M, Saad F, Luconi M, Lucchese M, Facchiano E, Sforza A, Forti  
574 G, Mannucci E, Maggi M. Body weight loss reverts obesity-associated hypogonadotropic  
575 hypogonadism: a systematic review and meta-analysis. *European journal of endocrinology / European*  
576 *Federation of Endocrine Societies* 2013;168:829-843.
- 577 30. Wang C, Jackson G, Jones TH, Matsumoto AM, Nehra A, Perelman MA, Swerdloff RS, Traish A,  
578 Zitzmann M, Cunningham G. Low testosterone associated with obesity and the metabolic syndrome  
579 contributes to sexual dysfunction and cardiovascular disease risk in men with type 2 diabetes.  
580 *Diabetes care* 2011;34:1669-1675.
- 581 31. Huhtaniemi IT, Tajar A, Lee DM, O'Neill TW, Finn JD, Bartfai G, Boonen S, Casanueva FF,  
582 Giwercman A, Han TS, Kula K, Labrie F, Lean ME, Pendleton N, Punab M, Silman AJ,  
583 Vanderschueren D, Forti G, Wu FC, Group E. Comparison of serum testosterone and estradiol  
584 measurements in 3174 European men using platform immunoassay and mass spectrometry; relevance  
585 for the diagnostics in aging men. *European journal of endocrinology / European Federation of*  
586 *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.

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

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

622

623

624

625

626

627

628

629

630

631

632

633

634

635

636

637

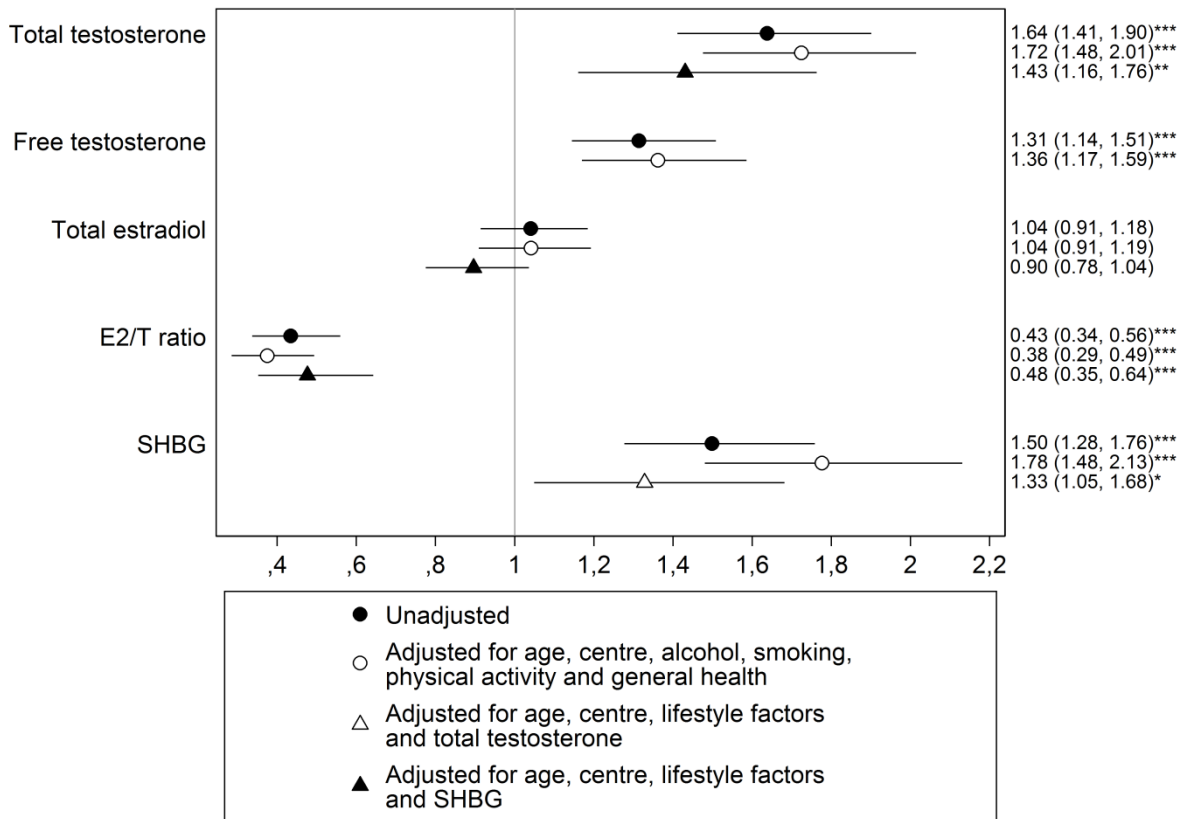
638

639

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		No MetS at follow-up (n = 1362, 82.5%)		Incident MetS at follow-up (n=289, 17.5%)		P-value
	Mean	SD	Mean	SD	Mean	SD	
<b>Physiological measures</b>							
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 <sup>2</sup> )	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
<b>Hormones</b>							
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
<b>MetS Components</b>							
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
<b>Medication use</b>							
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
<b>Total T</b>						
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)
<b>Free T</b>						
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)
<b>E2/T ratio</b>						
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)
<b>Total E2</b>						
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)
<b>SHBG</b>						
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  $\beta$  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.