Sex hormones and cardiometabolic risk

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Geslachtshormonen en cardiometabool risico

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Promotor: Prof.dr.ir. Y.T. van der Schouw

Bij onderzoek wijkt de horizon wanneer we vorderen... En onderzoek is altijd onvolledig.

Mark Pattison
Isaac Casaubon, 1875

Contents

Chapter 1

General introduction

Cardiovascular disease (CVD) is the primary cause of death in most parts of the world and a major determinant of chronic disability [1]. CVD affects men and women differently with the former having a higher incidence and earlier onset of disease [2,3]. The male predisposition to CVD is consistent across countries [4] and cannot fully be explained by differences in cardiovascular risk profiles [5-7]. The universality of this gender gap has produced considerable interest in the role of intrinsic factors, such as sex hormones, in the etiology of CVD. The lack of estrogens and abundance of androgens have traditionally been regarded as the main cause of the male predisposition in cardiovascular risk. This hypothesis was largely based on findings from basic research showing atheroprotective effects of estrogens [8,9] and case reports of acute myocardial infarction in men abusing anabolic steroids [10,11]. However, this hypothesis has turned out to be too simplistic, and there is now increasing evidence that the relationship between endogenous sex hormones and cardiovascular risk is complex and often sex-specific [12,13].

Sex hormones and cardiometabolic risk in women
For many years, the female advantage in CVD has been ascribed to premenopausal estrogen exposure, but epidemiological evidence for a cardioprotective role of estrogens is weak [14]. The lack of a beneficial effect of estrogen replacement in posmenopausal women [15,16], has generated interest in the role of endogenous sex hormones. Data on premenopausal women are limited, but suggest a tendency towards a beneficial effect of endogenous estrogen on cardiometabolic risk [17,18]. In postmenopausal women, however, an opposite association has been reported. In these women, high estrogen concentrations have been associated with an adverse cardiovascular risk profile [19,20], although results on cardiovascular events are mixed [21,22]. Epidemiological data on surrogate markers of estrogen exposure are also conflicting. The CVD rate, for instance, does not show a sharp increase at the time of menopause [23,24], as opposed to the midlife change in the incidence of breast cancer [14,24], an estrogen-related disease. Despite the lack of an inflection point, an earlier onset of menopause has consistently been associated with an increase in CVD morbidity and mortality [25]. Little is known about the impact of menopausal timing on metabolic disease risk. It is also unclear whether the observed increase in CVD risk with early menopause is related to reduced estrogen exposure or is due to other causal factors. It has been suggested that a high vascular risk profile may accelerate the onset of menopause [26], but few epidemiological studies have addressed this possibility of a reverse association. Due to the controversial data on endogenous

estrogens, research has gradually turned more focus to the role of androgens, and there are some indications that conditions of hyperandrogenism may adversely affect cardiometabolic health in women [27,28].

Sex hormones and cardiometabolic risk in men
While studies of anabolic steroid abuse have demonstrated a higher risk of myocardial infarction and sudden cardiac death [29,30], physiological concentrations of androgens do not appear to have a detrimental effect on the male cardiovascular system. In men, high circulating concentrations of testosterone have been associated with a more favourable cardiovascular risk profile [31] and experimental studies show beneficial effects of testosterone on lipid metabolism, insulin sensitivity and cardiac ischemia [32,33]. Nevertheless, a link with clinically manifest endpoints is less clear and little is known about the impact of testosterone and SHBG on subclinical CVD. Also, relatively few studies have investigated the impact of endogenous estrogens in this context. Recently, the main transporter protein of sex hormones, sex hormone-binding globulin (SHBG), has received increasing attention as a marker of metabolic risk [34,35]. SHBG binds androgens with higher affinity than estrogens. Traditionally SHBG has been regarded as a regulator of sex hormone bioavailability, but there is now growing evidence that SHBG may also facilitate the uptake of sex hormones in target tissues [36].

Aim of this thesis
In this thesis, we set out to investigate the complex relationship between endogenous sex hormones and cardiometabolic risk in men and women. The first part of this thesis is devoted to studies in women, and the second part describes the association between sex hormones and cardiometabolic risk in men.

In Chapter 2 we report on the relationship between menopausal age, reproductive lifespan and type 2 diabetes risk. We studied this association in the InterAct study, a case-cohort study nested in the European Prospective Investigation in Cancer and Nutrition (EPIC). In Chapter 3 we examined whether metabolic risk accelerates menopausal onset. Here, we investigated the impact of diabetes and age at diagnosis on timing of natural menopause in the EPIC cohort. As smoking is clearly associated with menopausal age and risk of cardiometabolic disease, we studied the association between smoking habits and sex hormone concentrations in postmenopausal women in Chapter 4. After reviewing the literature on testosterone, SHBG and

cardiovascular health in women (Chapter 5), we report on the results of a meta-analysis investigating the association of endogenous testosterone and SHBG with the metabolic syndrome in Chapter 6. In this literature-based meta-analysis, a significant amount of between-study heterogeneity was observed. Therefore, in Chapter 7 we conducted an individual participant data (IPD) meta-analysis to identify sources of this heterogeneity. Chapter 8 describes the association between testosterone, SHBG and glycated haemoglobin in men and Chapter 9 reports on the association with total and differential white blood cell count, a non-traditional CVD risk factor. Both studies have a cross-sectional design and were conducted in the EPIC-Norfolk cohort. Chapter 10 describes the association between endogenous sex hormones and markers of subclinical atherosclerosis in men. In Chapter 11, the main findings are discussed with recommendations for further research, and Chapter 12 summarizes the results reported in this thesis.

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Chapter 2

**Age at menopause, reproductive lifespan
and type 2 diabetes risk:
results from the EPIC-InterAct Study**

Abstract

Objective: Age at menopause is an important determinant of future health outcomes, but little is known about its relationship with type 2 diabetes. We examined the associations of menopausal age and reproductive lifespan (menopausal age minus menarcheal age) with diabetes risk.

Research design and methods: Data were obtained from the InterAct study, a prospective case-cohort study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC). A total of 3,691 postmenopausal type 2 diabetes cases and 4,408 subcohort members were included in the analysis, with a median follow-up of 11 years. Prentice-weighted Cox proportional hazards models were adjusted for age, known risk factors for diabetes and reproductive factors, and effect modification by BMI, waist circumference and smoking was studied.

Results: Mean (SD) age of the subcohort was 59.2 (5.8) years. After multivariable adjustment, hazard ratios (HRs) of type 2 diabetes were 1.32 (95% CI 1.04-1.69), 1.09 (95% CI 0.90-1.31), 0.97 (95% CI 0.86-1.10) and 0.85 (95% CI 0.70-1.03) for women with menopause at ages < 40 years, 40-44 years, 45-49 and $\geq$ 55 years, respectively, relative to those with menopause at age 50-54 years. The HR per SD younger age at menopause was 1.08 (95% 1.02-1.14). Similarly, a shorter reproductive lifespan was associated with a higher diabetes risk (HR per SD lower reproductive lifespan = 1.06 (95% 1.01-1.12)). No effect modification by BMI, waist circumference or smoking was observed (*P* interaction all > 0.05).

Conclusion: Early menopause is associated with a greater risk of type 2 diabetes.

Introduction

Menopause is an important transition in women's reproductive life, as it signals the end of fertility. Timing of menopause is also an important determinant of future disease risk. For example, an early age at menopause is associated with an increased risk of cardiovascular disease (CVD) (1) and bone fractures (2, 3). Conversely, an early menopause protects against breast (4, 5), endometrial (6) and ovarian cancer (7). Changes associated with the menopause transition, in particular loss of ovarian function and subsequent decline in endogenous estrogens, have been postulated as mediators of these differences in risk (8, 9).

While the relationship between menopausal age and CVD risk is well-established, its association with type 2 diabetes, one of the major risk factors for CVD, remains unclear. Oophorectomized women seem to have less favourable glucose and insulin levels (10, 11), which is suggestive of a link between premature menopause and diabetes risk. However, the few epidemiological studies that have investigated the relationship between menopausal age and diabetes, yielded conflicting results with either an inverse (12) or no association (13, 14). So far, no prospective studies have been performed and no studies have examined the relationship with reproductive lifespan (defined as menopausal age minus menarcheal age), which is a marker of total duration of endogenous estrogen exposure.

In the present study, we investigated the associations of menopausal age and reproductive lifespan with incident type 2 diabetes in InterAct. This large prospective case-cohort study with contributions of eight European countries is part of the European Prospective Investigation into Cancer and Nutrition (EPIC) and provides a unique opportunity to study these associations.

Methods

Study design
The InterAct study is a case-cohort study nested within the European Prospective Investigation into Cancer (EPIC). The participants, methods, study design and measurements have been described previously (15). Briefly, the InterAct consortium was initiated to investigate how genetic and lifestyle factors interact in their influence on the risk of type 2 diabetes. A case cohort study was established based on incident type 2 diabetes cases occurring between 1991 and 2007 in 26 centers from eight

of ten EPIC countries (Italy, Spain, United Kingdom, Netherlands, France, Germany, Sweden and Denmark) participating in InterAct. All participants gave written informed consent, and the study was approved by the local ethics committee in the participating countries and the Internal Review Board of the International Agency for Research on Cancer.

Case ascertainment and verification
Ascertainment and verification of incident diabetes have been described in detail elsewhere (15). New cases occurring up until 31 December 2007 were ascertained by multiple data sources including self-report, linkage to primary or secondary care registers, linkage to pharmacy databases, medication use, hospital admissions and mortality data. Verification of incident diabetes was undertaken, for participants with < 2 independent sources of information, by individual medical record checking or confirmation from another independent source of information.
Follow-up was censored at the date of diagnosis, 31 December 2007 or the date of death, whichever occurred first. In total, 12,403 verified incident cases were identified.

Subcohort
A subcohort of 16,835 individuals was randomly selected from those with available stored blood samples, stratified by centre. We oversampled the number of individuals in the subcohort for the proportion of prevalent diabetes cases in each center to account for later exclusion of individuals with prevalent diabetes from InterAct analyses. After exclusion of 548 individuals with prevalent diabetes and 133 individuals with unknown diabetes status, 16,154 subcohort individuals remained, of whom 778 had developed type 2 diabetes during follow-up.

Study population
The present analysis was restricted to postmenopausal women (Supplementary Figure 1). Women were considered postmenopausal when they reported not having had any menses over the past 12 months or when they reported bilateral oophorectomy. Women with missing or incomplete questionnaire data, or with reported previous hysterectomy, were considered postmenopausal only if they were older than 55 years. Age at menopause was defined as the self-reported age at the last menstrual period. Among the postmenopausal women (N = 9,054), we excluded those who had not reported their age at last menses (N = 1,190), leaving 3,691 incident type

2 diabetes cases and a subcohort of 4,408 postmenopausal women (including 235 incident diabetes cases) for the analyses.

Reproductive factors
At the baseline visit between 1991 and 2000, information on reproductive factors was collected using self-administered questionnaires. In all centers, participants were asked to report their age at first and last menstrual period, current and past use of oral contraceptives (OC) and hormone replacement therapy (HRT), and whether they had undergone a hysterectomy and/or oophorectomy. Except for the Bilthoven cohort (The Netherlands), all centers provided information on the number of full-term pregnancies (the sum of live and still births). In the Bilthoven cohort, the number of children was used as a proxy for the number of full-term pregnancies.
Age at menarche was defined as the age at the first menstrual period and was missing for 96 women. Reproductive lifespan was calculated by subtracting the age at menarche from the age at menopause.

Measurement of other baseline characteristics
Baseline questionnaires included questions on education level, smoking status, alcohol consumption and physical activity. Presence of hypertension and hyperlipidemia at baseline were based on self-reported diagnosis and/or use of medication. Information on hyperlipidemia was not collected in the Swedish cohorts. In most centers, trained health professionals measured weight, height and waist circumference during a visit to the study center. In part of the French cohort and Oxford cohort (UK), height, weight and waist circumference were self-reported, and in Umea (Sweden) only weight and height was measured. Body mass index (BMI) was calculated from the participant's weight (kilograms) divided by the square of their height (square meters).

Data analysis
Twenty percent of the women (N = 1,576) had missing values on one or more covariates. Because the missing values were likely to be missing at random and to avoid loss in efficiency, missing values for BMI, smoking status, alcohol consumption, physical activity, education level, number of full term pregnancies, ever OC and HRT use, and hysterectomy /oophorectomy status were imputed within countries using a multiple imputation technique (10 imputation sets) (16).
We used Cox proportional hazards models adapted to the case cohort design (Prentice weighted method (17)) to estimate hazard ratios (HRs) and 95% confidence

intervals (95% CIs) of type 2 diabetes for menopausal age and reproductive lifespan. In all analyses, age was used as underlying time variable (with entry and exit time defined as the participants age at recruitment and age at T2D diagnosis, death, loss-to follow-up or censoring at the end of the follow-up) and all models were stratified by center to account for study center effects such as follow-up procedures and covariate measurement.

In the analyses, age at menopause was entered as a categorical variable (<40, 40-44, 45-49, 50-54 and >55 years) with menopausal age between 50 and 54 years taken as a reference. Reproductive lifespan was divided into quartiles based on its distribution in the subcohort and HRs were calculated using the highest quartile as a reference. We also modeled menopausal age and reproductive lifespan as continuous variables to investigate the linear association with type 2 diabetes risk. For this purpose, we estimated HRs of type 2 diabetes per standard deviation (SD) younger age at menopause and per SD lower reproductive lifespan.

Analyses were adjusted for potential confounders in three consecutive models. The first model was adjusted for age. Next, we added known diabetes risk factors to the model, including BMI (continuous), smoking status (current, former, never), alcohol consumption ($\leq$ 10 g/day, 11-24g/day, $\geq$25 g/day), education level (none, primary school completed, technical or professional school, secondary school, longer education) and physical activity (inactive, moderately inactive, moderately active, active). In the final multivariable model we further adjusted the analyses for reproductive factors including number of full-term pregnancies (continuous) and ever OC and HRT use (yes vs. no).

We also performed a series of sensitivity analyses. To investigate whether associations were independent of other risk factors, we additionally adjusted the multivariable model for: 1. waist circumference (continuous) and 2. hypertension (yes vs. no) and hyperlipidemia (yes vs. no). Participants from respectively Umea and Sweden (Umea and Malmo) were excluded from these analyses, because of missing values for these variables.

Next, we excluded women who ever used HRT, as age at menopause may be difficult to determine under hormone use. Finally, we restricted the analysis to women who had not undergone a hysterectomy and/or oophorectomy in the past. In this sensitivity analysis, we were able to examine the potential influence of these surgical procedures as well as the effect of a reduction of circulating androgens that occurs with hysterectomy and oophorectomy.

Previously, it has been suggested that the association with menopausal age may vary depending on smoking (18), and obesity status (4, 19). Therefore we stratified the analyses according to BMI (< 25 kg/m², 25-29 kg/m², ≥ 30 kg/m²), waist circumference (< 88 cm, ≥ 88 cm) and smoking status (non-smokers, smokers) and tested for effect modification by including continuous interaction terms in the multivariable adjusted model.

To verify whether pooling of the data was justified, we calculated country-specific HRs using random effects meta-analysis (20) and assessed the amount of heterogeneity (I^2) between countries. For these analyses, we used the HRs of type 2 diabetes per SD younger age at menopause and per SD lower reproductive lifespan, using the multivariable adjusted model including center, age, diabetes risk factors and reproductive factors. All statistical analyses were performed using STATA, version 11.0 (Stata Corp., College Station, TX, USA).

Results

Table 1 shows the baseline characteristics of the subcohort across categories of menopausal age. The mean (SD) age at entry in the subcohort was 59.2 (5.8) years. The mean age at menopause was 48.6 (4.9) years and 4.8% of the women had their menopause before 40 years of age. As expected, women who went through menopause at a younger age had given birth to fewer children, were more likely to be smokers and more often reported having had a hysterectomy and/or oophorectomy. Earlier menopause was associated with a younger age at baseline and a younger age at menarche. Ever HRT-users and a low education level were also more common among these women.

Table 1. Baseline characteristics of the subcohort by categories of menopausal age - EPIC-InterAct study.

| | Total subcohort (N = 4408) | Age at menopause | | | | | P value [c] |
		< 40 (N = 210)	40-44 (N = 501)	45-49 (N = 1452)	50-54 (N = 1879)	≥ 55 (N = 366)	
Age at entry (years)	59.2 (5.8)	56.5 (6.9)	57.6 (6.7)	58.1 (6.0)	60.1 (5.0)	62.2 (3.8)	< 0.001
Body mass index (kg/m^2) *	26.3 (4.6)	26.6 (4.9)	26.3 (4.8)	26.2 (4.7)	26.3 (4.4)	26.6 (4.5)	0.42
Waist circumference (cm) * [a]	83.1 (11.5)	84.4 (12.4)	83.0 (11.8)	82.7 (11.3)	83.2 (11.4)	84.1 (11.5)	0.13
Smoking status % (n) *							< 0.001
Never	59.7 (2619)	51.4 (107)	54.5 (271)	55.0 (797)	64.6 (1207)	65.3 (237)	
Former	20.1 (880)	18.3 (38)	22.5 (112)	21.5 (311)	18.1 (339)	22.0 (80)	
Current	20.2 (887)	30.3 (63)	22.9 (114)	23.5 (340)	17.3 (324)	12.7 (46)	
Physical activity, % (n) *							0.64
Inactive	28.6 (1256)	32.2 (67)	30.9 (154)	27.6 (399)	28.8 (538)	27.1 (98)	
Moderately inactive	36.0 (1578)	37.5 (78)	37.7 (188)	35.3 (511)	35.6 (666)	37.3 (135)	
Moderately active	18.3 (801)	16.8 (35)	15.6 (78)	18.9 (273)	18.5 (345)	19.3 (70)	
Active	17.1 (752)	13.5 (28)	15.8 (79)	18.3 (265)	17.2 (321)	16.3 (59)	
Alcohol consumption, % (n) *							0.10
≤ 10 g/day	73.0 (3161)	77.2 (156)	73.5 (360)	72.4 (1033)	73.0 (1352)	72.2 (260)	
11-25 g/day	19.8 (858)	16.3 (33)	20.6 (101)	20.3 (289)	20.2 (375)	16.7 (60)	
> 25 g/day	7.2 (313)	6.4 (13)	5.9 (29)	7.4 (105)	6.8 (126)	11.1 (40)	
Education, % (n) *							0.01
None	11.0 (478)	11.7 (24)	15.0 (73)	10.5 (150)	11.1 (205)	7.2 (26)	
Primary school completed	39.4 (1711)	38.4 (79)	41.2 (201)	39.8 (570)	39.1 (724)	38.0 (137)	
Technical/professional school	23.5 (1020)	26.2 (54)	18.2 (89)	25.4 (363)	22.8 (422)	25.5 (92)	
Secondary school	12.9 (561)	15.1 (31)	13.1 (64)	11.3 (161)	13.6 (251)	15.0 (54)	
Longer education	13.1 (568)	8.7 (18)	12.5 (61)	13.1 (187)	13.5 (250)	14.4 (52)	
Hypertension, % (n) *	23.8 (1033)	22.8 (47)	21.1 (103)	23.3 (333)	23.9 (443)	29.8 (107)	0.05
Hyperlipidemia, % (n) * [b]	21.2 (686)	21.7 (34)	20.6 (80)	21.2 (228)	20.8 (287)	23.5 (57)	0.92
Number of full term pregnancies *	2.2 (1.4)	1.9 (1.2)	2.1 (1.4)	2.2 (1.3)	2.2 (1.4)	2.2 (1.5)	0.02
Age at menarche (years) *	13.4 (1.6)	13.2 (1.8)	13.1 (1.7)	13.4 (1.6)	13.5 (1.6)	13.8 (1.7)	< 0.001
Hysterectomy and/or oophorectomy, % (n) *	22.3 (858)	71.1 (133)	43.9 (195)	24.8 (618)	11.3 (183)	9.4 (29)	< 0.001
Ever OC use, % (n) *	40.8 (1792)	39.7 (83)	39.5 (197)	42.9 (620)	40.4 (758)	36.7 (134)	0.23
Ever HRT use, % (n) *	32.6 (1292)	43.9 (82)	31.0 (140)	36.1 (478)	27.9 (470)	38.1 (122)	< 0.001

Values are expressed as mean (SD), unless stated otherwise. Abbreviations: OC = oral contraceptive; HRT = hormone replacement therapy.

* variables with missing values (all < 5%, except for hysterectomy and/or oophorectomy status (% missing = 12.7))

[a] excludes Umea, where waist circumference was not measured.

[b] excludes Malmo and Umea where information on hyperlipidemia was not available.

[c] P values derived from statistical tests of between-group differences in menopausal age (Chi-square test for categorical variables and F-test for continuous variables)

During a median (IQR) follow-up of 10.7 (6.6-12.6) years, 3,691 women had developed incident type 2 diabetes. An earlier age at menopause was associated with a higher risk of diabetes (Table 2). After adjustment for known risk factors for diabetes and reproductive factors, HRs of type 2 diabetes were 1.32 (95% CI 1.04-1.69), 1.09 (95% CI 0.90-1.31), 0.97 (95% CI 0.86-1.10), and 0.85 (95% CI 0.70-1.03) for women with menopause at ages < 40 years, 40-44, 45-49 and ≥ 55 years, respectively, relative to those with menopause at age 50-54 years. The HR per SD younger age at menopause was 1.08 (95% 1.02-1.14). A shorter reproductive lifespan was also associated with a greater risk of type 2 diabetes ($HR_{Q1 \text{ vs. } Q4}$ = 1.17 (95% CI 0.98-1.39), $HR_{Q2 \text{ vs. } Q4}$ = 1.00 (95% 0.85-1.19) and $HR_{Q3 \text{ vs. } Q4}$ = 0.96 (95% 0.82-1.14)). The HR per SD lower reproductive lifespan was 1.06 (95% 1.01-1.12).

Analysis with additional adjustment for waist circumference, hypertension and hyperlipidemia yielded comparable results (Supplementary Table 1). When we restricted the analyses to women without a hysterectomy and/or oophorectomy and women not using HRT, effect estimates were also not materially different (Supplementary Table 1).

Interaction analysis showed that the associations of menopausal age and reproductive lifespan with type 2 diabetes did not differ by BMI, waist circumference or smoking status (*P*-values for interaction were 0.16, 0.80 and 0.97 for menopausal age and 0.30, 0.73 and 0.94 for reproductive lifespan respectively, see also Table 3).

Country-specific and pooled HRs of type 2 diabetes are shown in Figure 1. There was no evidence of heterogeneity in the associations between countries (I^2 = 0.0%, *P* = 0.74 for menopausal age and I^2 = 0.0%, *P* = 0.46 for reproductive lifespan), indicating that country-specific HRs were sufficiently similar to justify pooling across countries.

Table 2. Hazard ratios of type 2 diabetes according to menopausal age and reproductive lifespan - EPIC-InterAct study.

	N total / cases	Crude	Model 1	Model 2	Model 3
				HR (95% CI)	
Menopausal age (in categories)					
< 40 years	419 / 220	1.50 (1.22-1.85)	1.50 (1.22-1.85)	1.28 (1.00-1.64)	1.32 (1.04; 1.69)
40-44 years	887 / 424	1.19 (1.02-1.38)	1.18 (1.02-1.37)	1.08 (0.89-1.30)	1.09 (0.90; 1.31)
45-49 years	2570 / 1186	1.05 (0.95-1.17)	1.05 (0.95-1.17)	0.97 (0.86-1.10)	0.97 (0.86-1.10)
50-54 years	3333 / 1554	REF (1.00)	REF (1.00)	REF (1.00)	REF (1.00)
≥ 55 years	655 / 307	0.91 (0.77-1.08)	0.92 (0.77-1.08)	0.84 (0.69-1.02)	0.85 (0.70-1.03)
Menopausal age (per sd decrease)	7864 / 3691	1.11 (1.06-1.16)	1.11 (1.06-1.16)	1.07 (1.01-1.13)	1.08 (1.02-1.14)
Reproductive lifespan (in quartiles)					
Q1 (< 33 years)	1982 / 959	1.11 (0.97-1.28)	1.11 (0.97-1.28)	1.16 (0.97-1.38)	1.17 (0.98-1.39)
Q2 (33-36 years)	2364 / 1077	0.94 (0.82-1.08)	0.94 (0.82-1.08)	1.00 (0.85-1.19)	1.00 (0.85-1.19)
Q3 (37-39 years)	1979 / 897	0.91 (0.79-1.04)	0.91 (0.79-1.04)	0.97 (0.82-1.14)	0.96 (0.82-1.14)
Q4 (≥ 40 years)	1443 / 710	REF (1.00)	REF (1.00)	REF (1.00)	REF (1.00)
Reproductive lifespan (per sd decrease)	7768 / 3643	1.07 (1.02-1.12)	1.07 (1.02-1.12)	1.06 (1.00-1.12)	1.06 (1.01-1.12)

Prentice-weighted Cox proportional hazards models stratified by center
Model 1: adjusted for age at entry.
Model 2: Model 1 plus diabetes risk factors (body mass index, smoking, alcohol consumption, physical activity and education)
Model 3: Model 2 plus reproductive factors (number of full-term pregnancies, oral contraceptive use (ever) and hormone replacement therapy (ever))

Table 3. Multivariable adjusted hazard ratios of type 2 diabetes per sd decrease of menopausal age and reproductive lifespan, stratified by BMI, waist circumference and smoking status - EPIC-InterAct study.

		HR (95% CI)			
		N total / cases	Menopausal age (per sd decrease)	N total / cases	Reproductive lifespan (per sd decrease)
Body mass index	< 25 kg/m^2	2485 / 576	1.13 (1.03-1.25)	2444 / 566	1.12 (1.02-1.23)
	25-29 kg/m^2	3002 / 1446	1.09 (1.01-1.19)	2972 / 1426	1.08 (0.99-1.17)
	≥ 30 kg/m^2	2377 / 1669	1.08 (0.98-1.17)	2352 / 1651	1.07 (0.98-1.16)
	P value interaction [a]		0.16		0.30
Waist circumference	< 88 cm	4096 / 1221	1.08 (1.00-1.16)	4043 / 1202	1.06 (0.98-1.14)
	≥ 88 cm	3527 / 2347	1.09 (1.01-1.17)	3489 / 2321	1.07 (0.99-1.15)
	P value interaction [a]		0.80		0.73
Smoking status	Non-smokers	6262 / 2938	1.07 (1.01-1.14)	6191 / 2906	1.06 (1.00-1.13)
	Smokers	1602 / 753	1.08 (0.96-1.20)	1577 / 737	0.99 (0.97-1.00)
	P value interaction [a]		0.97		0.94

Model 3: Prentice-weighted Cox proportional hazards models stratified by center and adjusted for age at entry, diabetes risk factors (BMI, smoking status, alcohol consumption, physical activity, education) and reproductive factors (number of full-term pregnancies, ever OC use and ever HRT use)
[a] Effect modification was tested by adding interaction terms between these variables (body mass index (continuous), waist circumference (continuous), smoking status (smoker vs non-smoker)) and menopausal age (continuous) or reproductive lifespan (continuous) to the model.

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Figure 1: Country-specific hazard ratios of type 2 diabetes per sd decrease in menopausal age and reproductive lifespan - EPIC-InterAct study.
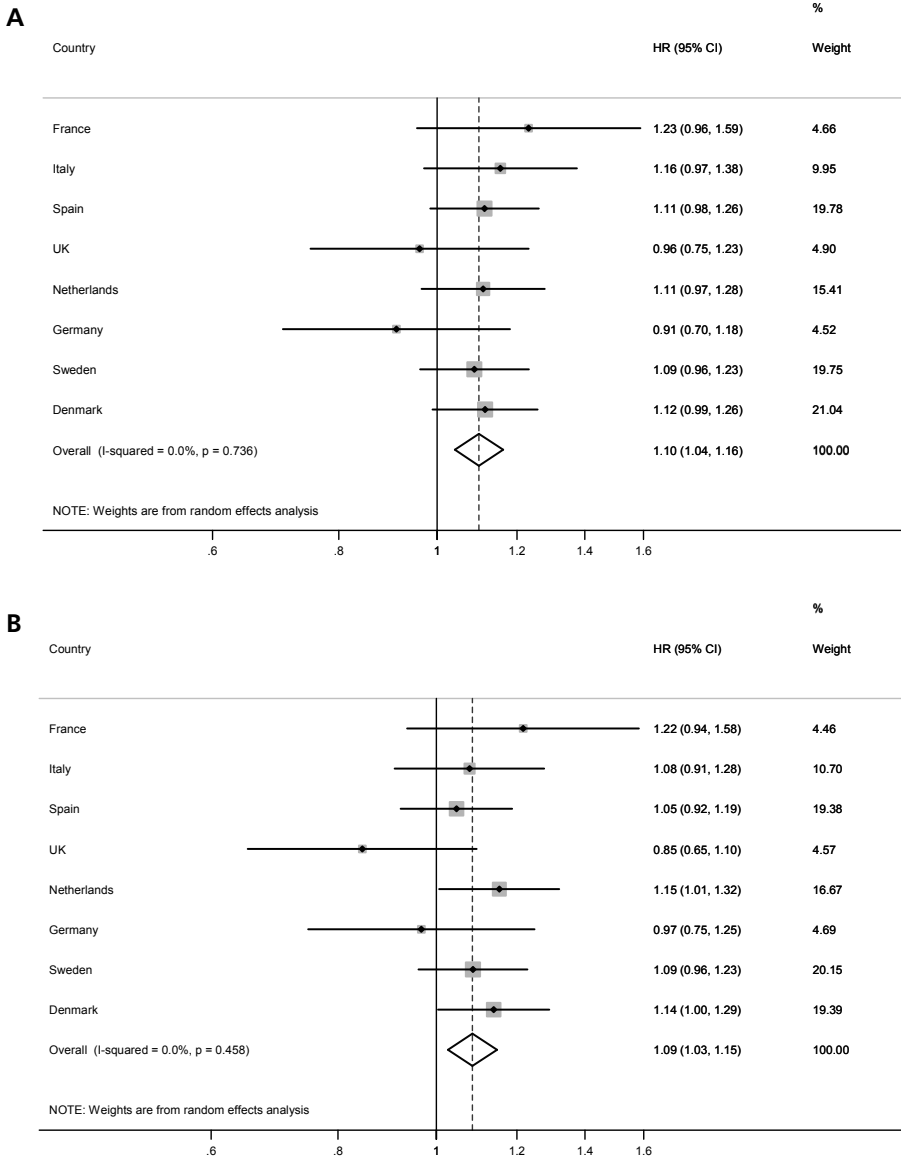
Hazard ratios and 95% CIs are derived from Prentice-weighted Cox proportional hazards models adjusted for center, age at entry, diabetes risk factors (BMI, smoking, alcohol consumption, physical activity and education) and reproductive factors (number of full-term pregnancies, ever OC use and ever HRT use). A = hazard ratio of type 2 diabetes per sd decrease in menopausal age. B = hazard ratio of type 2 diabetes per sd decrease in reproductive lifespan.

2

Discussion

In this prospective case-cohort study, we found that an earlier age at menopause was associated with a greater risk of type 2 diabetes. The hazard of type 2 diabetes was 32% higher in women who entered their menopause before 40 years of age compared with women having their menopause at 50-54 years. Similarly, a shorter reproductive lifespan was associated with a higher diabetes risk. All associations were robust to adjustment for a wide range of potential confounding factors and effect estimates were of similar magnitude after excluding women with a hysterectomy and/or oophorectomy and women using HRT. No effect modification by BMI, waist circumference or smoking was found.

The strengths of our study include its prospective design, the use of verified incident diabetes cases and adjustment in the analyses for a comprehensive set of potential confounders. Data collection on reproductive factors was fully standardized across cohorts, except for the number of full-term pregnancies, which was collected slightly different for one center. Nevertheless, our study also had some limitations. First of all, assessment of age at menarche and menopause was based on self-report, which is prone to recall bias, particularly in older women. However, previous studies have shown that the validity and reproducibility of self-reported age at menopause and menarche are fairly good (21-25). Because of the prospective design, any misclassification is most likely unrelated to the occurrence of diabetes, and such random misclassification if anything usually leads to an underestimation of risks. Second, the use of a clinical definition may have led to potential misclassification of individuals with undiagnosed diabetes. However, multiple sources were used for case ascertainment and even if some under-diagnosis may have occurred, this would have tended to attenuate associations rather than to produce spurious ones. Third, we adjusted the analyses for a large number of confounders, but we cannot rule out the possibility of residual confounding. Also, potential effect modifiers were not measured at the actual onset of menopause, but somewhere in between menopause and follow-up. This may have limited the interaction analyses and might explain the lack of interactions observed. Finally, despite the prospective design, the observed associations may partially reflect reverse causation. Women with type 1 diabetes enter menopause several years earlier than nondiabetic women (26), but so far no data exists on the effect of early-onset type 2 diabetes on menopausal timing. Glycosylation of functional proteins may cause ovarian dysfunction, but type 1 diabetes could also be linked to menopausal age through distinct mechanisms involving auto-immunity.

Several studies have examined the impact of menopause on diabetes risk. Most studies, however, investigated the relationship with menopause status rather than age at menopause onset, and did not find associations (27-29). Previous results regarding menopausal age are mixed (12-14). In a large study among women entering menopause clinics, no association between menopausal age and type 2 diabetes was found (13). In another cross-sectional study, diabetes was more prevalent among women with premature menopause, but this association was not statistically significant after multivariable adjustment (14). On the other hand, Malacara et al. (12) found a positive correlation between age at menopause and age at diabetes diagnosis. This is the first prospective study looking at menopausal age and type 2 diabetes risk. Associations with menopausal age and reproductive lifespan were of similar strength. Previous studies have linked an early age at menarche to a greater risk of type 2 diabetes (30, 31), which may suggest that earlier timing of menopause *per se*, rather than a shorter interval between menarche and menopause is the main determinant of diabetes risk. However, given the close correlation between menopausal age and reproductive lifespan (r = 0.95), it is difficult to truly distinguish between the relative contributions of these two factors.

Associations between menopausal age and risk of chronic diseases are usually attributed to a short or prolonged exposure to endogenous estrogens. In contrast to breast cancer, where the available evidence on reproductive factors, endogenous estrogen levels and exogenous estrogen supplementation all points to an important role of estrogen exposure (32), results are more equivocal for diabetes. Experimental data support a protective role for estrogens in glucose metabolism. Mechanistic studies have demonstrated beneficial long-term effects of exogenous estrogens on insulin secretion and glucose homeostasis (33), and in postmenopausal women estrogen replacement has been associated with a lower incidence of type 2 diabetes (34, 35). Observational data, however, argue against a simple protective effect of estrogens. In postmenopausal women, high endogenous estrogen levels have been associated with an increase rather than decrease in diabetes risk (36, 37). Moreover, an early start of estrogen exposure (i.e. an early age at menarche) appears to have an adverse effect on diabetes risk (30, 31). Thus, apart from the dramatic reduction in endogenous estrogen, other menopause-related factors may play a role in explaining the observed increase in diabetes risk with early menopause. The menopausal transition is also characterized by a shift towards androgen predominance including a decrease in sex hormone-binding globulin (SHBG) levels (38, 39). Increased androgenicity, in turn, has been linked to a higher risk of type 2

diabetes in postmenopausal women (36, 37). Alternatively, early menopause may represent a marker of premature ageing. A recent meta-analysis of genome-wide association studies found 17 loci for age at menopause that have previously been related to DNA damage repair and replication, important processes in determining longevity (40). More research is needed to unravel the mechanisms through which the timing of menopause influences metabolic disease risk.

The findings of the present study are of interest in light of the high prevalence of type 2 diabetes among postmenopausal women. The direct effect of early menopause may be relevant for the prevention of diabetes in women. For example, early menopause might be a factor to take into account when considering diabetes screening or direct preventive action. However, before embarking on this, more studies are needed to evaluate whether timing of menopause has any added value in diabetes prediction and prevention.

In conclusion, this is the first prospective study demonstrating that women with an early age at menopause are at higher risk of developing type 2 diabetes.

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Supplementary Figure 1. Flow chart of the study population – EPIC-InterAct study.

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                              │          EPIC           │
                              └─────────────────────────┘

 ┌──────────────────────────────────┐   ┌──────────────────────────────────┐
 │   Verified incident T2D cases    │   │            Subcohort             │
 │          (N = 12,403)            │   │          (N = 16,154)            │
 │         ┌ ─ ─ ─ ─ ─ ─ ─ ─ Overlap  (N = 778) ─ ─ ─ ─ ─ ─ ┐           │
 └─────────────────────────────────────────────────────────────────────────┘

                       ┌─────────────────────────┐      ┌──────────────────────────────────────────────┐
                       │      EPIC-InterAct       │      │ Exclusions:                                    │
                       │      (N = 27,779)        │─────▶│ Men (N = 11,892)                               │
                       │                          │      │ Pre- and perimenopausal women (N = 6,833)      │
                       └─────────────────────────┘      │ No age at menopause reported (N = 1,190)       │
                                                         └──────────────────────────────────────────────┘

                       ┌─────────────────────────┐
                       │     Study population     │
                       │       (N = 7,864)        │
                       └─────────────────────────┘

 ┌──────────────────────────────────┐   ┌──────────────────────────────────┐
 │   Verified incident T2D cases    │   │            Subcohort             │
 │           (N = 3,691)            │   │           (N = 4,408)            │
 │        ┌ ─ ─ ─ ─ ─ ─ Overlap  (N = 235) ─ ─ ─ ─ ─ ─ ┐                │
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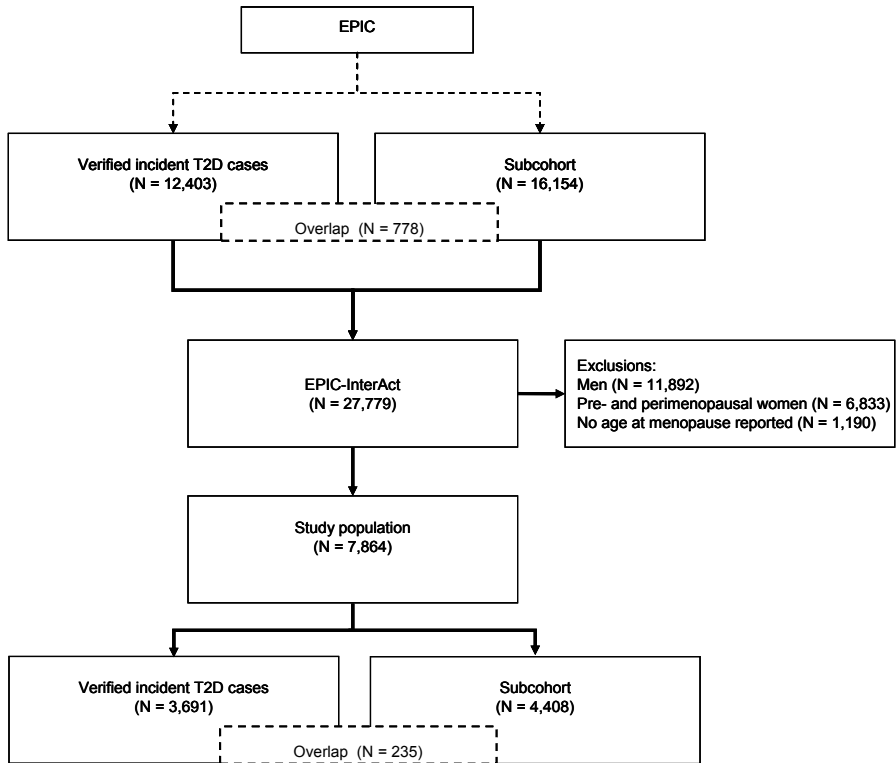
Flow chart showing the derivation of the study population for the present analysis from the EPIC-InterAct study. Details about the derivation of the original InterAct case-cohort sample from the total EPIC-cohort have been described in detail elsewhere (15)

Supplementary Table 1. Sensitivity analyses for risk of type 2 diabetes according to menopausal age and reproductive lifespan - EPIC-InterAct study.

	HR (95% CI)				
	Model 3	Model 4	Model 5	Model 6	Model 7
Menopausal age (in categories)					
< 40 years	1.32 (1.04-1.69)	1.28 (0.98-1.67)	1.39 (1.02-1.87)	1.43 (1.03-2.00)	1.34 (0.86-2.11)
40-44 years	1.09 (0.90-1.31)	1.07 (0.89-1.30)	1.21 (0.97-1.50)	1.13 (0.90-1.41)	1.09 (0.86-1.38)
45-49 years	0.97 (0.86-1.10)	0.99 (0.87-1.13)	1.01 (0.87-1.17)	1.06 (0.92-1.23)	0.94 (0.81-1.09)
50-54 years	REF (1.00)	REF (1.00)	REF (1.00)	REF (1.00)	REF (1.00)
≥ 55 years	0.85 (0.70-1.03)	0.81 (0.64-1.02)	0.94 (0.73-1.19)	0.94 (0.73-1.20)	0.86 (0.69-1.07)
Menopausal age (per sd decrease)	1.08 (1.02-1.14)	1.07(1.01-1.14)	1.09 (1.03-1.17)	1.08 (1.00-1.16)	1.07 (0.99-1.15)
Reproductive lifespan (in quartiles)					
Q1 (< 33 years)	1.17 (0.98-1.39)	1.15 (0.95-1.40)	1.19 (0.97-1.48)	1.17 (0.94-1.45)	1.13 (0.91-1.40)
Q2 (33-36 years)	1.00 (0.85-1.19)	1.05 (0.87-1.26)	0.97 (0.79-1.19)	1.00 (0.82-1.23)	0.93 (0.80-1.12)
Q3 (37-39 years)	0.96 (0.82-1.14)	0.99 (0.82-1.19)	0.91 (0.74-1.12)	0.95 (0.77-1.17)	0.96 (0.80-1.16)
Q4 (≥ 40 years)	REF (1.00)	REF (1.00)	REF (1.00)	REF (1.00)	REF (1.00)
Reproductive lifespan (per sd decrease)	1.06 (1.01-1.12)	1.06 (0.99-1.12)	1.08 (1.01-1.15)	1.07 (1.00-1.15)	1.04 (0.97-1.13)

Model 3: Prentice-weighted Cox proportional hazards models stratified by center and adjusted for age at entry, diabetes risk factors (BMI, smoking, alcohol consumption, physical activity and education) and reproductive factors (number of full-term pregnancies, ever OC use and ever HRT use)
Model 4: Model 3 plus waist circumference (N excluded = 241)
Model 5: Model 3 plus hypertension and hyperlipidemia (N excluded = 2,163)
Model 6: Model 3 excluding women who ever used hormone replacement therapy (N excluded = 2,589)
Model 7: Model 3 excluding women with a hysterectomy and/or oophorectomy (N excluded = 1,972)

Chapter 3

**Diabetes and timing of natural menopause
in the European Prospective Investigation into Cancer
and Nutrition (EPIC)**

Abstract

Background: Timing of natural menopause is influenced by both genetic and environmental factors. Recent evidence suggests that metabolic disease may also influence the onset of menopause.

Objective: We investigated the impact of diabetes and age at diagnosis on menopausal timing in 255,898 women from the European Prospective Investigation into Cancer and Nutrition (EPIC), collected between 1992 and 2000.

Methods: Information on menopause, diabetes and other covariates was obtained by self-administered questionnaires. Cox proportional hazards models were used to estimate the associations of diabetes and age at diagnosis (in categories: $\geq$ 50 years, 45-49 years, 40-44 years, 30-39 years, 20-29 years, 10-20 years, < 10 years) with age at natural menopause (ANM). Diabetes and confounders, where possible, were modeled as time-dependent variables and age from birth to menopause or censoring was used as the underlying time scale. All analyses were stratified by center and adjusted for age, smoking, reproductive factors and diabetes risk factors.

Results: Median ANM was 52.0 years. Overall, no significant association between diabetes and ANM was found (HR = 0.94; 95% CI 0.89-1.00). However, when looking at age at diagnosis, women with diabetes before the age of 20 years had an earlier menopause (10-20 years: HR = 1.45; 95% CI 1.03-2.03, < 10 years: HR = 1.59 (1.04-2.44)) compared to non-diabetic women, whereas women with diabetes at age 50 years and older had a later menopause (HR = 0.81; 95% CI 0.70-0.95). For the other four age groups no association with ANM was found (45-49 years: HR= 0.91; 95% CI 0.81-1.02, 40-44 years: HR = 1.00; 95% CI 0.86-1.16, 30-39 years: HR= 0.96, 95% CI; 0.84-1.10, 20-29 years: HR = 0.99, 95% CI 0.82-1.20).

Conclusions: Our results suggest that diabetes may influence ANM. Future longitudinal studies are needed to replicate these findings and to unravel the underlying mechanisms and clinical significance of the observed associations.

Introduction

Menopause is a universal event in women's reproductive life, but the timing of onset varies widely. In the Western world, age at natural menopause (ANM) typically ranges between 40 and 60 years, with an average age of onset of 51-52 years [1]. Although the exact underlying mechanisms are not completely understood, timing of menopause is considered to be a complex trait, being influenced by both genetic and environmental factors. Smoking is the best-established environmental factor affecting ANM, with menopause occurring 1-2 years earlier in smokers [2]. Other factors that have been linked to an earlier menopause are nulliparity and low socioeconomic status, while use of oral contraceptives tends to delay menopause [2]. Several studies have investigated an association with physical activity and dietary factors, but their impact seems to be small and not always consistent [2].

Next to genetic and environmental factors, chronic metabolic disease may also influence ANM. There is some evidence suggesting that diabetes may accelerate menopausal onset. Women with type 1 diabetes (T1D) have an earlier decline of inhibin B and anti-Müllerian hormone (AMH) levels, which is indicative of premature ovarian ageing [3]. Furthermore, women with T1D have been reported to enter menopause 5 years earlier than non-diabetic women [4], although this association was not confirmed in another study [5]. In addition, we have previously shown that women with an adverse metabolic risk factor profile enter menopause earlier [6], and recently a lower ovarian reserve was found in premenopausal women with type 2 diabetes (T2D) [7].

In the present study, we set out to examine the association of diabetes and age at diagnosis with ANM in the European Prospective Investigation into Cancer and Nutrition (EPIC).

Methods

Study population
The EPIC study is a multicenter prospective cohort study aimed at investigating the relations between diet, lifestyle, and genetic factors and the incidence of cancer and other chronic diseases. The cohort was initiated in the early 1990s in 23 centers from 10 European countries (France, Italy, Spain, United Kingdom, Netherlands, Greece,

Germany, Sweden, Denmark and Norway). Details of EPIC, in particular on the design, study population and baseline data collections have been described previously [8, 9]. In brief, 519,978 men and women, mostly aged 27 to 70 years, were mainly recruited from the general population between 1992 and 2000. Exceptions were the Oxford cohort, United Kingdom (vegetarian volunteers and healthy eaters); the Utrecht cohort, the Netherlands and the Florence cohort, Italy (women attending breast cancer screening); the French cohort (female members of the health insurance for state school employees); and components of the Italian and Spanish cohorts (members of local blood donor associations). Baseline questionnaires included questions on dietary, lifestyle, reproductive and medical factors. All participants provided written informed consent and the study was approved by the local ethics committees of the participating centers and the Internal Review Board of the International Agency for Research on Cancer. In most countries, participants were invited to a center for anthropometric measurements.

In total, 367,331 women participated in the EPIC study. For the present study, we excluded women from the Norwegian cohorts (N = 37,200) and the Swedish cohorts (N = 30,329), because of lack of data on hysterectomy and/or oophorectomy status. We further excluded women with missing data on menopause (N = 35,013) and diabetes (N = 5,891) status, leaving 258,898 women for the analyses.

Exposure assessment
Diabetes status was based on self-report and obtained through a questionnaire in which participants were asked if they had ever been diagnosed with diabetes and if so at what age. In the questionnaire, no distinction was made between type 1 and type 2 diabetes (T1D and T2D).

Assessment of menopausal status and age at menopause,
Menopausal status was defined according to information on menstruation status and age at enrolment. Women were considered as postmenopausal if they reported not having had any menses over the past 12 months. Women were considered premenopausal when they reported having had regular menses over the past twelve months. Women were considered perimenopausal if they reported having irregular menses over the past twelve months or if they indicated having had menses over the past 12 months, but were no longer menstruating at the time of enrolment. Women with missing or incomplete questionnaire data on menstruation status were classified as premenopausal when they were younger than 46 years, as perimenopausal when

they were between 46 and 55 years of age and as postmenopausal when they were older than 55 years at enrolment. Age at menopause was based on self-report and defined as age at last menses.

Histories of hysterectomy an oophorectomy and age at surgery were also obtained through self-administered questionnaires. We considered women surgically postmenopausal if they had had a hysterectomy and/or oophorectomy before reaching natural menopause.

Assessment of other covariates

Information on smoking, alcohol consumption, physical activity and education level was based on self-report. Baseline questionnaires also included questions on reproductive factors such as age at first menstruation, number of live and stillbirths, and current and past use of oral contraceptives (OC) and hormone replacement therapy (HRT). Except for the Bilthoven cohort (The Netherlands), all centers collected information on the number of full-term pregnancies (the sum of live and stillbirths). In the Bilthoven cohort, the number of children was used as a proxy for the number of full-term pregnancies. Women were also asked to give their age of starting and quitting smoking, and starting age and duration of OC use (in years). Information on the starting age of HRT use was collected slightly differently in each center. For this reason, start of HRT use was recoded in a uniform categorical variable (< 40 years, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, ≥ 55 years) to maximize comparability across centers.

In most centers, trained health professionals measured weight and height during a visit to the study center, except for part of the French cohort and Oxford cohort (UK) where height and weight were self-reported. Weight and height were measured to the nearest 0.1 kg and 0.1 or 0.5 cm, respectively, with subjects wearing no shoes. Weight was corrected for clothing worn during measurement in order to reduce heterogeneity due to protocol differences between centers [10], and the accuracy of self-reported measures was improved with the use of prediction equations derived from participants with both measured and self-reported measures [11]. Body mass index (BMI) was calculated from the participant's weight (kilograms) divided by the square of their height (square meters).

Data analysis

Missing values for covariates (all < 5%) were imputed using single imputation. Data were analyzed using a survival analysis approach, which allows for the inclusion of incomplete or censored observations in the estimation of ANM. Women who had had a hysterectomy and/or oophorectomy prior to menopause were censored at their age at surgery and pre- and perimenopausal women were censored at their age at enrolment. In all analyses, age from birth to menopause or censoring was used as the underlying time scale. We first used Kaplan-Meier analysis to estimate the median age at menopause for the entire cohort and after stratification for participant characteristics. We then used Cox proportional hazards models to estimate the associations between participant characteristics and ANM. Hazard ratios (HRs) derived from these models represent the risk of becoming naturally menopausal at a given age, with HRs less than 1 indicating a later menopause and HRs greater than 1 indicating an earlier menopause compared to the reference. Diabetes status was modeled as a time-dependent variable, changing from at-birth unexposed to exposed at the self-reported age at diagnosis. To investigate the impact of diabetes timing, we also entered age at diagnosis as a time-dependent categorical variable (age at diagnosis $\geq$ 50 years, 45-49 years, 40-44 years, 30-39 years, 20-29 years, 10-20 years, < 10 years) into the model.

Analyses were adjusted for potential confounders in three consecutive models and all models were stratified by center to account for study center effects (i.e. questionnaire design and covariate measurement). The first model was adjusted for age. Next, we added reproductive factors to the model: age at menarche (continuous), number of full-term pregnancies (0, 1, 2, $\geq$3) and OC and HRT use. In the final multivariable model, we further adjusted the analyses for known diabetes risk factors including BMI (continuous), smoking, alcohol consumption (< 10 g/day, 10-24 g/day, 25-50 g/day and > 50 g/day), physical activity (inactive, moderately inactive, moderately active, active) and education level (none, primary school, technical or professional school, secondary school, longer education). Similar to diabetes status, premenopausal exposures to smoking and oral contraceptives were modeled as time-dependent covariates with exposure starting at the self-reported age at smoking and OC initiation, and ending at cessation or censoring. HRT use before reaching natural menopause was modeled as a categorical variable (yes vs. no).

We also performed several sensitivity analyses. Hormone use prior to menopause may mask a woman's true menopausal age. To investigate the impact of this potential misclassification, we performed a separate analysis in which we excluded

women who used HRT prior to menopause and women who used OCs in the year before or after their menopause. Because the age at entry varied between 20 and 97 years, a cohort effect may have confounded the association. To investigate this, we additionally adjusted the analyses for birth cohort (before 1930; 1930 to 1939; 1940 to 1949; 1950 to 1959; 1960 and later). All statistical analyses were performed using STATA, version 11.0 (Stata Corp., College Station, TX, USA).

Results

Participant characteristics of the study population are presented in Table 1. The mean (SD) age was 50.6 (10.2) years and 131,923 (51.0%) women were still menstruating. The estimated median ANM in the entire study population was 52.0 years. Table 2 shows the crude ANM across strata of participant characteristics and the corresponding HRs after multivariable adjustment. Smoking and a low educational level were associated with an earlier natural menopause, while late menarche, OC use and being parous were associated with a later menopause. Menopause was also delayed in women who were physically active and women who consumed alcoholic drinks.

In total, 5,999 women had a self-reported diagnosis of diabetes of whom 2,752 had diabetes before menopause (defined as age at diabetes diagnosis < age at menopause or censoring). Table 3 shows the HRs for natural menopause according to diabetes status. Overall, no statistically significant association between diabetes and ANM was found (HR = 0.94; 95% CI 0.89-1.00). Analyses for age at diagnosis showed that women with diabetes before the age of 20 years were more likely to have an earlier menopause (10-20 years: HR = 1.45; 95% CI 1.03-2.03, < 10 years: HR = 1.59; 95% CI 1.04-2.44), whereas women with diabetes at age 50 years and older were more likely to enter menopause at a later age (HR = 0.81; 95% CI 0.70-0.95). None of the other categories of age at diagnosis were associated with ANM (Table 3, models 1-3). Results were not materially different when we excluded women who used HRT prior to menopause and/or oral contraceptives around menopause, although the association with diabetes before the age of 10 years was slightly strengthened (Supplementary Table 1, model 4). Results were also unchanged after adjusting the analyses for birth cohort (Supplementary Table 1, model 5).

Table 1. Characteristics of the study population (N = 255,898).

Participant characteristic	
Age at entry (years), mean (SD)	50.6 (10.2)
Body mass index (kg/m²), mean (SD)	25.2 (4.6)
Menopausal status, % (N)	
Pre- or perimenopausal	51.0 (131,923)
Natural postmenopausal	34.4 (88,992)
Surgical postmenopausal	14,7 (37,983)
Age at menarche *	13.0 (1.6)
Number of full-term pregnancies, % (N) *	
0	14.9 (38,575)
1	15.9 (41,273)
2	40.2 (104,128)
≥ 3	26.0 (67,381)
Ever OC use, % (N) *	58.4 (151,138)
Ever HRT use, % (N) *	19.2 (49,799)
Smoking status, % (N) *	
Never	58.5 (151,559)
Former	21.8 (56,556)
Current	18.0 (46,714)
Alcohol consumption, % (N) *	
< 10 g/day	69.3 (179,485)
10-24 g/day	20.8 (53,837)
25-50 g/day	7.5 (19,334)
> 50 g/day	1.5 (3,897)
Education, % (N) *	
None	5.8 (14,942)
Primary school	24.3 (62,982)
Technical or professional school	19.6 (50,814)
Secondary school	22.9 (59,356)
Longer education	23.7 (61,365)
Physical activity, % (N) *	
Inactive	24.6 (63,696)
Moderately inactive	34.9 (90,439)
Moderately active	23.7 (61,228)
Active	15.7 (40,569)
Diabetes, % (N)	2.3 (5,999)
Age at diabetes diagnosis, mean (SD)	47.3 (13.2)

Values are expressed as mean (SD), unless stated otherwise. Abbreviations: OC = oral contraceptive; HRT = hormone replacement therapy * variables with missing values (all < 5%)

Table 2. Associations of reproductive and lifestyle factors with age at natural menopause.

Participant characteristic	Median ANM (IQR) [a]	Adjusted HR (95% CI) [b]
Age at menarche		
< 12 years	52 (49-54)	REF (1.00)
≥ 12 years	52 (50-54)	0.95 (0.93-0.96)
Number of full-term pregnancies		
0	51 (49-54)	REF (1.00)
1	52 (49-54)	0.89 (0.87-0.92)
2	52 (50-55)	0.86 (0.84-0.88)
≥ 3	52 (49-54)	0.85 (0.83-0.87)
Ever use of oral contraceptives		
Never	51 (49-54)	REF (1.00)
Ever	52 (50-55)	0.93 (0.92-0.95)
Body mass index		
< 25 kg/m^2	52 (50-55)	REF (1.00)
25-30 kg/m^2	52 (49-54)	1.02 (1.01-1.04)
≥ 30 kg/m^2	52 (49-54)	1.01 (0.99-1.03)
Smoking status		
Never	52 (50-54)	REF (1.00)
Former	52 (50-55)	1.05 (1.03-1.07)
Current	51 (49-54)	1.35 (1.32-1.37)
Alcohol consumption		
< 10 g/day	52 (49-54)	REF (1.00)
10-25 g/day	52 (50-55)	0.94 (0.93-0.96)
25-50 g/day	52 (50-55)	0.91 (0.89-0.93)
> 50 g/day	52 (50-55)	0.91 (0.86-0.97)
Physical activity		
Inactive	51 (49-54)	REF (1.00)
Moderately inactive	52 (50-54)	0.96 (0.94-0.97)
Moderately active	52 (50-55)	0.92 (0.91-0.94)
Active	52 (50-55)	0.96 (0.94-0.98)
Education		
None	50 (48-53)	1.54 (1.49-1.58)
Primary school	51 (49-54)	1.31 (1.28-1.34)
Technical or professional school	52 (49-54)	1.18 (1.15-1.20)
Secondary school	52 (50-55)	1.04 (1.01-1.06)
Longer education	52 (50-55)	REF (1.00)

Abbreviations: ANM = age at natural menopause; HR = hazard ratio; 95% CI = 95% confidence interval.

[a] Median age at natural menopause was estimated using Kaplan Meier analyses.

[b] Hazard ratios derived from multivariable adjusted models including reproductive factors (age at menarche, number of full-term pregnancies, ever use of oral contraceptives) and diabetes risk factors (body mass index, smoking status, alcohol consumption, physical activity and education)

Table 3. Hazard ratios of natural menopause according to diabetes status and age at diagnosis.

	HR (95% CI)		
	Model 1 (N = 255,898)	Model 2 (N = 255,898)	Model 3 (N = 255,898)
Diabetes before menopause			
No (N = 256,146)	REF (1.00)	REF (1.00)	REF (1.00)
Yes (N = 2,752)	0.97 (0.91-1.03)	0.95 (0.89 -1.01)	0.94 (0.89-1.00)
Age at diabetes diagnosis			
No diabetes before menopause	REF (1.00)	REF (1.00)	REF (1.00)
≥ 50 (N = 317)	0.85 (0.73-0.99)	0.82 (0.70-0.96)	0.81 (0.70-0.95)
45-49 (N = 649)	0.91 (0.81-1.02)	0.90 (0.81-1.01)	0.91 (0.81-1.02)
40-44 (N = 444)	1.02 (0.87-1.19)	1.01 (0.86-1.17)	1.00 (0.86-1.16)
30-39 (N = 715)	1.00 (0.87-1.14)	0.97 (0.85-1.11)	0.96 (0.84-1.10)
20-29 (N = 394)	1.07 (0.89-1.30)	1.03 (0.85-1.24)	0.99 (0.82-1.20)
10-20 (N = 161)	1.20 (0.85-1.69)	1.43 (1.02-2.02)	1.45 (1.03-2.03)
< 10 (N = 72)	1.56 (1.01-2.39)	1.67 (1.09-2.56)	1.59 (1.04-2.44)

Cox proportional hazards models stratified by center and including diabetes status and age at diabetes diagnosis as time-dependent variables.
Model 1: adjusted for age
Model 2: Model 1 plus reproductive factors (age at menarche, number of full-term pregnancies, oral contraceptive use and hormone replacement therapy)
Model 3: Model 2 plus diabetes risk factors (body mass index, smoking, alcohol consumption, education and physical activity)

Discussion

In the present study, we found no statistically significant association between diabetes and ANM. When looking at age of diagnosis, women with diabetes before the age of 20 years reached menopause earlier, whereas menopausal onset was delayed in women having diabetes after 50 years of age. For diabetes onset between the ages 20-50 years, no association with ANM was found. All associations were independent of age, smoking, reproductive factors and diabetes risk factors.

The strengths of our study include its large sample size and the measurement of a broad set of potential confounders. Except for BMI and number of full-term pregnancies, covariate measurement was fully standardized across centers. Well-known associations with smoking status, education and parity were replicated, which can be interpreted as an internal validation of our data. We also used comprehensive statistical methods including time-dependent variables. This is of special importance

to our particular design in which women who enter menopause at older ages had more time to develop diabetes prior to menopause. The analysis of time-dependent data, such as diabetes, may lead to immortal time bias if the dynamic nature of this variable is not accounted for in the analyses [12, 13]. We avoided this bias by modelling diabetes as a time-dependent variable. This approach acknowledges the change in diabetes status and does not credit the premenopausal time until diabetes (i.e. the immortal time) toward the effect of diabetes.

There are also several limitations that need to be considered when interpreting our findings. First of all, the assessment of both diabetes and menopause status was based on self-report, not verified by medical records. However, previous studies have shown that the validity and reproducibility of self-reported age at diabetes and menopause are acceptable [14-16]. Although the probability of correct recall is lower when more time has passed [14, 15], it seems unlikely that recall of menopausal age is differential with respect to diabetes status.

Second, we cannot be certain about the sequence of diabetes and menopause in women where both events occur in a relatively short time period. The menopause transition is a process that takes at least 1 to 3 years [17] and diabetes occurs at least 4 to 7 years prior to diagnosis [18]. Thus, we cannot rule out the possibility that women with a short interval between menopause and diabetes were misclassified in our study. The most common approach to investigate this is to exclude participants with a short time interval between the exposure and outcome. However, this approach would introduce bias in this particular design due to the selective exclusion of exposed women with a relatively early menopause. For this reason, we cannot draw any conclusions about the impact of this potential misclassification.

Third, our study may have suffered from survivor bias. Our study population is a volunteer sample that consists of individuals willing to fill in questionnaires. The prevalence of diabetes in our study sample is somewhat lower compared to previously reported estimates in the same time period [19, 20]. It is also known that women with early-onset diabetes have a higher risk of future disease and poorer survival rates, and are therefore less likely to enter the study at an older age [21]. This may have led to reduced statistical power and an underestimation of the observed associations.

Fourth, we could perform time-dependent analyses for our main exposure and for some confounders (e.g. smoking and OC-use), but for BMI, physical activity, alcohol and HRT use this was not possible. BMI, physical activity and alcohol were measured at enrolment in EPIC and for HRT the age at stopping was not available in most centers. For women who were postmenopausal at enrolment (49,1%), we thus

relied on postmenopausal measurements as an approximation of premenopausal exposures, which might be incorrect. Previous studies show conflicting results for the association of BMI with menopausal timing [22-24]. In a Japanese study it was found that in women with a late menopause, the average BMI was higher at any time point both pre- and postmenopausally [22]. Similarly, a recent study showed that women who gain weight between the age of 20 and 40 years reach menopause later [24]. However, in a large longitudinal study where BMI was measured at different time points before menopause no association with ANM was found [23]. As for BMI, data on the association with physical activity [25, 26] and alcohol use [24, 25, 27] are inconsistent. Thus, residual bias due to BMI, physical activity, and alcohol use is expected to be minimal as previous studies do not show a consistent relationship of these risk factors with ANM.

The interpretation of our findings is further complicated by the fact that we could not distinguish between the effects of T1D and T2D, as information on diabetes type was not collected in EPIC. The decreasing age at T2D onset is a relatively recent phenomenon [28, 29]. Since women of the EPIC cohort were recruited in the mid 1990s, it seems reasonable to assume that the younger ANM observed in women with diabetes before the age of 20 years is a T1D effect. However, we cannot explain the finding of a null association in women with diabetes diagnosed between the age of 20 and 30, where T1D is also the most likely type of diabetes.

Based on the literature, an accelerating effect of T1D on ANM could be considered more plausible than a delaying effect of T2D. Dorman et al [4] were the first to report an earlier onset of menopause in women with T1D. In a recent cross-sectional study among women with T1D, ANM was not associated with age at diagnosis, but T1D patients with microvascular complications (retinopathy and nephropathy) were more often postmenopausal [5]. However, both studies were limited by a low response rate and like ours were based on cross-sectionally collected data. Despite these inconsistencies and limitations, there are several epidemiological and experimental data supporting a link between T1D and ovarian ageing. Disturbances in ovarian function (e.g. menstrual irregularities, infertility and late menarche) are common among women with T1D [30, 31], and become more prevalent with poor metabolic control [31-33]. Furthermore, animal studies have shown that abnormal insulin signaling influences the functioning of the hypogonadal-pituitary-gonadal (HPG) axis and the reproductive cycle [34, 35]. Finally, T1D and early menopause may share a common autoimmune origin. Premature ovarian failure (POF) has been associated with other autoimmune diseases, such as hypothyroidism and Addison's disease [36], and in a

recent meta-analysis of genome wide association studies a link between autoimmune susceptibility genes and ANM was found [37].

Diabetes diagnosed after the age of 45 is most likely T2D. A delaying effect of T2D on ANM has not been reported before, and is not in agreement with a recent study showing a lower ovarian reserve in premenopausal T2D patients [7]. Since we did not have detailed information on BMI and weight changes before menopause, the observed association with late-onset diabetes could also partly reflect the effect of obesity.

In conclusion, our results suggest that diabetes may influence ANM. Given the methodological limitations, future longitudinal studies with continued follow-up of premenopausal women and measurement of explanatory variables (genetic and biological markers) are needed to replicate these findings and to explore the underlying mechanisms and clinical significance of the observed associations.

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Supplementary Table 1. Hazard ratios of natural menopause according to diabetes status and age at diagnosis - sensitivity analyses.

	HR (95% CI)	
	Model 4 (N = 234,240)	Model 5 (N = 258,898)
Diabetes before menopause		
No (N = 256,146)	REF (1.00)	REF (1.00)
Yes (N = 2,752)	0.93 (0.87-0.99)	0.94 (0.88-1.00)
Age at diabetes diagnosis		
No diabetes before menopause	REF (1.00)	REF (1.00)
≥ 50 (N = 317)	0.83 (0.71-0.98)	0.81 (0.70-0.94)
45-49 (N = 649)	0.87 (0.77-0.99)	0.90 (0.80-1.01)
40-44 (N = 444)	1.00 (0.85-1.18)	0.99 (0.85-1.16)
30-39 (N = 715)	0.92 (0.80-1.07)	0.96 (0.84-1.10)
20-29 (N = 394)	0.99 (0.81-1.21)	1.00 (0.82-1.20)
10-20 (N = 161)	1.43 (0.93-2.19)	1.46 (1.04-2.05)
< 10 (N = 72)	1.81 (1.14-2.87)	1.56 (1.02-2.39)

Model 4: Model 3 after excluding women using hormone replacement therapy prior to menopause and/or women using oral contraceptives around menopause
Model 5: Model 3 plus 10-year birth cohorts

Chapter 4

**Cigarette smoking and endogenous sex hormones
in postmenopausal women**

Abstract

Context: Sex hormones play a key role in women's health, but little is known about lifestyle factors that influence their levels.

Objective: To investigate the association between cigarette smoking habits and endogenous sex hormone levels in postmenopausal women.

Design and participants: Cross-sectional study among 2030 postmenopausal women aged 55-81 from the Norfolk population of the European Prospective Investigation into Cancer (EPIC-Norfolk). All women were at least 1 year postmenopausal and not currently using hormone replacement therapy. General linear models were used to examine the association between smoking habits and sex hormone levels.

Results: Among current smokers, the daily number of cigarettes smoked was associated with increased levels of testosterone (19-37%), free testosterone (19-34%), 17-hydroxprogesterone (17-22%), androstenedione (2-23%), SHBG (6-10%) and estradiol (-2-15%). Stratified analysis for BMI revealed an interaction such that the association with SHBG was restricted to lean women, while a smoking-related increase in free estradiol was only found in overweight women. No clear dose-response relationship was observed for estrone, although its levels were highest in heavy smokers. Current smoking habit was associated with a larger difference in sex hormone levels than lifetime cigarette exposure as measured by pack years. Among former smokers, sex hormones were at levels of never smokers within 1-2 years of smoking cessation.

Conclusions: Cigarette smoking is associated with higher circulating levels of androgens, estrogens, 17-hydroxprogesterone and SHBG in postmenopausal women. The almost immediate lower levels with smoking cessation may indicate that hormone related disease risks could potentially be modified by changing smoking habits.

Introduction

Sex hormones play a key role in women's health. High circulating levels of estrogens are associated with an increased risk of breast and endometrial cancer in postmenopausal women [1, 2], while they are protective against osteoporosis and bone fractures [3, 4]. Besides estrogens, elevated androgen levels have also been implicated as a potential risk factor for breast and endometrial cancer [1, 2] and appear to protect against fractures [5]. Epidemiological data on metabolic outcomes are sparse, but suggest a role for sex hormones. In prospective studies, high circulating levels of testosterone and estradiol have been linked to an increased risk of type 2 diabetes [6-8]. There are also some indications for a cardiovascular effect of sex hormones, with high levels of estradiol and low levels of testosterone being protective [9, 10]. Recent data further suggest a role for estrogens in lung cancer growth, as supported by the presence of estrogen receptors in lung cancer tissue [11, 12].

Despite the importance of sex hormones in chronic disease etiology, little is known about lifestyle determinants of circulating sex hormone levels. Cigarette smoking is a well established risk factor for chronic disease such as cancer, cardiovascular disease and diabetes. Cigarette smoking also affects the functioning of hormone producing organs, including the pituitary, adrenals and ovaries [13] and may thereby influence chronic disease risk. Several studies have investigated the relationship between smoking and endogenous sex hormones in postmenopausal women [14-26]. While most studies found no relationship between smoking and estrogen levels [16, 17, 19-26], reported associations for androgens have been less consistent, with either an increase in testosterone levels [14, 15, 26] or no association [16, 22, 25]. Few studies have reported on dose-response relationships, once again with variable findings [14, 15, 23]. These inconsistencies may reflect differences in sample size, study design, and assay characteristics between studies. It is also unclear to what degree the effects of smoking are reversible and how long it takes for sex hormone levels to return to normal values after smoking cessation. To further examine the association between cigarette smoking habit and endogenous sex hormone levels, we conducted a cross-sectional study in a population-based sample of postmenopausal women.

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Methods

Study participants
Participants were from the Norfolk cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC), a prospective population-based study investigating the relationship between diet, cancer and other chronic diseases. Recruitment details have been described previously [27]. Briefly, study participants were identified from collaborating general practice age-sex registers and invited to participate. Between 1993 and 1997, ~ 25 000 men and women aged 40 to 79 years completed detailed Health and Lifestyle Questionnaires and attended a baseline Health Check. Of these, ~16 000 participants attended a second Health Check between 1997 and 2000. At both health checks, anthropometric measurements and non-fasting blood samples were collected. The present study is based on a subset of 2114 postmenopausal women who were controls in a previous nested case-control study on genetic factors and breast cancer risk. These women were all postmenopausal for at least 1 year and were not using hormone replacement therapy (HRT) 3 months prior to the study.

Sex hormone analyses
Non-fasting blood samples were collected at the second Health Check and stored frozen in separate serum and plasma aliquots until analysis. Sex hormones were analysed sequentially based on the available plasma or serum per participant. More than 94% of the participants had sufficient plasma or serum for measurement of estradiol, testosterone, sex hormone-binding globulin (SHBG) and 17 hydroxyprogesterone (17-OHP), 71% of the participants had adequate plasma or serum for androstenedione analysis and in only 59% there were sufficient samples for estrone measurement.
Estradiol was measured by radioimmunoassay after ether extraction [28], and estrone was measured using radioimmunoassay after ether and liquid column chromatography on a Lipidex 5000 (PerkinElmer, Boston, MA) with elution using chloroform:hexane:methanol (50:50:1). The within and between batch coefficients of variations (CVs) for estradiol were 8.6% and 13% respectively at 18pmol/L. The sensitivity limit was 3.0 pmol/L. Within and between-batch coefficients of variation for estrone were 14% and 22% at 55 pmol/L and the sensitivity limit was 15 pmol/L. 17-OHP concentrations were measured by radioimmunoassay using a modification of the DSL 6800 kit: the antibody was diluted 1:1 giving a detection limit of 0.06 nmol/L and within and between assay CVs of 4.2% and 6.2% respectively at a concentration of 1.5 nmol/L. Androstenedione was measured using a solid phase radioimmunoassay

(DSL 3800, Beckman Coulter UK Ltd). The sensitivity was 0.1 nmol/L and the within and between batch CVs were 5.6 and 11% respectively at a concentration of 3.5 nmol/L. Testosterone was measured using a solid phase radioimmunoassay kit (TKTT2, Siemens Healthcare diagnostics UK). The within and between batch CVs were 6.1% and 10%, respectively at a concentration of 3.1 nmol/L. The sensitivity limit was 0.14 nmol/L. SHBG was measured using a liquid phase immunoradiometric kit from Orion Diagnostica, Finland. The sensitivity limit was 0.5 nmol/L and the within and between batch CVs were 2.1% and 7.4%, respectively, at a concentration of 11 nmol/L.

Free testosterone and estradiol levels were calculated using the measured values for testosterone or estradiol, SHBG and assuming a fixed albumin concentration of 43 g/L [29].

Lifestyle and reproductive information

Information on cigarette smoking habit was derived from the response to two questions, "Have you ever smoked as much as one cigarette a day for as long as a year?", and "Do you smoke cigarettes now?" Participants were categorised as either 'current', 'former' or 'never' smokers, based on the yes/no responses to these questions. The numbers of cigarettes smoked each day at ages 20, 30, 40 and 50 years and at the time of study were recorded and the cumulative cigarette consumption in pack years was derived from these data. One pack year was defined as 20 cigarettes/day for 1 year. For former smokers, information was also collected on the age at which they gave up smoking, from which the time since quitting was calculated.

Information on alcohol consumption was derived from the question: "How many alcohol drinks do you have each week?" with four separate categories of drinks. Total alcohol consumption was estimated as the total units consumed per week. Alcohol intake was categorized as non-drinker, 1-7, 8-14, >14 units/week. Physical activity level was assessed with a detailed questionnaire previously validated against objective methods and coded using the following four-level index: inactive, moderately inactive, moderately active and active [30].

Participants' use of HRT was identified based on their responses to the questions: "Have you ever taken hormone replacement therapy?", "Are you currently taking this treatment?" and "If you are no longer taking hormone replacement therapy, at what age did you stop?". Similar questions were asked for oral contraceptives (OC). Participants were also asked to report their age at menarche, age at menopause, number of children (parity) and whether they had undergone a hysterectomy and/or ovariectomy in the past.

Anthropometric measures

At the second Health Check, anthropometric measurements were taken by trained nurses based on standardized protocols. Standing height and weight were taken with participants in light clothing without shoes on and to the nearest 0.1 cm and 0.2 kg using a free-standing stadiometer and digital scales, respectively. BMI was calculated from the participant's weight (kg) divided by the square of their height (m^2). Waist circumference was measured at the smallest circumference between the ribs and iliac crest with participants standing with abdomen relaxed, or at the level of the umbilicus if there was no natural waistline.

Statistical analysis

Statistical analyses were performed using SPSS statistical package for Windows version 17.0 (SPSS Inc., Chicago, IL, USA). We excluded 21 women with hormone levels outside the reference range and 63 women with inconsistencies in smoking report, leaving 2030 participants for the analyses. 314 women (15.5%) had missing values on one or more covariates. Because missing values were likely to be missing at random, and to avoid loss in efficiency, missing values for age at menarche, age at menopause, parity, ever HRT use, ever OC use and hysterectomy/ovariectomy status were imputed using a multiple imputation technique [31]. As the distributions of sex hormone and SHBG concentrations were skewed, log-transformed values were used to approximate the normal distribution.

First, participants were classified as never smokers, former smokers or current smokers with the latter group subdivided by the number of cigarettes smoked daily. Differences in participant characteristics were tested using univariate analysis of variance (ANOVA) for continuous variables and Chi Square tests for categorical variables. Crude, age-adjusted, age- and BMI-adjusted, and multivariable-adjusted geometric means of hormone and SHBG levels were calculated for each smoking category and differences across categories were tested using analysis of covariance. Multivariable adjusted geometric means were adjusted for age, BMI, alcohol consumption, physical activity, past use of HRT, past use of OC, age at menarche, age at menopause, parity and hysterectomy/ovariectomy status. Tests for linear trend were conducted by entering the smoking categories as continuous terms. To assess potential effect modification by BMI, we further stratified the analyses by BMI (< 25 kg/m^2 vs ≥ 25 kg/m^2).

To investigate the impact of lifetime cigarette exposure, we divided ever smokers (current and former smokers) into tertiles of total pack years of smoking. Multivariable adjusted geometric means of sex hormone and SHBG levels were then calculated for each tertile. Finally, we investigated the effect of smoking cessation by subdividing former smokers into 4 categories of time since quitting. Linear regression analyses including never smokers as indicator variable were conducted to assess the association between time since quitting and sex hormone and SHBG levels. Since estradiol and testosterone levels are correlated with SHBG levels, we mutually adjusted for estradiol, testosterone and SHBG in the analyses to identify independent associations.

Ethics
Ethics approval of the EPIC-Norfolk Study was obtained from the Norwich Local Research Ethics Committee and all participants gave written informed consent.

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Results

Descriptive characteristics of the study population are summarized in Table 1. Multiple imputation returned results similar to those of complete case analysis. The mean age of the study population was 67 ± 6.7 years and 5.9% were current smokers. Current smokers were, on average, younger than former and never smokers and had a lower BMI and waist circumference. Current smokers also had an earlier menopause, consumed more alcohol per week and were more likely to have used oral contraceptives in the past.

Table 1. Characteristics of the study population by smoking status (N = 2030).

| | | | Smoking status | | | |
| | | | | Current smokers | | |
	Never smokers (N = 1208)	Former smokers (N = 702)	< 10 cigarettes/day (N = 32)	10-15 cigarettes/day (N = 59)	>15 cigarettes/day (N = 29)	P (F-test)
Mean (SD)						
Age (years)	66.9 (6.4)	67.3 (7.1)	65.8 (6.8)	64.3 (6.3)	64.5 (5.9)	0.002
Body mass index (kg/m^2)	26.6 (4.1)	27.1 (4.6)	25.0 (3.7)	25.0 (3.9)	26.5 (5.0)	< 0.001
Waist circumference (cm)	82.9 (10.5)	83.9 (10.6)	80.0 (9.9)	79.8 (9.5)	84.7 (12.9)	0.01
Age at menarche (years)	13.1 (2.1)	13.1 (2.0)	12.8 (2.0)	13.2 (2.0)	13.1 (2.0)	0.76
Age at menopause (years)	50.2 (5.8)	49.5 (5.8)	49.8 (5.8)	48.1 (5.8)	48.1 (5.8)	0.01
% (N)						
Alcohol intake						< 0.001
0 units/week	20.2 (244)	12.3 (86)	15.6 (5)	23.7 (14)	17.2 (5)	
1-7 units/week	65.5 (791)	57.4 (403)	59.4 (19)	59.3 (35)	44.8 (13)	
8-14 units/week	11.0 (133)	18.9 (133)	18.8 (6)	6.8 (4)	20.7 (6)	
> 14 units/week	3.3 (40)	11.4 (80)	6.3 (2)	10.2 (6)	17.2 (5)	
Physical activity						0.75
Inactive	41.3 (499)	41.0 (288)	43.8 (14)	42.4 (25)	51.7 (15)	
Moderately inactive	34.6 (418)	33.2 (233)	25.0 (8)	30.5 (18)	20.7 (6)	
Moderately active	17.1 (206)	18.7 (131)	18.8 (6)	13.6 (8)	17.2 (5)	
Active	7.0 (85)	7.1 (50)	12.5 (4)	13.6 (8)	10.3 (3)	
Parity						0.52
None	28.6 (345)	28.1 (197)	18.8 (6)	25.4 (15)	20.7 (6)	
1-2	40.3 (487)	40.3 (283)	43.8 (14)	32.2 (19)	34.5 (10)	
> 2	31.1 (376)	31.6 (222)	37.5 (12)	42.4 (25)	44.8 (13)	
Past use of hormone replacement therapy	16.4 (198)	19.4 (136)	21.9 (7)	16.9 (10)	20.7 (6)	0.50
Past use of oral contraceptives	26.8 (324)	33.2 (233)	28.1 (9)	39.0 (23)	55.2 (16)	< 0.001
Ovariectomy/hysterectomy status						0.66
Intact	76.3 (922)	75.6 (531)	87.5 (28)	81.4 (48)	72.4 (21)	
Hysterectomy (without ovariectomy)	10.3 (125)	11.4 (80)	9.4 (3)	10.2 (6)	6.9 (2)	
Ovariectomy	13.3 (161)	13.0 (91)	3.1 (1)	8.5 (5)	20.7 (6)	

Table 2 shows the age adjusted, age -and BMI adjusted and multivariable adjusted geometric means of sex hormone and SHBG levels by smoking status. Former smokers had hormone levels comparable to never smokers and the greatest differences were primarily observed between current smokers and non-smokers. Testosterone levels were higher in current smokers than non-smokers and increased with increasing cigarette exposure. This difference by smoking habit remained significant after adjusting for age, BMI, physical activity, alcohol use and reproductive history. 17-OHP, androstenedione and free testosterone levels showed a similar increase with smoking exposure with the highest levels being observed among those smoking the most cigarettes. While crude estradiol levels did not differ by smoking status, an increasing trend with smoking exposure was observed after adjusting for BMI. A similar positive trend was found for SHBG and free estradiol, although the latter was not significant. There was no clear pattern for estrone, but its levels were highest among women smoking > 10 cigarettes/day. The observed associations between smoking and sex hormones were not materially changed after mutual adjustment for other hormones and when we adjusted for waist circumference instead of BMI (data not shown).

We further explored whether BMI modified the effect of cigarette smoking on sex hormone levels. Stratified analyses showed that the association with SHBG was restricted to lean women, while the increasing trend in free estradiol only reached significance in overweight women. The association with free testosterone levels was also stronger in overweight women. (Supplementary Table 1).

Of the ever smokers, 623 had data available for the calculation of pack years. Supplementary Table 2 shows the geometric means of sex hormone and SHBG levels by tertiles of total pack years. Although free testosterone levels tended to increase with increasing pack years, there was no clear relationship between lifetime cigarette exposure and sex hormone levels.

Among former smokers, data on time since quitting was available on 563 participants. Levels of 17-OHP and androstenedione showed a sharp decline after smoking cessation. In women quitting smoking within the previous year, 17-OHP and androstenedione levels were the same as in never smokers. Levels of estradiol, testosterone, free testosterone and free estradiol returned to levels of never smokers after 1 year of smoking cessation. No clear pattern was observed for SHBG, although SHBG levels were slightly higher among women quitting smoking for more than 10 years (Table 3).

Table 2. Geometric means (plus 95% CIs) of hormones and sex hormone-binding globulin by smoking status, crude and adjusted for covariates.

| | N | Never smokers | Former smokers | Smoking status | | | | |
| | | | | Current smokers | | | | |
				< 10 cigarettes/day	10-15 cigarettes/day	>15 cigarettes/day	P (F-test)	P (trend) [1]
Estradiol (pmol/L)								
Crude	2006	15.9 (15.4-16.4)	16.0 (15.3-16.6)	14.5 (12.0-17.7)	16.1 (13.9-18.6)	18.6 (15.1-22.8)	0.56	0.37
Model 1	2006	15.9 (15.4-16.4)	15.9 (15.3-16.6)	14.6 (12.0-17.7)	16.2 (14.0-18.7)	18.6 (15.2-22.9)	0.55	0.34
Model 2	2006	16.0 (15.5-16.5)	15.6 (15.0-16.2)	16.0 (13.4-19.1)	17.7 (15.5-20.2)	18.8 (15.6-22.7)	0.17	0.02
Model 3	2006	16.0 (15.6-16.5)	15.6 (15.0-16.2)	15.7 (13.2-18.8)	17.8 (15.6-20.3)	18.8 (15.6-22.7)	0.13	0.02
Model 4	1984	16.0 (15.6-16.5)	15.6 (15.0-16.2)	15.7 (13.1-18.8)	17.8 (15.6-20.4)	18.8 (15.5-22.7)	0.13	0.02
Estrone (pmol/L)								
Crude	1135	75.3 (72.5-78.0)	75.2 (71.8-78.7)	50.5 (40.0-63.8)	82.0 (69.8-96.3)	81.9 (64.3-104.3)	0.01	0.65
Model 1	1135	75.3 (72.6-78.1)	75.0 (71.7-78.6)	51.0 (40.4-64.4)	82.4 (70.3-96.8)	82.6 (64.9-105.1)	0.01	0.57
Model 2	1135	75.6 (73.0-78.3)	73.8 (70.5-77.1)	55.6 (44.4-69.6)	88.6 (75.9-103.3)	85.0 (67.5-107.2)	0.01	0.11
Model 3	1135	76.0 (73.4-78.7)	73.0 (69.8-76.4)	55.2 (44.0-69.1)	90.6 (77.6-105.7)	85.8 (68.0-108.3)	0.003	0.07
17-OHP (pmol/L)								
Crude	1936	1.05 (1.02-1.07)	1.04 (1.01-1.08)	1.23 (1.04-1.44)	1.29 (1.15-1.46)	1.31 (1.10-1.56)	< 0.001	< 0.001
Model 1	1936	1.05 (1.02-1.07)	1.04 (1.00-1.08)	1.23 (1.04-1.45)	1.30 (1.15-1.47)	1.32 (1.11-1.57)	< 0.001	< 0.001
Model 2	1936	1.05 (1.02-1.07)	1.04 (1.00-1.07)	1.24 (1.06-1.46)	1.31 (1.16-1.48)	1.32 (1.11-1.57)	< 0.001	< 0.001
Model 3	1936	1.04 (1.02-1.07)	1.04 (1.00-1.08)	1.25 (1.06-1.46)	1.32 (1.17-1.49)	1.34 (1.13-1.60)	< 0.001	< 0.001
Androstenedione (nmol/L)								
Crude	1443	3.15 (3.05-3.26)	3.03 (2.90-3.16)	3.15 (2.57-3.86)	3.38 (2.88-3.97)	3.96 (3.16-4.94)	0.11	0.03
Model 1	1443	3.15 (3.04-3.26)	3.03 (2.90-3.17)	3.14 (2.57-3.85)	3.35 (2.85-3.94)	3.92 (3.14-4.89)	0.15	0.04
Model 2	1443	3.15 (3.05-3.26)	3.02 (2.89-3.16)	3.20 (2.61-3.92)	3.43 (2.92-4.03)	3.96 (3.17-4.94)	0.09	0.02
Model 3	1443	3.14 (3.03-3.25)	3.03 (2.90-3.17)	3.20 (2.62-3.92)	3.50 (2.98-4.12)	4.10 (3.28-5.12)	0.05	0.01

Testosterone (nmol/L)

	N						p	p
Crude	1895	0.67 (0.65–0.69)	0.71 (0.68–0.74)	0.82 (0.67–1.01)	0.86 (0.74–1.00)	1.02 (0.82–1.26)	< 0.001	< 0.001
Model 1	1895	0.67 (0.65–0.69)	0.71 (0.68–0.74)	0.83 (0.68–1.02)	0.87 (0.75–1.01)	1.03 (0.84–1.28)	< 0.001	< 0.001
Model 2	1895	0.67 (0.65–0.69)	0.70 (0.67–0.73)	0.86 (0.70–1.05)	0.90 (0.78–1.05)	1.04 (0.84–1.28)	< 0.001	< 0.001
Model 3	1895	0.67 (0.65–0.69)	0.70 (0.67–0.74)	0.83 (0.68–1.01)	0.90 (0.77–1.04)	1.07 (0.87–1.32)	< 0.001	< 0.001
Model 4	1875	0.67 (0.65–0.69)	0.71 (0.67–0.74)	0.85 (0.70–1.04)	0.89 (0.77–1.03)	1.06 (0.86–1.31)	< 0.001	< 0.001

SHBG (nmol/L)

	N						p	p
Crude	2003	41.2 (40.1–42.3)	41.5 (40.0–42.9)	46.4 (39.4–54.7)	47.5 (42.2–53.5)	43.8 (37.0–51.8)	0.12	0.02
Model 1	2003	41.2 (40.2–42.3)	41.3 (39.9–42.7)	46.9 (39.9–55.2)	48.9 (43.5–54.9)	45.0 (38.1–53.1)	0.03	0.004
Model 2	2003	41.1 (40.1–42.1)	41.9 (40.6–43.3)	44.0 (37.8–51.0)	45.7 (40.9–50.9)	44.7 (38.2–52.1)	0.26	0.05
Model 3	2003	40.9 (40.0–41.9)	42.1 (40.8–43.5)	43.5 (37.5–50.5)	45.4 (40.7–50.7)	45.5 (39.0–53.1)	0.18	0.04
Model 5	1871	40.7 (39.7–41.8)	41.9 (40.5–43.3)	42.8 (36.8–49.8)	45.7 (40.9–51.0)	43.6 (37.3–51.0)	0.24	0.07

Free testosterone (pmol/L)

	N						p	p
Crude	1875	9.8 (9.5–10.2)	10.4 (9.9–10.9)	11.7 (9.3–14.8)	11.3 (9.5–13.3)	14.7 (11.6–18.7)	0.003	0.001
Model 1	1875	9.8 (9.5–10.2)	10.4 (9.9–10.9)	11.7 (9.3–14.8)	11.3 (9.5–13.3)	14.7 (11.6–18.7)	0.003	0.001
Model 2	1875	9.9 (9.5–10.3)	10.2 (9.7–10.7)	12.6 (10.1–15.6)	12.2 (10.4–14.3)	14.8 (11.8–18.5)	< 0.001	< 0.001
Model 3	1875	9.9 (9.5–10.2)	10.2 (9.7–10.7)	12.2 (9.9–15.2)	12.2 (10.4–14.2)	15.0 (12.0–18.8)	< 0.001	< 0.001

Free estradiol (pmol/L)

	N						p	p
Crude	1984	0.39 (0.38–0.40)	0.39 (0.37–0.41)	0.35 (0.28–0.43)	0.37 (0.32–0.44)	0.45 (0.36–0.56)	0.55	0.78
Model 1	1984	0.39 (0.38–0.40)	0.39 (0.37–0.41)	0.35 (0.28–0.43)	0.37 (0.32–0.43)	0.45 (0.36–0.55)	0.55	0.85
Model 2	1984	0.39 (0.38–0.40)	0.38 (0.37–0.40)	0.38 (0.32–0.46)	0.42 (0.36–0.48)	0.45 (0.37–0.55)	0.31	0.09
Model 3	1984	0.39 (0.38–0.41)	0.38 (0.36–0.39)	0.38 (0.32–0.46)	0.42 (0.37–0.48)	0.45 (0.37–0.54)	0.21	0.09

Model 1: age adjusted
Model 2: Model 1 plus body mass index
Model 3: Model 2 plus alcohol consumption, physical activity, past use of hormone replacement therapy, past use of oral contraceptives, age at menarche, age at menopause parity and hysterectomy/ovariectomy status.
Model 4: Model 3 plus SHBG
Model 5: Model 3 plus testosterone and estradiol

[1] Linear trend analyses entering smoking categories as continuous terms. For never and former smokers, cigarettes daily smoked was 0.

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Table 3. Time since quitting and hormone and SHBG levels.

	Beta (95% CI)							
	Estradiol	Estrone	17-OHP	Androstenedione	Testosterone	SHBG	Free testosterone	Free estradiol
Smoking status								
Never smokers	0 (REF)	0 (REF)	0 (REF)	0 (REF)	0 (REF)	0 (REF)	0 (REF)	0 (REF)
Current smokers	0.08 (-0.01; 0.18)	0.04 (-0.08; 0.16)	0.22 (0.13-0.31)	0.12 (0.00-0.23)	0.31 (0.20-0.42)	0.09 (0.01-0.17)	0.26 (0.14-0.38)	0.05 (-0.05; 0.15)
Former smokers								
≤ 1 year since quitting	0.27 (0.00-0.55)	0.14 (-0.20; 0.48)	0.03 (-0.21; 0.27)	0.02 (-0.30; 0.33)	0.35 (0.05-0.65)	-0.12 (-0.34; 0.11)	0.39 (0.07-0.71)	0.29 (0.01-0.57)
2-5 years since quitting	-0.05 (-0.22; 0.13)	-0.04 (-0.22; 0.14)	-0.01 (-0.16; 0.14)	0.06 (-0.13; 0.25)	0.07 (-0.13; 0.26)	-0.08 (-0.23; 0.07)	0.11 (-0.10; 0.32)	-0.03 (-0.20; 0.15)
5-10 years since quitting	0.01 (-0.13; 0.15)	-0.01 (-0.16; 0.15)	-0.03 (-0.16; 0.09)	-0.01 (-0.17; 0.15)	0.05 (-0.11; 0.21)	-0.08 (-0.19; 0.04)	0.08 (-0.09; 0.25)	0.03 (-0.12; 0.18)
> 10 years since quitting	-0.03 (-0.09; 0.03)	-0.03 (-0.10; 0.04)	-0.03 (-0.08; 0.02)	-0.05 (-0.12; 0.01)	0.03 (-0.04; 0.09)	0.07 (0.02; 0.12)	-0.01 (-0.08; 0.06)	-0.06 (-0.12; -0.003)

Beta coefficients represent change in log hormone and SHBG levels. Linear regression analyses are adjusted for age, BMI, alcohol consumption, physical activity, past use of hormone replacement therapy, past use of oral contraceptives, age at menarche, age at menopause, parity and hysterectomy/ovariectomy status.

Discussion

In this population-based sample of 2030 postmenopausal women, we found that current smokers had higher circulating levels of testosterone (by 19-37%), free testosterone (by 19-34%), 17-hydroxprogesterone (by 17-22%), androstenedione (by 2-23%), SHBG (by 6-10%) and estradiol (by -2-15%) compared with never smokers. A dose-response relationship was apparent for these hormones with the highest levels being observed in women who smoked the most cigarettes. All associations were independent of age, BMI, physical activity, alcohol use and reproductive history. We also observed an increasing trend in free estradiol levels with increasing cigarette exposure. No clear dose-response relationship was found for estrone, but its levels were highest in heavy smokers. Although stratified analyses were limited by the small number of current smokers, there were indications for an interaction by BMI. The association with SHBG was restricted to lean women, while the smoking-related increase in free estradiol only reached significance in overweight women. The association with free testosterone was also stronger in overweight women. Interestingly, former smokers had hormone levels that were comparable to those of never smokers. The smoking cessation and pack year data further seem to indicate that the effects of cigarette smoking on sex hormone levels are not permanent and that hormone-related disease risks could potentially be modified by changing smoking habits.

Previous studies on cigarette smoking and testosterone levels have produced conflicting results. Our findings are consistent with studies reporting higher testosterone levels in postmenopausal [14, 15, 26] and premenopausal smokers [32]. In contrast, several studies [16, 22, 25] found no significant association between smoking and testosterone levels. Since these latter studies were relatively small, differences in statistical power may explain some of the discrepancy. Previous data on dose-response relationships are mixed with either a positive association [15] or no association [14, 23]. We further observed an increase in free testosterone levels with increasing cigarette exposure. This association has not been described previously in women, but does agree with observations in men [33]. In line with previous studies [14-16, 18, 20, 22, 23, 25], we found an increase in androstenedione levels with increasing cigarette exposure. Consistent with the elevated androstenedione and testosterone levels in smokers, we observed an increase in 17-OHP, which is a precursor of androstenedione and downstream testosterone in the hormone biosynthesis pathway. Few studies have examined the association between smoking and this androgen precursor. Friedman

et al. [26] reported higher 17-OHP levels in postmenopausal smokers compared with non-smokers and Baron et al. [18] found an acute rise in 17-OHP levels after short-term smoking.

There are several mechanisms through which smoking could affect androgen levels. Nicotine can cross the blood-brain barrier and act centrally by stimulating the release of adrenocorticotropic hormone (ACTH) from the pituitary [34], which in turn leads to an increase in the adrenal production of 17-OHP and downstream androgens (androstenedione and testosterone). There is also some evidence that smoking influences luteinizing hormone (LH) levels. In rats, a transient increase in circulating LH levels has been reported after nicotine exposure [35]. In postmenopausal women, a similar trend toward higher LH levels has been observed in smokers compared with non-smokers [20]. Smoking may also increase androgen levels by decreasing their metabolic clearance. *In vitro* studies [36, 37] have shown that nicotine inhibits aromatase, an enzyme responsible for the peripheral conversion of androgens to estrogens.

Epidemiological studies suggest that smoking exerts an anti-estrogen effect. Cigarette smoking has been associated with estrogen deficient conditions in postmenopausal women such as a lower bone mineral density and an increased fracture risk [38]. Likewise, endometrial cancer, a condition related to estrogen excess, is less common among smokers [39]. Inconsistent with the anti-estrogen theory, we observed a trend toward higher estrogen levels with increasing cigarette consumption. This trend, however, is in line with the higher androgen levels found in smokers. A similar increase in estradiol has been reported in large-scale studies among men [33] and premenopausal women [17]. Previous studies in postmenopausal women [16, 17, 19-26] found no difference in estrogen levels. These studies, however, were limited by a small sample size and/or lack of control for obesity which may have offset the effect of cigarette smoking on estrogen levels. In postmenopausal women, an important source of estrogens is the peripheral conversion in fat cells from androgen precursors. The relationship with estradiol and free estradiol became only significant after adjusting for BMI and when analyses were restricted to overweight women. These findings suggest that a certain amount of fat issue is required to translate the smoking-related increase in testosterone into higher estradiol levels. The observed interactions between smoking and BMI may also reflect the higher imprecision of hormone assays towards the lower end of the hormone distribution. Women with a high BMI have higher circulating levels of estradiol and testosterone, but lower levels of SHBG [40]. The resulting changes in intra-assay variability with increasing BMI may explain the significant increase in free estradiol and the stronger association with free testosterone found in overweight

women, and the lack of an association with SHBG in these women. Estrone levels were highest in heavy smokers, but women smoking < 10 cigarettes/day had lower estrone levels than never and former smokers. Reasons for this observation are not clear and we cannot exclude the possibility that this is a chance finding. Further research is needed to determine the exact relationship between cigarette smoking and estrone levels.

Our study has strengths including its large population based sample and the use of radioimmunoassay techniques sensitive and specific in detecting low concentrations of estrogens and androgens. Nevertheless, the validity and reproducibility of hormone measurements in postmenopausal women have been questioned. Measurement errors resulting from this imprecision are expected to be random and therefore only likely to have attenuated relationships. Furthermore, as sex hormones were measured only once they may not be representative of a long-term hormone profile. Future studies may consider serial hormone measurements in time to further clarify the effects of smoking on endogenous sex hormone concentrations. Our measurement of smoking exposure was based on self-report, not validated against biochemical measures. However, misclassification resulting from this is most likely to be non-differential with respect to hormone status, and could therefore only have attenuated associations. Another limitation is the cross-sectional design of the study which does not allow us to make causal inferences between smoking exposure and hormone outcome. Although hormones may influence an individual's desire to smoke, differences in postmenopausal hormones are unlikely to affect smoking habits acquired premenopausally. The presence of dose-response relationships and the almost immediate reduction in hormone levels following smoking cessation also increase the likelihood of causality. We adjusted our analyses for several factors that potentially confound the smoking-hormone association, but we cannot exclude residual confounding from other, unknown factors though these are unlikely to explain the substantial difference in testosterone and 17-OHP levels found. Finally, despite of being population-based, our study population is a volunteer sample that consists of individuals willing to fill in questionnaires and attend health checks. Comparison of the EPIC-Norfolk cohort with a national sample from the Health Survey of England shows that our cohort is a representative sample in terms of anthropometric variables, blood pressure and serum lipids, but has fewer current smokers. Although this may indicate some degree of selection, bias is unlikely since the participants were unaware of their hormone status prior to the study. The small number of current smokers may have limited the dose-response analyses and interaction analyses for BMI. Further

studies are needed to investigate dose-response effects between smoking and sex hormones, taking possible interactions by BMI into account.

In conclusion, this study indicates that postmenopausal women who smoke have higher testosterone, androstenedione, estradiol, estrone, 17-OHP and SHBG levels. These findings may have implications. Given the importance of sex hormones in chronic disease risk, a change in smoking habits may modify sex hormone-related disease risks in a favourable direction. Nowadays there is increasing interest in the role of endogenous testosterone in chronic disease risk in postmenopausal women. Though direct comparisons are difficult, the magnitude of the increase in total testosterone concentrations in our study - approximately 0.25 nmol/L higher testosterone in never smokers compared to current smokers - is comparable to the reported mean difference of 0.21 nmol/L in testosterone levels between those with diabetes and controls in a meta-analysis of cross-sectional studies [8], and the observed nearly two fold increased risk of breast cancer for postmenopausal women in the highest compared to lowest quintiles of testosterone [1]. Endogenous sex hormones may thus provide one plausible mechanism through which cigarette smoking influences chronic disease risk. Future studies are needed to clarify the biological mechanisms through which cigarette smoking influences hormone levels in women.

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Supplementary Table 1. Multivariable adjusted geometric means (plus 95% CIs) of hormones and sex hormone-binding globulin by smoking status, stratified by BMI.

	N	Never smokers	Former smokers	Current smokers			P (t-test)	P (trend) [1]	P (interaction)
				< 10 cigarettes/day	10-15 cigarettes/day	>15 cigarettes/day			
Estradiol (pmol/L)									
< 25 kg/m²	779	12.9 (12.3-13.5)	12.5 (11.7-13.4)	12.4 (9.8-15.9)	14.3 (11.9-17.2)	14.0 (10.6-18.4)	0.67	0.24	0.08
≥ 25 kg/m²	1227	18.4 (17.7-19.1)	17.9 (17.1-18.8)	18.6 (14.2-24.3)	20.4 (16.8-24.7)	23.9 (18.5-31.0)	0.19	0.03	
Estrone (pmol/L)									
< 25 kg/m²	439	65.8 (62.2-69.8)	64.5 (59.8-69.6)	48.5 (36.8-63.8)	85.4 (69.1-105.5)	66.2 (47.8-91.7)	0.03	0.25	0.63
≥ 25 kg/m²	696	83.1 (79.5-86.9)	79.1 (74.7-83.7)	58.7 (39.3-87.8)	89.5 (71.0-112.8)	110.6 (78.7-155.7)	0.09	0.14	
17-OHP (pmol/L)									
< 25 kg/m²	755	1.04 (0.99-1.08)	0.97 (0.91-1.03)	1.23 (0.99-1.53)	1.30 (1.11-1.54)	1.22 (0.95-1.56)	0.002	0.001	0.24
≥ 25 kg/m²	1181	1.05 (1.01-1.09)	1.09 (1.04-1.14)	1.23 (0.96-1.57)	1.33 (1.12-1.59)	1.48 (1.16-1.88)	0.004	< 0.001	
Androstenedione (nmol/L)									
< 25 kg/m²	558	3.02 (2.85-3.19)	2.75 (2.55-2.96)	2.79 (2.11-3.68)	4.02 (3.25-4.96)	3.77 (2.78-5.12)	0.01	0.003	0.07
≥ 25 kg/m²	885	3.22 (3.08-3.36)	3.22 (3.05-3.40)	3.50 (2.60-4.71)	2.78 (2.15-3.58)	4.64 (3.34-6.44)	0.17	0.31	
Testosterone (nmol/L)									
< 25 kg/m²	722	0.61 (0.57-0.64)	0.63 (0.58-0.68)	0.75 (0.57-0.98)	0.88 (0.72-1.08)	0.89 (0.66-1.21)	0.001	< 0.001	0.13
≥ 25 kg/m²	1173	0.71 (0.68-0.74)	0.75 (0.71-0.80)	0.93 (0.69-1.25)	0.86 (0.69-1.06)	1.24 (0.93-1.65)	0.001	< 0.001	
SHBG (nmol/L)									
< 25 kg/m²	772	48.4 (46.7-50.2)	50.0 (47.6-52.5)	49.7 (41.2-59.9)	56.5 (49.1-64.9)	55.7 (45.2-68.4)	0.19	0.03	0.03
≥ 25 kg/m²	1231	36.9 (35.7-38.1)	37.9 (36.3-39.5)	39.8 (31.6-50.1)	37.7 (32.0-44.5)	40.7 (32.5-50.9)	0.76	0.45	
Free testosterone (pmol/L)									
< 25 kg/m²	709	8.0 (7.6-8.5)	8.2 (7.6-8.9)	10.3 (7.7-13.8)	10.4 (8.4-12.9)	11.3 (8.1-15.6)	0.03	0.002	0.02
≥ 25 kg/m²	1166	11.2 (10.7-11.7)	11.6 (10.9-12.3)	14.1 (10.3-19.4)	13.1 (10.4-16.5)	18.4 (13.5-25.1)	0.01	0.001	
Free estradiol (pmol/L)									
< 25 kg/m²	765	0.30 (0.28-0.31)	0.29 (0.27-0.31)	0.29 (0.22-0.37)	0.31 (0.26-0.38)	0.31 (0.23-0.41)	0.86	0.55	0.02
≥ 25 kg/m²	1219	0.47 (0.45-0.49)	0.45 (0.43-0.47)	0.47 (0.35-0.62)	0.51 (0.42-0.63)	0.59 (0.45-0.78)	0.21	0.04	

Multivariable adjusted geometric means were adjusted for age, BMI, alcohol consumption, physical activity, past use of hormone replacement therapy, past use of oral contraceptives, age at menarche, age at menopause parity and hysterectomy/ovariectomy status.

Supplementary Table 2. Multivariable adjusted geometric means (plus 95% CIs) of hormones and sex hormone-binding globulin by pack years in ever smokers

	N	Geometric mean (95% CI)			P (F-test)	P (trend) [1]
		pack years tertile 1	pack years tertile 2	pack years tertile 3		
Estradiol (pmol/L)	618	16.6 (15.4-17.8)	15.6 (14.5-16.7)	15.9 (14.8-17.1)	0.46	0.42
Estrone (pmol/L)	361	79.6 (73.1-86.7)	72.7 (66.9-79.0)	75.1 (69.2-81.6)	0.32	0.35
17-hydroxyprogesterone (pmol/L)	599	1.04 (0.98-1.11)	1.04 (0.97-1.11)	1.11 (1.04-1.19)	0.23	0.17
Androstenedione (nmol/L)	457	3.27 (3.02-3.55)	3.05 (2.81-3.31)	3.02 (2.77-3.28)	0.33	0.17
Total testosterone (nmol/L)	577	0.72 (0.66-0.78)	0.71 (0.65-0.76)	0.77 (0.71-0.83)	0.32	0.26
SHBG (nmol/L)	615	44.5 (41.9-47.3)	40.4 (38.0-42.9)	41.8 (39.3-44.4)	0.07	0.15
Free testosterone (pmol/L)	571	10.0 (9.1-10.9)	10.5 (9.6-11.5)	11.2 (10.3-12.2)	0.20	0.07
Free estradiol (pmol/L)	612	0.39 (0.37-0.42)	0.38 (0.36-0.41)	0.39 (0.36-0.42)	0.88	0.67

Multivariable adjusted geometric means were adjusted for age, BMI, alcohol consumption, physical activity, past use of hormone replacement therapy, past use of oral contraceptives, age at menarche, age at menopause parity and hysterectomy/ovariectomy status.

Chapter 5

**Testosterone, SHBG and cardiovascular health
in postmenopausal women**

Abstract

Cardiovascular disease (CVD) affects men and women differently with women having a lower incidence and later onset of disease. Research has recently refocused interest on the cardiovascular role of androgens. The purpose of this review is to summarize the evidence available on the association between testosterone, sex hormone-binding globulin (SHBG) and cardiovascular health in postmenopausal women. Studies included in this review suggest that increased androgenicity, characterized by high testosterone and low SHBG levels, is associated with an adverse CVD risk factor profile in postmenopausal women. However, evidence for an association with cardiovascular events is lacking and it is uncertain whether the observed associations with endogenous testosterone have clinical implications regarding the use of postmenopausal testosterone therapy. Large-scale, longitudinal studies relating testosterone and SHBG levels to cardiovascular risk factors and endpoints are needed to determine the temporal relationship between androgenicity and cardiovascular risk and to ascertain the long-term efficacy and safety of testosterone therapy in postmenopausal women.

Introduction

Cardiovascular disease (CVD) is the leading cause of death among women worldwide. Although the incidence of CVD is much lower in premenopausal women compared with men of similar age, the CVD rate in women rises steadily after the age of 50 years [1,2]. Initially, this increase in CVD incidence later in life was ascribed to the menopausal decline of estrogen levels [3]. However, throughout the years, several observations have brought this theory into question. Studies examining associations between endogenous estrogen levels and CVD risk factors have yielded conflicting results [4–6] and available data on CVD events indicate a lack of association [7,8]. Furthermore, in contrast to other estrogen related diseases, the CVD rate does not show a sharp rise at time of menopause [1,2,9]. In addition, several trials have failed to show a beneficial effect of estrogen replacement therapy in postmenopausal women [10–12], further weakening the estrogen protection hypothesis.

Owing to the controversial role of estrogens in women's cardiovascular health, recent research has gradually turned more focus to the potential effects of androgens. Indirect evidence for a role of androgens comes from findings of clinical studies showing an unfavourable cardiovascular risk profile in hyperandrogenic conditions such as hirsutism [13] and the polycystic ovary syndrome [14]. Several studies in postmenopausal women have also shown a positive correlation between testosterone and various CVD risk factors [5,15–17]. These findings have raised questions regarding the safety of testosterone administration to postmenopausal women [18]. However, inverse associations between testosterone and markers of atherosclerosis have been reported as well [19,20]. This review aims to summarize the current evidence on the role of endogenous testosterone in cardiovascular health in postmenopausal women and to examine potential adverse effects of testosterone therapy.

Search strategy and selection criteria

For this review we searched for English language articles in PubMed and EMBASE using the key words 'androgens', 'sex hormones', 'sex steroids', 'testosterone' and 'sex hormone-binding globulin' in combination with 'cardiovascular' and 'women'. In addition, we checked reference lists of retrieved articles. To exclude confounding by postmenopausal hormone therapy, we excluded observational studies that included postmenopausal women on hormone therapy. For reviewing the association

between hormones and cardiovascular risk factors, we further focused on studies that excluded subjects with prevalent metabolic and cardiovascular conditions (for example, diabetes, heart disease and stroke) or studies that adjusted for these conditions in their analyses.

Testosterone production and action

In women, testosterone circulates in levels that are about 5% of those observed in men. There are three main sources of testosterone production in women: (1) the ovary, (2) the adrenal cortex and (3) peripheral conversion of androgen precursor hormones (androstenedione, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEA-S). The secretion of testosterone from the ovary is stimulated by luteinizing hormone, with estradiol exerting negative feedback. In the adrenal cortex, the secretion of testosterone is stimulated by adrenocorticotropic hormone with negative feedback by cortisol. In premenopausal women, 25% of testosterone is derived from ovaries, 25% from adrenals and 50% is produced by peripheral conversion. These three sources of circulating testosterone are slightly redistributed in postmenopausal women, because of the menopause and age-related atrophy of the adrenal cortex [21]. With the follicular depletion during menopause, estradiol production decreases rapidly, leading to a loss of negative feedback at the pituitary. The resulting increase in luteinizing hormone levels drives the ovarian production of testosterone [22,23]. Although the ovaries remain active in naturally postmenopausal women, the adrenocortical secretion of testosterone and androgen precursor hormones decreases gradually because of the age-related atrophy of the adrenal cortex. This explains the lower proportion of testosterone that is derived from the adrenal cortex (10%) and peripheral conversion (40%) in postmenopausal women (Figure 1).

In men, circulating testosterone levels decline with aging [24]. Whether a similar age-related trend occurs in women remains controversial. Cross-sectional studies have reported an age-related decrease in testosterone levels starting from the early reproductive years to the age of 65 years, followed by a small increase in late years [25,26]. However, recent insights from a longitudinal study suggest that circulating testosterone levels increase rather than decrease in women aged 38–50 years.[27] Testosterone exerts its biologic effects directly or through its metabolites (dihydrotestosterone (DHT) and estradiol). In local tissues, a small fraction of testosterone is metabolized by the enzyme 5a-reductase to DHT, which has a higher molar potency because of its higher affinity for the androgen receptor. The androgenic action is diversified by the aromatase (CYP19) mediated conversion of testosterone to estradiol.

Figure 1. Testosterone production in postmenopausal women.
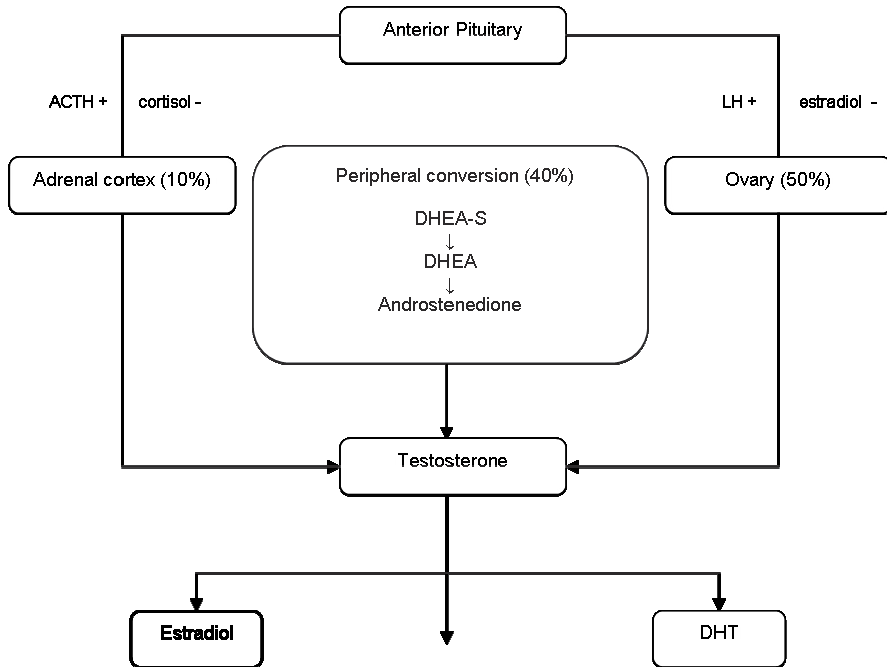
Abbreviations: ACTH, adrenocorticotropic hormone; DHT; dihydrotestosterone; LH, luteinizing hormone; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulphate. +, stimulatory; -, inhibitory.

Measuring testosterone levels

In the circulation, testosterone is specifically bound to sex hormone-binding globulin (SHBG) (66%) and non-specifically to albumin (33%), leaving only a small fraction unbound or free (1–2%). Historically, free testosterone (FT) and bioavailable testosterone (BT), which includes both free and albumin-bound testosterone, have been considered to be the bioactive fractions able to diffuse across cellular membranes. However, increasing evidence suggests that SHBG also mediates the cellular uptake and biological actions of testosterone [28,29]. As SHBG is present in such a large excess in postmenopausal women, FT and BT levels are primarily driven by SHBG. The hepatic production of SHBG is regulated positively by estradiol and negatively by testosterone. Therefore, increased testosterone levels in hyperandrogenic conditions not only raise FT levels directly, but also indirectly by lowering SHBG levels.

Given the low circulating levels of testosterone, highly sensitive and accurate assays are required to obtain reliable results in women. Mass spectrometry is the gold standard for the measurement of total testosterone (TT). However, most laboratories use direct immunoassays, as they are more rapid and less time consuming. FT and BT levels can be measured directly or calculated by algorithms (for example, Sodergard [30] and Vermeulen [31]). The limitations in measuring testosterone have been well reviewed recently [32]. To obtain more reliable TT measurements in female samples and to ensure accurate estimation of FT and BT levels, extraction and chromatography before the testosterone assay have been recommended.

Cardiovascular risk factors and disease

Over the past years, several observational studies have examined the relationship between endogenous testosterone and CVD and its risk factors. Tables 1–5 summarize the reported associations of TT, SHBG, BT and FT with various indicators of CVD risk. It is important to emphasize the limitations of the observational studies being summarized here. Study populations were heterogeneous and selection criteria diverse. For example, the type of menopause (surgical, natural or mixed) varied between study populations. Furthermore, adjustment for potential confounding factors was not always adequate. Another limitation is that most studies used direct immunoassays (without extraction), which are suboptimal for the measurement of TT levels in postmenopausal women.

Body composition and blood pressure
Obesity and hypertension are important predictors of cardiovascular morbidity and mortality in postmenopausal women [76,77]. Cross-sectional findings on the relationship between TT and markers of obesity are somewhat conflicting with high TT levels being associated with either an increase or no change in body mass index (BMI), waist circumference and waist-to-hip ratio (WHR) (Table 1). None of the studies, however, suggest that low TT levels are associated with obesity. Associations with SHBG, BT and FT seem to be more consistent. High BT and FT and low SHBG levels have been associated with an increased BMI, waist circumference and WHR. It has been suggested that testosterone and SHBG are more strongly related to abdominal obesity than general obesity [33,44]. In the study of Kaye et al. [44], the association between SHBG and WHR remained significant after adjusting for BMI. Few studies have examined the

relationship with measures of body fat distribution. Phillips et al. [39] found a positive correlation between FT and visceral fat mass in healthy postmenopausal women and two studies reported an inverse association between SHBG and visceral fat tissue [41,47]. In another study, including pre-, peri- and postmenopausal women, high BT and low SHBG levels were also found to be associated with an increase in visceral fat, independently of age, insulin resistance and estradiol [78].

Since all studies used a cross-sectional design, the causal direction of the reported associations cannot be ascertained. Studies showing a decrease in testosterone levels after weight and body fat loss in overweight women suggest the presence of reverse causation [79,80]. Furthermore, adipose tissue with its 17b-hydroxysteroid dehydrogenase activity is an important site of peripheral testosterone production [81,82], but adipose tissue is also the site where testosterone is converted into estradiol. High dose testosterone therapy has been associated with changes in body composition, including a redistribution of fat depots. In female-to-male (FM) transsexuals, testosterone administration (250 mg intramuscular testosterone ester injections every 2–3 weeks) causes a decrease in subcutaneous fat, while increasing BMI and visceral fat mass [83–85]. In obese postmenopausal women, a similar increase in visceral fat and decrease in subcutaneous fat have been reported after administration of a weak androgen (nandrolone decanoate) [86]. In female cynomolgus monkeys, high doses of exogenous testosterone have also been associated with an increase BMI and visceral fat mass [87]. A role of testosterone in the development of visceral adiposity is further supported by observational data. In healthy middle-aged women, high BT predicts the accumulation of visceral fat 5 years later, independently of estradiol and weight changes [88]. Recent findings from Zang et al.[89] also support an impact of testosterone on fat metabolism. In subcutaneous fat tissue from postmenopausal women, a decrease in hormone sensitive lipase expression was found after oral administration with testosterone undecanoate. This may explain the shift of fat accumulation towards the visceral abdominal area. In this study, testosterone undecanoate also increased the expression of phosphodiesterase-3B, an enzyme involved in the anti-lipolytic action of insulin in adipocytes.

The relation between androgenicity and blood pressure has been studied less frequently and with less consistent results (Table 1). Two studies [5,43] failed to show an association between testosterone and blood pressure. In contrast, Haffner et al. [17] found a positive association of TT with systolic and diastolic blood pressure. High FT and low SHBG levels have also been associated with hypertension [54,58]. Testosterone may influence blood pressure through the induction of obesity. However, several

studies [17, 58] have shown that the association between testosterone and blood pressure is independent of BMI, suggesting a direct effect on the renin–angiotensin–aldosterone system (RAAS). Experimental data also support a direct effect of testosterone. In female rats, testosterone treatment causes an increase in renin activity and angiotensinogen expression [90,91]. Moreover, high testosterone levels have been associated with vasoconstriction in sucrose-fed female rats [92] and increased levels of endothelin, a potent vasoconstrictor, in postmenopausal women [15].

Table 1. Associations of endogenous testosterone and SHBG levels with blood pressure and measures of body composition: results from observational studies.

	BMI	Waist circumference	WHR	DBP	SBP
TT	↑ [15,33-38]	↑ [15,33,34,39]	↑ [34]	↑ [15,17]	↑ [15,17,40]
	= [41-46]	= [35,42,43,45]	= [42-45]	= [5,43]	= [5,43]
SHBG	↓ [33-37,42,44-54]	↓ [33-35,42,45,48,49]	↓ [34,42,44,45,48,49,52,55]	↓ [5,17]	↓ [5]
	= [41]	= [39]	= [50]	= [56]	= [17,56]
BT	↑ [34,42,43,46,50,57]	↑ [34,42,43]	↑ [34,42,50]	↑ [43]	= [5,43]
			= [43,57]	= [5]	
FT	↑ [35,36,38,45]	↑ [35,39]	= [44,45]	= [17]	↑ [40,58]
	= [41,44,46]	= [45]			= [17]

Abbreviations: TT, total testosterone; SHBG, sex hormone-binding globulin; BT, bioavailable testosterone; FT, free testosterone; BMI, body mass index; WHR, waist to hip ratio; DBP, diastolic blood pressure; SBP, systolic blood pressure. ↑, positive association; ↓, negative association; =, no significant association.

Glucose and insulin metabolism

Table 2 summarizes the cross-sectional and longitudinal studies that examined the association between testosterone, SHBG and markers of glucose and insulin metabolism. In most studies no significant association with TT was found. Some of these studies, however, may have been limited by a small sample size and lack of control for confounders. Lambrinoudaki et al. [5] found an independent association between TT and insulin resistance in a large study of 598 postmenopausal women. Similarly, another study including a large number of incident diabetes cases showed a significant association between TT and type 2 diabetes after multivariable adjustment [38]. Evidence for an association with SHBG and bioactive fractions of testosterone appears to be stronger, but is not fully consistent (Table 2).

The underlying nature of the observed associations seems to be complex. Associations may in part be mediated by obesity. Although associations between SHBG and glucose metabolism appear to be independent of obesity [16,46,48,54], this may not be the case for

BT and FT. In several studies, associations of BT and FT with type 2 diabetes [38,43,62] and insulin resistance [5,43,46] remained significant after controlling for measures of obesity (for example, BMI, waist circumference), whereas others could not demonstrate an independent association [16,60]. The temporal nature of the associations is also not completely resolved. Experimental studies support a causal role for testosterone and SHBG in glucose and insulin metabolism. Androgen administration reduces insulin sensitivity in young, regularly menstruating women [93] and peripheral glucose uptake in FM transsexuals [94]. Similarly, anti-androgen therapy partially improves insulin sensitivity in hyperandrogenic women [95,96]. In addition, testosterone impairs insulin mediated glucose uptake at the skeletal muscle by reducing the expression of glycogen synthase [97,98].

There is, however, also some evidence that hyperinsulinemia could give rise to increased androgen levels. Insulin inhibits hepatic SHBG production in vitro [99]. Insulin also stimulates ovarian testosterone production [100] and luteinizing hormone release from pituitary cells [101]. Likewise, suppression of insulin levels by metformin therapy reduces androgen levels in women with the polycystic ovary syndrome [102]. These observations suggest that the high testosterone and SHBG levels being found in type 2 diabetes may be an epiphenomenon, with insulin determining circulating levels. However, in middle-aged women lower SHBG levels have been associated with type 2 diabetes independent of fasting insulin levels [103]. Recent findings from a Mendelian randomization study show an association between SHBG polymorphisms and type 2 diabetes, further supporting a causal role of SHBG in glucose and insulin metabolism.[54]

Table 2. Associations of endogenous testosterone and SHBG levels with measures of glucose and insulin metabolism: results from observational studies.

	Fasting glucose	Fasting insulin	Insulin resistance	HbA$_{1c}$	Type 2 diabetes
TT	= [16,41-43,59,60] a	↑ [39] = [41-43,46,52,60] a	↑ [5] = [16,42,43,46,60] a	= [17,38,61]	↑ [38] a = [16,40,43,50,62] a
SHBG	↓ [16,41,42,56,59] = [47,48,50,51]	↓ [41,42,44,46,51,52,56] = [47,48,50]	↓ [5,16,42,46,53] = [47]	↓ [17,61] = [54]	↓ [16,48,50,54,62] = [40]
BT	↑ [42,50,59] = [16,43,57,60] a	↑ [42,43,50,57,60] a = [46]	↑ [5,16,42,43,46,57,60] a	↑ [61]	↑ [16,43,50,62] a
FT	= [41,60]	↑ [39,60] = [41,46,52]	↑ [60] = [46]	= [17,38]	↑ [38,40] a

Abbreviations: TT, total testosterone; SHBG, sex hormone-binding globulin; BT, bioavailable testosterone; FT, free testosterone.; HbA1c, glycated hemoglobin. ↑, positive association; ↓, negative association; =, no significant association; *, longitudinal study. a Longitudinal studies: [38,43]

Lipid profile

Results from observational studies do not support a major role for testosterone in lipid metabolism. A few studies have reported an inverse association of TT with high-density lipoprotein cholesterol (HDL-C) and a positive association with total cholesterol, low-density lipoprotein cholesterol (LDL-C) and triglycerides, but in most studies no significant association between TT and lipid parameters was found (Table 3). Available data regarding an association with BT and FT are inconsistent (Table 3). In contrast, low levels of SHBG have consistently been associated with a pro-atherogenic lipid profile, namely increased triglyceride and decreased HDL-C levels.

Despite the lack of a clear association, clinical observations support a role for testosterone in dyslipidemia. Women with polycystic ovary syndrome have an abnormal lipid profile, characterized by elevated triglyceride and reduced HDL-C levels [104,105]. In FM transsexuals, testosterone administration has been associated with a reduction in HDL-C and an increase of triglyceride levels [83]. In obese, postmenopausal women administration of nandrolone decanoate [86] causes a decrease in HDL-C and an increase in LDL-C levels. These clinical and experimental data suggest that a higher level of androgenicity is required to induce an atherogenic lipid profile. The mechanisms through which testosterone and SHBG affect lipid metabolism are not completely understood, although direct regulatory effects on hepatic lipase (HL) and lipoprotein lipases (LPLs) have been reported. HL and LPL are key enzymes involved in the regulation of triglyceride and HDL-C levels. LPL activity causes a decrease in triglyceride and an increase in HDL-C levels, whereas HL activity is associated with a decrease in HDL-C. In FM transsexuals, testosterone therapy causes an increase in HL activity [83]. The sensitivity of lipolytic enzymes for androgens is further supported by findings from the HERITAGE study showing a strong inverse association between SHBG and HL activity and a positive association between SHBG and LPL activity [106].

Apart from direct regulatory effects, testosterone and SHBG may also influence lipid metabolism indirectly through their associations with obesity. Yasui et al.[60] found that associations of SHBG with HDL-C and triglycerides were no longer significant after controlling for BMI. In another study, a similar lack of independence was observed for the association with HDL-C [64]. Conversely, in several studies associations between SHBG and triglycerides [5,6,55,64] and HDL-C [5,17] persisted after adjustment for BMI or WHR. Phillips et al.[39] found that after controlling for visceral fat mass, the positive association between FT and triglyceride levels remained significant, whereas the association between FT and HDL-C lost significance. Another possible mediator is insulin. Insulin is known to exert a direct regulatory effect on HL and LPL [107,108].

Interestingly, only a few studies [6,52,56] adjusted for the effect of insulin. Mudali et al.[6] showed that adjustments for insulin and BMI did not significantly influence associations of SHBG with HDL-C and triglycerides. In contrast, Soler et al.[52] found that associations between SHBG and triglycerides were lost after controlling for WHR, insulin and estradiol. In the study of Haffner et al.[56] the association between SHBG and HDL-C was independent of fasting insulin levels, but the relationship with triglycerides was no longer significant after adjusting for insulin.

Table 3. Associations of endogenous testosterone and SHBG levels with plasma lipids: results from observational studies.

	Total cholesterol	LDL cholesterol	HDL cholesterol	Triglycerides
TT	↑ [5,17,40] = [6,34,52,55,60,63]	↑ [5,40] = [6,34,55,60,63,64]	↓ [5] = [6,17,34,39,52,55,60,63,64]	↑ [5] = [6,39,43,52,55,60,63,64]
SHBG	↓ [6,17] = [5,34,47,52,55,56,60,65]	↓ [6,54] = [5,34,47,55,56,60,64,65]	↑ [5,6,17,34,47,52,54-56,60,65] = [64]	↓ [5,6,52,54-56,60,64] = [65]
BT	↑ [5,34] = [6,57,60]	↑ [5,6,34] = [57,60]	↓ [5,34] = [6,43,57,60]	↑ [5,57] = [6,43,60]
FT	↑ [40] = [17,52,60,65]	= [60,64,65]	↓ [17,39] = [52,60,64,65]	↑ [39] = [52,60,64,65]

Abbreviations: TT, total testosterone; SHBG, sex hormone-binding globulin; BT, bioavailable testosterone; FT, free testosterone; LDL cholesterol, low density lipoprotein cholesterol; HDL cholesterol, high density lipoprotein cholesterol. ↑, positive association; ↓, negative association; =, no significant association.

Other cardiovascular risk factors

Besides traditional risk factors, a growing number of studies have started to examine associations with other markers of cardiovascular risk such as C-reactive protein (CRP), fibrinogen and white blood cell count (Table 4). CRP is an inflammatory marker and independent predictor of cardiovascular events in postmenopausal women [109]. In several studies, high BT and low SHBG levels [34,54,66,69] have been associated with an increase in CRP levels. In addition, a positive association between TT and CRP has been reported in healthy postmenopausal women [15,34]. Conversely, Joffe et al.[67] found an opposite association in women referred to coronary angiography, with low testosterone levels being associated with an increase in CRP levels. Interestingly, this inverse association with testosterone was not present in women who remained CVD-free. These findings suggest that the association between testosterone and CRP depends on CVD status, with a potential confounding effect of subclinical CVD.

Early atherogenic changes may affect testosterone production by the ovaries and adrenals through restriction of the blood supply. This may explain the presence of an inverse association among women with subclinical CVD. Data on the association between testosterone and the clotting factor fibrinogen are inconclusive. In two small studies [15,68], TT levels were not associated with plasma fibrinogen levels. However, in a larger study including 317 postmenopausal women [66], fibrinogen levels were approximately 10% higher in highest TT quartile compared with the lowest quartile, but no association between SHBG and fibrinogen was found. Interestingly, high TT levels in this study were also associated with an increase in white blood cell count, another non-traditional CVD risk factor.

Table 4. Associations of endogenous testosterone and SHBG with C-reactive protein, fibrinogen and white blood cell count: results from observational studies.

	C-reactive protein	Fibrinogen	White blood cell count
TT	↑ [15,34] ↓ [67] = [64,66,69]	↑ [66] = [15,68]	↑ [66]
SHBG	↓ [34,54,64,66,67,69]	= [66]	= [66]
BT	↑ [34,67,69]		
FT	= [64]		

Abbreviations: TT, total testosterone; SHBG, sex hormone-binding globulin; BT, bioavailable testosterone; FT, free testosterone. ↑, positive association; ↓, negative association; =, no significant association.

Atherosclerosis

Studies investigating the relation between testosterone and atherosclerotic indices (carotid, aortic, coronary and peripheral atherosclerosis) have yielded contradictory results (Table 5). Phillips et al.[75] examined the correlation between testosterone and the degree of coronary atherosclerosis in a cross sectional study among 60 patients with coronary artery disease. In this study, high FT levels were positively associated with the severity of disease, independent of estradiol, BMI and other cardiovascular risk factors. In the WISE study [72] positive associations of TT and FT with coronary artery disease were significant after adjustment for estradiol levels. Ouyang et al.[70] studied a population of women without clinically evident CVD and found that high levels of TT and BT and low levels of SHBG were associated with subclinical carotid atherosclerosis. Associations with TT and BT were independent of age, BMI and cardiovascular risk factors, but the association with SHBG was no longer significant

after adjustment for HDL and LDL cholesterol. In the Rotterdam Study [71], high TT levels were found to be associated with aortic atherosclerosis, but this association was weakened after adjustments for cardiovascular risk factors. On the other hand, several studies have reported inverse associations. Bernini et al. [19] showed a negative association between FT and carotid intima-media thickness (cIMT) in 44 postmenopausal women. Similar findings were reported by Debing et al. [20] who found that cases with carotid atherosclerosis had lower levels of FT than atherosclerotic free controls. In another case–control study [74], carotid atherosclerosis was also found to be more common in women with low TT levels. Low testosterone levels have also been associated with impaired endothelial function [110]. On the other hand, increased carotid atherosclerosis and endothelial dysfunction have been reported in women with the polycystic ovary syndrome women, i.e. women having supranormal testosterone levels [111,112]. Reverse causation may explain the contradictory findings. Atherosclerosis, for instance, may affect testosterone production by impairing the blood flow to androgen-producing organs. Alternatively, the contradictory results may suggest the presence of a U-shaped relationship in which very low testosterone and very high testosterone levels are associated with reduced arterial function.

Table 5. Associations of endogenous testosterone and SHBG levels with indices of atherosclerosis: results from observational studies.

	Carotid atherosclerosis	Aortic atherosclerosis	Coronary atherosclerosis	Peripheral atherosclerosis
TT	↑ [70] ↓ [20,74] = [19]	↑ [71] a	↑ [72] = [75]	= [73]
SHBG	↓ [70,74] = [19,20]		= [72,75]	= [73]
BT	↑ [70] = [74]	= [71] a		= [73]
FT	↓ [19,20]		↑ [72,75]	

Abbreviations: TT, total testosterone; SHBG, sex hormone-binding globulin; BT, bioavailable testosterone; FT, free testosterone. ↑, positive association; ↓, negative association; =, no significant association; a, longitudinal study: [71]

Results from experimental studies show a similar pattern. Bruck et al.[113] found an increase in plaque size when female rabbits on an atherogenic diet were treated with testosterone. This increase was independent of changes in plasma lipids. In cynomolgus monkeys, a similar effect of testosterone on coronary atherosclerosis was found [87]. Testosterone also induces vasoconstriction in sucrose fed female rats, suggesting an adverse effect of testosterone on endothelial function [92]. In vitro data further suggest that testosterone may increase monocyte adhesion to the vascular endothelium [114]. On the other hand, testosterone has been reported to induce relaxation in rabbit coronary artery and aorta rings [115]. In addition, administration of physiological testosterone levels to androgen-deficient female rats improves the vasodilatory reserve of the vascular endothelium [116].

Cardiovascular events and mortality
Relatively few data are available on the relationship between endogenous testosterone and cardiovascular morbidity and mortality. In the Rancho Bernardo Study [8], TT and BT levels did not differ between women with and without CVD at baseline and did not predict cardiovascular mortality over a 19-year follow-up. Although the number of cardiovascular deaths was relatively high in this study (n = 176), stratification for estrogen replacement therapy may have reduced power to detect an association. Contrary to the data on CVD risk factors, Haffner et al. [117] found that diabetic women in the lowest TT quartile had an increased risk of ischemic heart disease mortality, although this association was no longer significant in multivariable adjusted analyses. In a large nested case control study [7] high BT levels were associated with an increased risk of cardiovascular events, although this association was not independent of BMI, hypertension and diabetes.
Data on the relationship between SHBG and CVD are also mixed. Haffner et al.[117] failed to show an association between SHBG and ischemic heart disease mortality in diabetic women. In the Gothenburg Study[118] a U-shaped association between SHBG and myocardial infarction was found, with a high incidence of myocardial infarction in the lowest decile of SHBG. In the Rancho Bernardo study [119], which adjusted for BMI, no significant association between SHBG and CVD mortality was found; however, women with higher SHBG levels had slightly lower CVD mortality rates. Similarly, Rexrode et al.[7] found that low SHBG levels were associated with an increased risk of CVD events, although this association was not independent of BMI.

Postmenopausal hormone therapy

There is increasing interest in the use of testosterone as part of postmenopausal hormone therapy. In several studies addition of testosterone to estrogen therapy has been reported to improve sexual function and well-being [120,121]. In addition, beneficial effects on bone mineral density have been described [122]. Despite the large number of studies investigating the effect of testosterone coadministration, there are limited long-term data regarding the cardiovascular safety of this combination therapy. Treatment duration ranges from 1 month to 2 years and there are not data available on the effect on cardiovascular events (Table 6). Two studies [139,141] reported adverse effects on atherosclerotic indices in naturally postmenopausal women. Penotti et al. [139] showed that 8-month coadministration of high-dose testosterone undecanoate (40 mg per day) causes a small, but significant increase in the pulsatile index of the middle cerebral artery, but not in the internal carotid artery. In a retrospective study, Hak et al.[141] found an adverse effect of long-term, high-dose intramuscular estrogen–testosterone therapy on aortic atherosclerosis. In addition, an increase in fibrinogen levels has been reported with oral methyl testosterone therapy [132]. However, no significant changes in clotting factors have been found in surgically postmenopausal women treated with low-dose testosterone patches [124–126].

The effect of testosterone supplementation on lipids seems to depend on the route of administration (Table 6). Coadministration with oral methyltestosterone or testosterone undecanoate causes a decrease in HDL-cholesterol levels, whereas no significant change in lipid parameters is observed with testosterone patches or implants. Furthermore, oral preparations of methyltestosterone cause a favourable decrease in triglyceride levels, an effect which is not observed with transdermal testosterone. These differential effects are thought to be attributed to a first-pass liver effect, which is bypassed by implants and transdermal patches.

Available data on body composition suggest both beneficial and adverse effects. An increase in lean body mass and decrease in fat mass have been observed after coadministration of methyltestosterone [133] and testosterone implants [122]. In contrast, Leao et al.[136] reported an increase in body weight and visceral fat mass after the addition of 1.25 mg methyltestosterone to transdermal estradiol therapy. Regarding glucose metabolism, no adverse effects have been reported.

Altogether these findings indicate that short-term cardiovascular effects of testosterone coadministration depend on the dose and route of administration, with low-dose testosterone patches being the safest option. However, it is important to emphasize that co-treatment with estrogens may counteract potential adverse

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effects of testosterone. Therefore, safety data from testosterone coadministration studies cannot be used to draw firm conclusions about the cardiovascular effects of exogenous testosterone in postmenopausal women. Studies monitoring the effects of testosterone therapy only provide a better model for studying these effects. Recently, the effect of testosterone only was investigated in a 52-week double-blind placebo-controlled trial [140] in which postmenopausal women received a patch delivering 150 or 300 mg of testosterone per day or placebo. In this study, circulating levels of TT, FT and BT showed a dose-related increase with testosterone treatment, but no difference in lipid profile and glucose metabolism was found between women treated with testosterone compared with placebo. These findings do not correspond with the associations found in observational studies and the effects of exogenous testosterone reported in FM transsexuals and animal models. There are several explanations for these discrepant results. First of all, the dosages administered were lower than those used by FM transsexuals. Secondly, transdermal administration may exert different effects than the intramuscular injections administered to FM transsexuals and animal models. The duration of exposure may also not be sufficient to detect an effect. Finally, postmenopausal women in this study had symptoms of androgen deficiency and may therefore have a lower baseline CVD risk and be less susceptible to adverse effects of testosterone.

Table 6. Trials examining the effect of exogenous testosterone on cardiovascular risk parameters in healthy postmenopausal women.

Trial	Menopause	Drug	Route	Dose	Follow-up	Cardiovascular effect
Co-administered with estrogen/progesterone replacement therapy						
Shifren et al, 2000 [121]	Surgical	T	Transdermal (patch)	150/300 µg/day	12 weeks	=
Buster et al, 2005 [123]	Surgical	T	Transdermal (patch)	300 µg/day	24 weeks	=
Braunstein et al, 2005 [124]	Surgical	T	Transdermal (patch)	150/300/450 µg/day	24 weeks	=
Simon et al, 2005 [125]	Surgical	T	Transdermal (patch)	300 µg/day	24 weeks	=
Davis et al, 2006 [126]	Surgical	T	Transdermal (patch)	300 µg/day	24 weeks	=
Shifren et al, 2006 [120]	Natural	T	Transdermal (patch)	300 µg/day	24 weeks	=
Nathorst-Boost et al, 2006 [128]	Natural	T	Transdermal (gel)	10 mg/day	3 months	=
Burger et al, 1987 [129]	Natural/surgical	T	Implant	50 mg x 1	6 weeks	=
Davis et al, 1995 [122]	Natural/surgical	T	Implant	50 mg/3 months	24 months	↓ fat mass
Farish et al, 1984 [129]	Surgical	T	Implant	100 mg x 1	6 months	=
Hickok et al, 1993 [130]	-	MT	Oral	1.25 mg/day	6 months	↓ HDL-C
Watts et al, 1995 [131]	Surgical	MT	Oral	2.5 mg/day	24 months	↓ HDL-C, triglycerides
Basaria et al, 2002 [132]	Natural/surgical	MT	Oral	2.5 mg/day	16 weeks	↓ HDL-C, triglycerides ↑ fibrinogen
Dobs et al, 2002 [133]	Natural/surgical	MT	Oral	2.5 mg/day	16 weeks	↓ HDL-C, triglycerides, fat mass
Lobo et al, 2003 [134]	Natural/surgical	MT	Oral	1.25 mg/day	16 weeks	↓ HDL-C, triglycerides
Warnock et al, 2005 [135]	Surgical	MT	Oral	1.25 mg/day	8 weeks	↓ HDL-C, triglycerides
Leao et al, 2006 [136]	Surgical	MT	Oral	1.25 mg/day	12 months	↓ HDL-C ↑ visceral fat mass
Barrett-Connor et al, 1999 [137]	Surgical	MT	Oral	1.25 mg/day	24 months	↓ HDL-C, triglycerides
Raisz et al, 1996 [138]	Natural/surgical	MT	Oral	2.5 mg/day	9 weeks	↓ HDL-C, triglycerides
Penotti et al, 2001 [139]	Natural	TU	Oral	40 mg/day	8 months	↓ HDL-C, ↑ pulsatile index (PI)
Without estrogen/progesterone replacement therapy						
Davis et al, 2008 [140]	Natural/surgical	T	Transdermal (patch)	150/300 µg/day	52 weeks	=

Abbreviations: T, testosterone; MT, methyl testosterone; TU, testosterone undecanoate; HDL-C, high density lipoprotein-cholesterol; CRP, C-reactive protein.
↑, positive association; ↓, negative association. = no association.

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Conclusions

Studies reviewed in this article suggest that increased androgenicity (high endogenous testosterone and low SHBG levels) is associated with an adverse CVD risk factor profile in postmenopausal women. However, firm conclusions about the association between testosterone, SHBG and clinical CVD events cannot be drawn. Many studies were limited by a cross-sectional design and longitudinal data regarding the association with cardiovascular events are scarce and inconclusive.

Existing data from testosterone supplementation studies further suggest that the use of low-dose transdermal testosterone is safe. However, studies examining potential adverse effects were often restricted by a short follow-up period. As testosterone replacement therapy is increasingly being used for the treatment of sexual dysfunction in postmenopausal women, its long-term effects on cardiovascular risk markers need to be studied more thoroughly. Although the effects of testosterone may be less detrimental than in FM transsexuals, because of the lower dosages being administered, the long-term safety of testosterone use needs to be assessed carefully as minor deleterious effects on CVD may outweigh the benefits of testosterone therapy.

There are several potential mechanisms linking increased androgenicity to an adverse CVD risk profile, including both direct and indirect effects. Experimental data suggest direct effects of testosterone on body composition, lipid levels and glucose metabolism. We postulate that increased androgenicity contributes to the accumulation of visceral fat and impairment of glucose metabolism, creating a vicious circle, whereby the increase in insulin and fat tissue in turn promote the production of testosterone. Apart from these direct effects, associations of androgen excess with glucose and lipid metabolism may be strengthened through the effects of testosterone on body composition. Associations between testosterone, SHBG and atherosclerotic indices seem to be mediated in part through their association with CVD risk factors. Atherosclerosis in turn may lead to lower circulating testosterone levels by decreasing the blood supply to androgen-producing organs. Research into non-traditional CVD risk factors is growing and may identify alternative pathways through which androgens affect cardiovascular health.

Of particular interest is that SHBG is often more strongly related to the CVD risk profile than testosterone itself. As SHBG is correlated with testosterone, the independent contribution of SHBG to the CVD risk profile is unclear. The lack of a strong association with testosterone may also reflect the reliability of the testosterone assays currently

being used. If SHBG does have an independent effect, its precise role requires further investigation. SHBG may influence CVD risk indirectly by modulating the biologic effects of testosterone or exert more direct effects through its own SHBG receptor. There is need for more large-scale longitudinal studies examining the temporal relationship between endogenous testosterone and SHBG levels and cardiovascular risk and studies evaluating the long-term safety of testosterone replacement in postmenopausal women. In addition, Mendelian randomization studies may help to determine the likelihood of causality. Finally, more research is needed to elucidate the mechanisms through which testosterone and SHBG affect CVD risk and to indicate potential means of prevention and intervention.

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5

Chapter 6

**Testosterone, SHBG and the metabolic syndrome:
a systematic review and meta-analysis of observational
studies**

Abstract

Background: Accumulating evidence suggests a sex-dependent role of circulating testosterone in the metabolic syndrome (MetS).

Methods: We conducted a meta-analysis of observational studies (PubMed and EMBASE – 1 May 2010) relating MetS to determinants of testosterone status [total testosterone (TT), free testosterone (FT) and sex hormone-binding globulin (SHBG)].

Results: A total of 52 studies were identified, comprising 22 043 men and 7839 women and presenting relative risk (RR) estimates or hormone levels for subjects with and without MetS. Endogenous TT and FT levels were lower in men with MetS (TT mean difference = -2.64 nmol/L; 95% CI, -2.95, -2.32, FT standardized mean difference = -0.26 pmol/L; 95% CI, -0.39, -0.13) and higher in women with MetS (TT mean difference = 0.14 nmol/L; 95% CI, 0.07, 0.20, FT standardized mean difference = 0.52 pmol/L; 95% CI, 0.33, 0.71) compared with those without. Similarly, men with higher TT levels had a lower MetS risk (RR estimate = 0.38; 95% CI, 0.28, 0.50), whereas higher TT levels increased the risk of MetS in women (RR estimate = 1.68; 95% CI, 1.15, 2.45). In both sexes, higher SHBG levels were associated with a reduced risk (men: RR estimate = 0.29; 95% CI, 0.21, 0.41, women: RR estimate = 0.30; 95% CI, 0.21, 0.42).

Conclusion: This meta-analysis supports the presence of a sex-dependent association between testosterone and MetS: TT and FT levels are lower in men with MetS, whilst they are higher in women with MetS. There are no indications for a sex-specific association between SHBG and MetS. In both men and women, MetS is associated with lower SHBG levels.

Introduction

The metabolic syndrome (MetS) is a constellation of metabolic risk factors (including hypertension, dyslipidemia, abdominal obesity and impaired glucose metabolism), that is associated with a two-fold increased risk of cardiovascular disease, and an even higher risk of type 2 diabetes [1,2]. Over the past years, various definitions of MetS have been introduced, of which those proposed by the National Cholesterol Education Program - Adult Treatment Panel III (NCEP ATP III) [3], the World Health Organization (WHO) [4] and the International Diabetes Federation (IDF) [5] are the most widely used. The prevalence of MetS increases with age and is higher in men than in women [6]. MetS associated risks seem to vary according to sex, with MetS being a stronger risk factor for cardiovascular disease in women than men [7,8].

Besides sex differences in prevalence and prognosis, factors associated with the occurrence of MetS may also vary by gender. Previous studies have suggested a role for sex hormones in the development of MetS. Androgen-deprivation therapy in prostate cancer patients [9], and low total testosterone levels (TT) in hypogonadal men [10,11] have been associated with the metabolic syndrome. On the other hand, MetS and its individual components are common in hyperandrogenic conditions in women, such as the polycystic ovary syndrome (PCOS) [12,13]. Sex hormone-binding globulin (SHBG), a testosterone transport protein that affects the circulating levels of free testosterone (FT), has also been linked to MetS. Low SHBG levels have been observed in both men and women with MetS [14,15]. However, little is known about possible sex differences in this association. Furthermore, several studies have examined the relationship between FT and MetS, but their findings have been inconsistent in men [16-18] and women [15,19,20].

To systematically asses the associations of MetS with TT, SHBG and FT, and to investigate possible sex differences in these associations, we conducted a meta-analysis of observational studies relating endogenous TT, SHBG and/or FT levels to the metabolic syndrome in men and women separately.

6

Methods

Data sources and searches
We performed this meta-analysis according to the guidelines of the Meta-analysis of Observational Studies in Epidemiology group [21]. A systematic search of PubMed and EMBASE (1966 – 1 May 2010) was conducted for English-language articles using the key words 'metabolic syndrome', 'insulin resistance syndrome' and 'syndrome X' combined with 'testosterone', 'sex hormone-binding globulin', 'shbg', 'androgens', 'sex hormones' and 'sex steroids'. In addition, reference lists of retrieved articles were searched.

Study selection
Studies were selected by two investigators (J.S.B., Y.T.v.d.S.), using the following criteria: (i) Observational studies including TT, SHBG and/or FT as 'determinant' and MetS as 'outcome'. (ii) MetS defined as the presence of at least three of the following five components: obesity (based on waist circumference, waist to hip ratio or body mass index (BMI)), elevated triglyceride levels, low high density lipoprotein cholesterol levels, impaired glucose metabolism (based on fasting glucose or insulin levels, presence of insulin resistance or diagnosis of diabetes) and hypertension (based on systolic and diastolic blood pressure measurements). (iii) Studies conducted in adults or adolescents. (iv) Availability of a measure of association (mean plus SD of hormone levels in subjects with and without MetS and/or a relative risk estimate (odds ratio (OR), relative risk (RR), hazard ratio (HR), prevalence ratio (PR)). (v) Studies not selecting participants on the basis of existing diabetes mellitus or cardiovascular disease.
If multiple reports used the same population for calculating association measures, we only included the analysis based on the largest number of participants.

Data extraction and quality assessment
The following data were extracted from each included study: (i) Study characteristics (first author, year of publication, country of data collection, study design, length of follow-up if longitudinal, MetS definition (and if applicable its modification), method of free testosterone assessment, exclusion criterion regarding type 2 diabetes and variables incorporated in multivariable analyses). (ii) Study sample characteristics (sex, mean age and BMI, PCOS status in women, number of subjects with and without MetS, mean and SD (derived if SE or 95% CI reported) of TT, SHBG and FT in subjects with and without MetS and RR estimates).

The primary measure of association was the mean difference in TT, SHBG and FT levels between subjects with and without MetS. For the calculation of mean differences, medians and geometric means were assumed to equal means. If studies provided ranges or interquartile ranges instead of SDs, approximate SDs were derived using data extraction methods of Higgins [22] and Hozo [23] et al.

For studies relating TT, SHBG and FT to MetS risk, RR estimates were included as a secondary measure of association. ORs, RRs, HRs and PRs adjusted for the largest number of confounders were extracted. Adjustments for other hormones and components part of the MetS definition were omitted, as these might obscure true associations. Since individual studies reported RR estimates based on various cut-off levels (tertiles, quartiles or specific thresholds) or as a 1 SD increase in testosterone and SHBG, RR estimates were transformed to a uniform scale (comparing the highest versus lowest tertile of TT, SHBG and FT) using the method of Danesh et al. [24]. According to this method, the log relative risk estimate comparing the highest versus lowest tertile can be estimated as 2.18/2.54 times the log relative risk estimate comparing the highest versus lowest quartile, or assuming a normal distribution, as 2.18 times the log relative risk estimate for a 1 SD increase in TT, SHBG or FT. From the study of Laaksonen et al [25] log ORs for the highest versus lowest tertile were obtained by multiplying the dichotomized log ORs by 2.18/1.695.

The quality of each study was assessed against the following criteria: (i) Population-based sample. (ii) Exclusion of subjects on hormonal therapy. (iii) Use of fasting blood samples for assessment of MetS components. (iv) Adjusted analysis for potential confounders. An extra criterion was added for studies including men: (v) Blood sample collection for hormonal assessment in the morning. Studies with a population based sample were defined as those including subjects from the community, who were not institutionalized, clinic based or known to have MetS. Each criterion was graded as 'yes', 'no' or 'unclear'.

Attemps were made to contact authors when further information was needed for meta-analytic calculations. We contacted 13 authors for missing data of whom 9 provided additional data [15,26-33]

Data synthesis and analysis

Measures of association were analysed for men and women separately, unless results showed no clear indications for an interaction by sex. To compare TT and SHBG levels between subjects with and without MetS, pooled analyses were performed using unstandardized mean differences of TT and SHBG. For the comparison of FT levels, standardized mean differences (mean differences divided by the pooled

standard deviation) were used, because individual studies used various methods for FT assessment.

Between-study heterogeneity was quantified by the I^2 statistic [22]. Random-effects models of DerSimonian and Laird [34] were applied in obtaining pooled estimates of association measures.

Univariable metaregression analyses including sex as covariate were conducted to assess sex differences in TT, SHBG and FT levels between subjects with and without MetS. Within each sex, univariable metaregression analyses for predetermined variables (age, BMI, MetS criteria, exclusion of type 2 diabetes, PCOS status, study design, adjustment for covariates and method of FT assessment) were performed to investigate their impact on the association measures and between-study heterogeneity. For these analyses, studies were stratified according to mean age (< 55 years vs $\geq$ 55 years), mean BMI (< 25 kg/m^2 vs ≥ 25 kg/m^2), MetS definition used (NCEP ATP III vs other criteria (WHO, IDF, EGIR)), exclusion of diabetic patients (yes vs no), study design (cross-sectional (CS) vs longitudinal (LO)), adjustment for covariates (yes vs no) and method of FT assessment (direct measurement vs algorithms). Age and BMI were also entered as continuous terms in metaregression analyses. In women, studies were further classified according to the number of PCOS patients included ($< 50\%$ vs $\geq 50\%$). The prevalence of PCOS ranges from 5 to 10% in reproductive women, depending on ethnicity and the criteria being used [35]. In studies not excluding PCOS patients explicitly, the relative number of PCOS patients was assumed not to exceed this percentage range.

Multivariable metaregression analyses including sex and each of the predetermined variables (except for PCOS status) were conducted to investigate whether the interaction effect of sex changed after adjusting for age, BMI and control for age. Univariable and multivariable metaregression analyses were not considered when there were fewer than 10 studies available.

To investigate the impact of each quality parameter separately, sensitivity analyses were conducted in which studies not meeting the individual criteria were excluded. Since direct radioimmunoassay (RIA) is a less reliable method for measuring free testosterone levels [36], the impact of this assay was also investigated in sensitivity analyses. To asses the presence of possible publication bias, funnel plots were drawn and correlations between standardized association measures and their corresponding SEs were analyzed using Egger's test [37]. In case of publication bias, the "trim and fill" method of Duval and Tweedie [38] was used to correct for this bias. All analyses were conducted using STATA 11.1 (StataCorp., College Station, Tex., U.S.A.).

Results

Study selection

The study selection process is described in Figure 1. Our initial search yielded 596 articles. Of these, 428 articles were excluded based on abstract review. After full text review, an extra 116 studies were excluded because of lack of measure of interest (n = 91), lack of standard MetS definition (n = 7), inappropriateness of reported association measure for inclusion (n = 8), multiple publication (n = 7), unavailability of full text (n = 2) and no correct stratification of MetS (n = 1), leaving 52 studies eligible for inclusion, 32 including men, 19 including women and 1 study including both men and women.

Figure 1. Flow diagram outlining the study selection process.
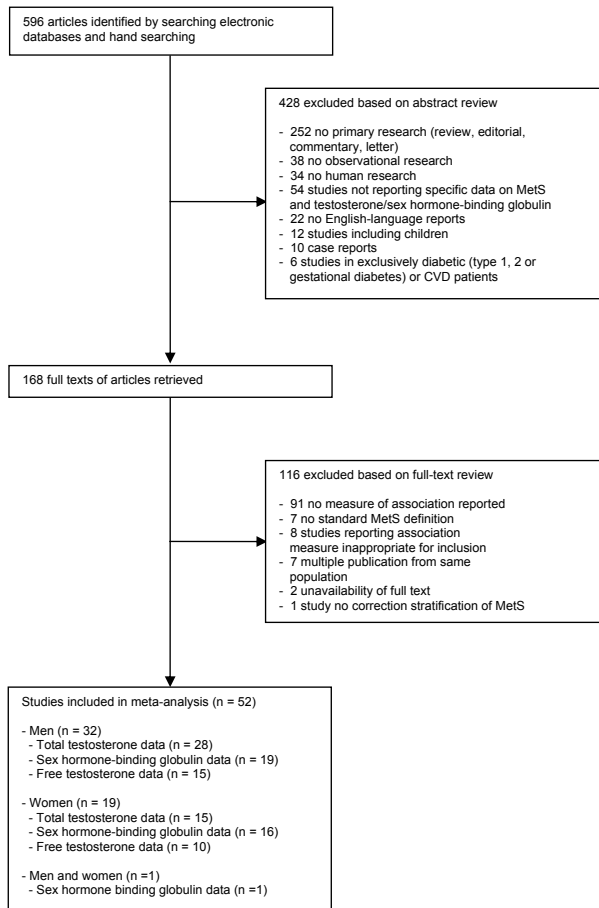
Abbreviations: Mets, metabolic syndrome; CVD, cardiovascular disease.

Table 1. Characteristics of studies reporting on TT, SHBG and/or FT levels in men with and without MetS.

Nr.	Source	Country	Study design	Participants	Mean age	Mean BMI	Adjusted for age	MetS No.		Mean (SD), nmol/L				Mean (SD), pmol/L	
										Total testosterone		SHBG		Free testosterone	
								Yes	No	MetS+	MetS-	MetS+	MetS-	MetS+	MetS-
1	Katabami et al, 2010 [39]	Japan	CS	Nondiabetic men	46	23.7	No	70	204					40.6 (13.9) [c]	51.01 (16.0) [c]
2	de Oya et al, 2010 [28 d]	Spain	CS	Adolescent boys	14	21.9	No	13	377			28.8 (15.2)	51.0 (34.6)		
3	Atlantis et al, 2009 [40] (1) [b]	Australia	CS	Men from the Florey Adelaide Male Ageing Study	53		No	445	737	12.2 (4.9)	15.3 (5.6)	30.8 (14.0)	37.1 (17.1)		
	Atlantis et al, 2009 [40] (2) [b]	Australia	CS	Men from the Florey Adelaide Male Ageing Study	53		No	498	691	12.1 (4.8)	15.5 (5.6)	31.2 (15.5)	37.2 (16.5)		
4	Coviello et al, 2009 [41] (1) [d]	U.S.A.	CS	Fathers of women with PCOS	57	30.2	No	89	122	12.8 (5.2)	15.6 (4.3)	72 (40)	80 (37)		
	Coviello et al, 2009 [41] (2) [d]	U.S.A.	CS	Brothers of women with PCOS	29	28.7	No	13	45	15.0 (4.6)	18.8 (7.0)	46 (22)	55 (26)		
5	Demir et al, 2009 [42]	Turkey	CS	Men with lower urinary tract symptoms	60 [a]	27.4 [a]	No	60	130	14.0 (5.2)	16.0 (6.1)				
6	Haring et al, 2009 [43]	Germany	LO	Men from the Study of Health in Pomerania	49	26.4	No	480	524	15.5 (4.8)	17.7 (5.3)				
7	Chubb, 2008 [10]	Australia	CS	Nondiabetic men from the Health in Men Study	76 [a]	26.2 [a]	No	602	1900	14.0 (4.9)	16.7 (5.7)	36.8 (14.0)	45.5 (17.0)	274.6 (88.2)	291.2 (90.8)
8	Emmelot-Vonk et al, 2008 [44]	Netherlands	CS	Nondiabetic men with low normal testosterone levels	67	27.3	No	62	160	12.7 (2.3)	13.5 (2.4)	28.9 (8.9)	34.4 (10.5)	376.5 (104.0)	345.4 (128.5)
9	Goncharov et al, 2008 [45] (1) [b]	Russia	CS	Nondiabetic obese men	31	32.6	No	34	26	11.2 (4.0)	16.3 (6.8)	29.7 (21.5)	45.2 (32.0)	249.0 (94.0)	294.0 (129.0)
	Goncharov et al, 2008 [45] (2) [b]	Russia	CS	Nondiabetic obese men	31	32.6	No	23	37	10.9 (4.4)	15.0 (6.3)	33.9 (27.2)	37.4 (28.0)	230.0 (95.0)	295.0 (120.0)
	Goncharov et al, 2008 [45] (3) [b]	Russia	CS	Nondiabetic obese men	31	32.6	No	27	33	11.2 (4.6)	15.3 (6.3)	31.4 (22.3)	40.4 (30.7)	236.0 (85.0)	296.0 (125.0)
10	Laughlin et al, 2008 [31]	U.S.A.	CS	Men from the Rancho Bernardo Study	71	25.7	Yes	143	651	8.5 (2.8)	10.8 (3.4)				
11	Suetomi et al, 2008 [46]	Japan	CS	Men with erectile dysfunction	60	23.9 [a]	No	25	108	15.3 (5.5)	16.0 (5.9)			33.7 (12.8)	36.1 (11.8)
12	Yeh et al, 2008 [47]	Taiwan	CS	Men with erectile dysfunction	58	24.9 [a]	No	38	65	12.4 (5.8)	16.2 (5.9)				
13	Corona et al, 2007 [17] (1) [b]	Italy	CS	Male patients with sexual dysfunction	52		No	348	738	13.6 (6.0)	17.4 (7.2)			34.8 (14.0) [c]	
	Corona et al, 2007 [17] (2) [b]	Italy	CS	Male patients with sexual dysfunction	52		No	485	601	14.7 (7.4)	18.2 (6.0)			36.2 (14.1) [c]	
14	Guay et al, 2007 [33] (1) [b]	U.S.A.	CS	Men with erectile dysfunction	54	29.4	No	88	66					42.7 (18.4) [c]	49.3 (22.9) [c]
	Guay et al, 2007 [33] (2) [b]	U.S.A.	CS	Men with erectile dysfunction	54	29.4	No	54	100					39.6 (10.4) [c]	51.4 (22.9) [c]
15	Rodriguez et al, 2007 [48]	U.S.A.	CS [e]	Caucasian men from the Baltimore Longitudinal Study of Aging	63	26.0	Yes	113	505	12.8 (0.2)	14.9 (0.1)	62.9 (2.8)	82.1 (1.6)		

No.	Study	Country	Type	Description	Age	BMI	MetS	n MetS+	n MetS–	T MetS+	T MetS–	SHBG MetS+	SHBG MetS–	Total T MetS+	Total T MetS–
16	Tang et al, 2007 [49]	Taiwan	CS	Men residing in a veterans' nursing home	79	23.8	No	101	280	13.3 (0.6)	16.2 (0.4)	39.9 (1.6)	53.9 (1.2)	194.5 (76.9)	205.4 (74.7)
17	Chen et al, 2006 [29]	Australia	CS	Nondiabetic men from the Australian Longitudinal Study of Aging	76	26.0	No	20	140	12.1 (3.6)	14.2 (4.7)				
18	Gannagé-Yared et al, 2006 [50]	Lebanon	CS	Nondiabetic men	59	27.3	No	94	59	12.5 (3.8)	14.3 (4.0)	34.0 (13.7)	41.0 (15.5)		
19	Kaplan et al, 2006 [51]	U.S.A.	CS	Men with dyslipidemia	52	27.4	No	265	597	14.0 (4.7)	16.1 (4.9)				
20	Kupelian et al, 2006 [18]	U.S.A.	CS [e]	Men from the Massachusetts Male Aging Study	53 [a]	27.1 [a]	No	146	950	15.6 (6.4)	18.4 (5.9)	26.1 (11.8)	33.6 (16.1)	430.0 (190.0)	470.0 (180.0)
21	Maggio et al, 2006 [52]	Italy	CS	Men from the InCHIANTI study	75	26.6 [a]	No	73	389	13.8 (4.8)	15.0 (4.5)	83.6 (30.8)	104.0 (46.1)	145.7 (48.8)	131.9 (56.6)
22	Mousavinasab et al, 2006 [53]	Finland	LO	Military service men on a high-caloric high-fat diet	17-28	24.3 [a]	No	11	169			15.1 (6.6)	19.1 (10.2)		
23	Robeva et al, 2006 [54]	Bulgaria	CC	Nondiabetic, hyperinsulinaemic men with MetS and healthy-age matched controls	30	30.6	Yes	10	10	12.1 (3.7)	21.5 (7.5)				
24	Kalme et al, 2005 [55]	Finland	CS	Men from the Finish part of the Seven Countries Study	70-89		Yes	94	241	16.4 (9.4)	23.2 (9.9)	54.4 (27.1)	74.4 (31.0)		
25	Muller et al, 2005 [14]	Netherlands	CS	Independently living men	60	26.3	No	96	304	15.7 (4.5)	19.4 (5.3)	34.7 (12.4)	42.4 (14.6)	321.1 (90.7)	364.7 (98.2)
26	Nuver et al, 2005 [56]	Netherlands	CS	Testicular cancer patients treated with chemotherapy	38	25.4	No	22	62	18.3 (5.0)	20.0 (8.0)	20.0 (6.0)	26.0 (9.0)	442.0 (115.0)	495.0 (153.0)
27	Tong et al, 2005 [30] (1) [d]	China	CS	Men from the Hong Kong Diabetes Family Study without a family history of diabetes	44	24.7	Yes	30	98	15.8 (4.0)	18.4 (6.1)	27.1 (9.3)	30.8 (13.2)		
	Tong et al, 2005 [30] (2) [d]	China	CS	Men from the Hong Kong Diabetes Family Study with a family history of diabetes	39	25.9	Yes	70	109	16.0 (3.7)	18.3 (5.6)	21.2 (8.6)	27.4 (14.4)		
28	Laaksonen et al, 2003 [16]	Finland	CS	Nondiabetic men from the Kuopio Ischaemic Heart Disease Risk Factor Study	53 [b]	26.8 [b]	No	345	1551	17.6 (6.8)	21.6 (7.4)	31.2 (13.0)	38.1 (15.6)	273.0 (79.0)	307.0 (75.0)

Abbreviations: BMI, body mass index; CC, case-control; CS, cross-sectional; LO, longitudinal; MetS, metabolic syndrome; MetS+, subjects with the metabolic syndrome; MetS–, subjects without the metabolic syndrome; No., number; SHBG, sex hormone-binding globulin.

[a] mean age/BMI of study sample based on weighted means of age/BMI of subjects with and without MetS.

[b] studies using multiple criteria to define the metabolic syndrome (Atlantis et al, 2009: (1) NCEP ATP III, (2) WHO, (3) IDF; Goncharov et al, 2008: (1) NCEP ATP III, (2) WHO, (3) IDF; Corona et al, 2007: (1) NCEP ATP III, (2) IDF; Guay et al, 2007: (1) NCEP ATP III modified, (2) WHO).

[c] free testosterone measured by radioimmunoassay

[d] mean differences reported for two separate populations (1) and (2).

[e] longitudinal study providing data on hormonal levels in subjects with and without MetS at baseline.

SI conversion factors: to convert testosterone (total testosterone/free testosterone) to ng/dL divide by 0.0347. To convert SHBG to µg/mL divide by 8.896.

6

Characteristics and quality of studies

Study characteristics are summarized in Tables 1-4. In men, 26 studies were cross-sectional, 5 were longitudinal and 1 study used a case-control design. In women, 19 studies were cross-sectional and 1 study used a case-control design. Nine studies included PCOS patients. Of these, 5 studies used the NICHD criteria to define PCOS [73], 3 studies used the Rotterdam criteria [74] and in 1 study PCOS criteria were not specified. Mean differences were derived from 45 studies and 17 studies provided RR estimates. Ten studies reported both measures of association and 4 studies provided mean differences for two populations separately. In analyses, these populations were considered as individual studies.

Most of the studies used the NCEP ATP III criteria to define MetS and some applied modified versions of criteria (Supplementary Table 1). Four studies reported mean differences for more than one MetS definition. From these studies, only the NCEP ATP III definition was considered in the pooled estimate of the mean difference. In univariable metaregression analyses, mean differences corresponding with all definitions were included. An overview of the study quality and methods of FT measurement is presented in Supplementary Tables 2 and 3.

Table 2. Characteristics of studies reporting on TT, SHBG and/or FT levels in women with and without MetS.

| Nr. | Source | Country | Study design | Participants | Mean age | Mean BMI | Adjusted for age | No. | | Mean (SD), nmol/L | | | | Mean (SD), pmol/L | |
| | | | | | | | | | | Total Testosterone | | SHBG | | Free Testosterone | |
								MetS+	MetS-	MetS+	MetS-	MetS+	MetS-	MetS+	MetS-
1	Alemzadeh et al, 2010 [57]	U.S.A.	CS	Obese adolescent girls with PCOS	16	36,2	No	35	68	1.14 (0.51)	1.07 (0.6)	49.4 (24.6)	57.0 (26.2)	48.6 (17.7)	38.5 (15.9)
2	Healy et al, 2010 [58]	Ireland	CS	Postmenopausal women with newly diagnosed breast cancer	68	28.3	No	42	63						40.0 (15.0)
3	de Oya et al, 2010 [28][c]	Spain	CS	Adolescent girls	14	21.8	No	4	424			24.6 (34.9)	64.6 (34.9)		
4	de Sousa et al, 2010 [60]	Germany	CS	Obese postmenarcheal adolescent girls	15	32.6	No	48	112	1.8 (0.7)	1.5 (0.7)	19.1 (7.9)	37.9 (8.5)	49.0 (7.0)	40.0 (15.0)
5	Ni et al, 2009 [59]	China	CS	Women with PCOS	27	21.9	No	97	481	2.1 (0.8)	2.2 (0.9)	27.8 (25.6)	55.4 (38.7)	152.7 (97.7)	111.1 (72.0)
6	Janssen et al, 2008 [61]	U.S.A.	CS	Women from the SWAN study at time of their final menstruation period	51	26.9	No	130	819	1.5 (0.6)	1.3 (0.6)	34.1 (19.4)	45.0 (24.2)		
7	Maggio et al, 2007 [15]	Italy	CS	Women from the InCHIANTI Study 65 years and older	76	27.6[a]	No	145	367	2.3 (1.1)	2.1 (0.9)	97.5 (51.6)	131.2 (66.9)		
8	Park et al, 2007 [62]	Korea	CS	Women with PCOS	26	23.6	No	16	97	2.3 (0.9)	2.4 (1.1)	18.8 (8.9)	49.6 (40.6)	9.0 (2.8)[b]	5.9 (3.1)[b]
9	Coviello et al, 2006 [63]	U.S.A.	CS	Postmenarcheal adolescent girls with PCOS	17	32.0	No	18	31	2.8 (0.8)	2.5 (0.9)	33.0 (13.0)	77.0 (53.0)		
10	Ehrmann et al, 2006 [12]	U.S.A.	CS	Nondiabetic PCOS women who participated in a large multicenter national trial	28[a]	36.0[a]	No	123	245	2.2 (1.2)	2.2 (1.1)	32.8 (15.5)	43.8 (21.9)	41.8 (17.7)	37.8 (20.4)
11	Leibel et al, 2006 [19]	U.S.A.	CS	Postmenarcheal adolescent girls with PCOS	16	32.4[a]	No	10	26			8.4 (6.3)	15.4 (9.6)	90.2 (35.7)	67.0 (23.9)
12	Pasanisi et al, 2006 [64]	Italy	CS	Postmenopausal women operated for breast cancer	57		No	16	94	1.7 (0.5)	1.4 (0.5)	46.3 (28.1)	67.8 (29.8)		
13	Weinberg et al, 2006 [32]	U.S.A.	CS	Postmenopausal women from the Women's Health Study (WHS)	65[a]	26.2[a]	Yes	108	104	0.8 (0.6)	0.6 (0.4)	32.6 (29.2)	55.8 (17.3)		
14	Apridonidze et al, 2005 [65]	U.S.A.	CS	Women with PCOS	30[a]	36.1[a]	No	46	60	2.5 (1.0)	2.1 (1.0)	26.2 (31.5)	36.5 (19.8)	55.9 (26.3)[b]	37.1 (28.1)[b]
15	Dokras et al, 2005 [66]	U.S.A.	CS	Women with PCOS	28	-	No	45	84	1.9 (1.0)	1.9 (1.2)	20.0 (11.1)	32.0 (31.5)	27.4 (15.2)	27.1 (26.0)
16	Golden et al, 2004 [67](1)[c]	U.S.A.	CS	Postmenopausal women from the ARIC study with minimal carotid atherosclerosis	62[a]	27.4[a]	No	60	121	0.9 (0.8)	0.7 (0.6)				
	Golden et al, 2004 [67](2)[c]	U.S.A.	CS	Postmenopausal women from the ARIC study with significant atherosclerosis	62[a]	27.9[a]	No	94	87	0.8 (0.6)	0.7 (0.5)				
17	Korhonen et al, 2003 [20]	Finland	CC	Premenopausal women from a community-based study	43[a]	28.3[a]	Yes	63	88	1.4 (0.5)	1.3 (0.6)	37.4 (22.2)	52.9 (25.3)	21.5 (9.5)	16.8 (6.6)

Abbreviations: ARIC, Atherosclerosis Risk in Communities Study; BMI, body mass index; CS, cross-sectional; LO, longitudinal; MetS, metabolic syndrome; MetS+, subjects with the metabolic syndrome; MetS-, subjects without the metabolic syndrome; No., number; PCOS, polycystic ovary syndrome; SHBG, sex hormone-binding globulin; SWAN, Study of Women's Health Across the Nation.

[a] mean age/BMI of study sample based on weighted means of age/BMI of subjects with and without MetS;

[b] free testosterone measured by radioimmunoassay

[c] mean differences reported for two separate populations (1) and (2).

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Table 3. Characteristics of studies presenting relative risk estimates for MetS according to TT, SHBG and/or FT levels in men.

Nr.	Source	Country	Study design	Mean follow-up	Participants	N	Variables adjusted for	RR estimate TT [a] (95% CI)	RR estimate SHBG [a] (95% CI)	RR estimate FT [a] (95% CI)
1	Akishita et al, 2010 [27]	Japan	CS	-	Nondiabetic men	194	Age	OR 0.26 (0.11-0.59)		
2	Li et al, 2010 [68]	U.S.A	CS	-	Men from the Third National Health and Nutrition Examination Survey (NHANES-II)	1226	Age, smoking, alcohol consumption, physical activity, race, CRP, LDL cholesterol, HOMA-IR	PR 0.52 (0.38-0.69)	PR 0.51 (0.34-0.79)	PR 0.87 (0.63-1.20)
3	Haring et al, 2009 [43][b]	Germany	LO	5.0 yr	Men from the Study of Health in Pomerania (SHIP) study	1004		RR 0.70 (0.59-0.83)		
4	Schneider et al, 2009 [26]	Germany	CS	-	Men from the Diabetes Cardiovascular Risk-Evaluation: Targets and Essential DATA for Commitment of Treatment (DETECT)	2719		OR 0.26 (0.21-0.32)		
5	Chubb et al, 2008 [10][b]	Australia	CS	-	Nondiabetic men from the Health in Men study	2052		OR 0.28 (0.22-0.36)	OR 0.21 (0.16-0.28)	
6	Emmelot-Vonk et al, 2008 [44][b]	Netherlands	CS	-	Nondiabetic men with low normal testosterone levels	222	Age, smoking, alcohol consumption	OR 0.45 (0.21-0.95)	OR 0.25 (0.11-0.56)	OR 2.15 (1.00-4.57)
7	Kupelian et al, 2008 [69]	U.S.A.	CS	-	Men from the Boston Area Community Health (BACH) survey	1885	Age, smoking, alcohol consumption, physical activity, ethnicity	OR 0.16 (0.10-0.27)	OR 0.13 (0.08-0.23)	OR 0.22 (0.13-0.37)
8	Rodriguez et al, 2007 [48][b]	U.S.A.	LO	5.8 yr	Men from the Baltimore Longitudinal Study of Aging	417	Age, BMI	HR 0.46 (0.25-0.84)	HR 0.30 (0.17-0.58)	
9	Kupelian et al, 2006 [18][b]	U.S.A.	LO	14.4 yr	Men from the Massachusetts Male Aging Study	950		RR 0.75 (0.55-0.97)	RR 0.50 (0.37-0.68)	RR 1.06 (0.81-1.41)
10	Muller et al, 2005 [14][b]	Netherlands	CS	-	Independently living men	400	Age, smoking, alcohol consumption, physical activity	OR 0.20 (0.10-0.38)	OR 0.17 (0.08-0.34)	OR 0.31 (0.15-0.63)
11	Tong et al, 2005 [30][b]	China	CS	-	Men from the Hong Kong Diabetes Family Study	307	Age, smoking, family history of diabetes, CRP (TT and SHBG), IGF-1 (SHBG only)	OR 0.25 (0.12-0.52)	OR 0.17 (0.08-0.38)	
12	Laaksonen et al, 2004 [25]	Finland	LO	11.0 yr	Nondiabetic men from the Kuopio Ischaemic Heart Disease Risk Factor Study	702	Age, BMI, smoking, alcohol consumption, presence of CVD, socioeconomic status	OR 0.43 (0.25-0.76)	OR 0.37 (0.21-0.64)	OR 0.56 (0.31-0.99)
13	Laaksonen et al, 2003 [16][b]	Finland	CS	-	Nondiabetic men from the Kuopio Ischaemic Heart Disease Risk Factor Study	1896	Age, BMI, smoking, alcohol consumption, presence of CVD, socioeconomic status	OR 0.52 (0.36-0.75)	OR 0.54 (0.37-0.77)	OR 0.58 (0.41-0.83)

Abbreviations: BMI, body mass index; CI, confidence interval; CS, cross-sectional; CVD, cardiovascular disease; CRP, C-reactive protein; FT, free testosterone; HR, hazard ratio; HOMA-IR, homeostasis model assessment of insulin resistance; IGF-1, insulin-like growth factor 1; LO, longitudinal; MetS, metabolic syndrome; OR, odds ratio; PR, prevalence ratio; RR, relative risk; SHBG, sex hormone-binding globulin; TT, total testosterone; yr, year; -, not applicable.

[a] relative risk estimates of MetS comparing highest versus lowest tertiles of TT, SHBG and FT. [b] studies reporting both measures of association (relative risk estimates and mean differences).

Table 4. Characteristics of studies presenting relative risk estimates for MetS according to TT and/or SHBG levels in women.

Nr.	Source	Country	Study design	Participants	N	Variables adjusted for	RR estimate TT [a] (95% CI)	RR estimate SHBG [a] (95% CI)	RR estimate FT [a] (95% CI)
1	Patel et al, 2009 [70]	U.S.A.	CS	Nondiabetic women 65 years and older from the Cardiovascular Health Study (CHS)	301	Age, race, estrogen use and number of ovaries removed	OR 2.49 (1.30-4.76)		OR 1.24 (0. 67-2.32)
2	Maggio et al, 2007 [15 b]	Italy	CS	Women from the InCHIANTI Study 65 years and older	589		OR 1.40 (0.91-2.16)	OR 0.31 (0.19-0.49)	
3	Chen et al, 2006 [71]	Taiwan	CS	Women with PCOS not undergoing treatment	106	Age		OR 0.10 (0.01-0.89)	
4	Weinberg et al, 2006 [32 b]	U.S.A.	CS	Postmenopausal women from the Women's Health Study	212	Age, BMI, smoking, alcohol consumption, physical activity, and the presence of CVD at follow-up	OR 3.20 (1.40-7.30)	OR 0.14 (0.05-0.37)	
5	Santoro et al, 2005 [72]	U.S.A.	CS	Nondiabetic women from the Study of Women's Health Across the Nation (SWAN)	2961	Age, smoking, ethnicity, site	OR 1.25 (1.12-1.40)	OR 0.36 (0.29-0.43)	

Abbreviations: BMI, body mass index; CI, confidence interval; CS, cross-sectional; CVD, cardiovascular disease; FT, free testosterone; MetS, metabolic syndrome; OR, odds ratio; PCOS, polycystic ovary syndrome; SHBG, sex hormone-binding globulin; SWAN, Study of Women's Health Across the Nation; TT, total testosterone; -, not applicable. [a] relative risk estimates of MetS comparing highest versus lowest tertiles of TT, SHBG and FT. [b] studies reporting both measures of association (relative risk estimates and mean differences).

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Total testosterone

Studies presenting TT levels in subjects with and without MetS included 14 319 men and 3904 women in total. Men with MetS had lower levels of TT (mean difference = -2.64 nmol/L; 95% CI, -2.95 to -2.32), whereas women with MetS had higher levels of TT (mean difference = 0.14 nmol/L; 95% CI, 0.07 - 0.20) compared with those without (Figure 2A). In multivariable metaregression analyses this sex-dependent association remained significant (P < 0.001) after adjusting for study level differences in age, BMI, diabetes status and control for age.

In men, there was evidence of substantial between-study heterogeneity (I^2 = 89.1%), which was not explained by BMI, diabetes status, control for age or study design. However, in stratified and metaregression analyses TT mean differences were smaller in studies applying NCEP ATP III criteria (*P* = 0.03) (Table 5). Furthermore, metaregression analyses including age as continuous term showed a trend (*P* = 0.08) towards a stronger association in younger men. In women, no significant heterogeneity was observed (I^2 = 28.5%), though the association between TT and MetS appeared to be stronger in women without PCOS (*P* = 0.02) (Table 3). In sensitivity analyses, differences in study quality did not influence associations between TT and MetS in both men and women.

Studies incorporating RR estimates for TT comprised 13 974 men and 4063 women. Pooled analyses of RR estimates showed a reduced MetS risk with increasing TT levels (RR estimate highest versus lowest TT tertile = 0.38; 95% CI, 0.28, 0.50) (Figure 3A). An opposite association was observed in women (RR estimate highest versus lowest TT tertile = 1.68; 95% CI, 1.15, 2.45). Although the number of studies on which the pooled RR estimates are based are small, these data are consistent with a sex difference in the association of MetS with TT. Substantial heterogeneity was observed among RR estimates in both men (I^2 = 88.5%) and women (I^2 = 66.6%). In men, analyses stratified for study design showed that associations were stronger in cross-sectional studies (RR estimate highest versus lowest TT tertile = 0.31; 95% CI, 0.23, 0.41) than longitudinal studies (RR estimate highest versus lowest TT tertile = 0.64; 95% CI, 0.53, 0.79). In women, no sources of heterogeneity could be identified.

Funnel plots did not disclose publication bias among studies reporting mean differences (men: Egger's test = -1.21; 95% CI, -2.49 to 0.06 and women: Egger's test = -0.09; 95% CI, -1.88 to 1.70) and RR estimates (men: Egger's test = -2.03; 95% CI, -5.81 to 1.75 and women: Egger's test = 2.05; 95% CI, -0.60 to 4.70) (Supplementary Figures 1A and 1B). Although there was no strong evidence for publication bias in RR estimates, visual inspection of the funnel plot showed some

asymmetry in women. Because of the small number of studies (N = 4), this plot was difficult to interpret.

Figure 2A. Random effects pooled mean difference of TT levels between subjects with and without MetS, stratified by sex.

Source	TT mean difference (95% CI) (nmol/L)
Men	
Haring et al, 2010	-2.20 (-2.82, -1.58)
Atlantis et al, 2009	-3.10 (-3.71, -2.49)
Coviello et al, 2009 (1)	-2.85 (-4.17, -1.53)
Coviello et al, 2009 (2)	-3.89 (-7.15, -0.63)
Demir et al, 2009	-1.97 (-3.66, -0.29)
Chubb et al, 2008	-2.70 (-3.17, -2.23)
Emmelot-Vonk et al, 2008	-0.78 (-1.46, -0.10)
Goncharov et al, 2008	-5.10 (-8.04, -2.16)
Laughlin et al, 2008	-2.32 (-2.85, -1.80)
Suetomi et al, 2008	-0.69 (-3.14, 1.75)
Yeh et al, 2008	-3.82 (-6.16, -1.49)
Corona et al, 2007	-3.80 (-4.62, -2.98)
Rodriguez et al, 2007	-2.14 (-2.19, -2.10)
Tang et al, 2007	-2.88 (-3.00, -2.76)
Chen et al, 2006	-2.10 (-3.85, -0.35)
Gannage-Yared et al, 2006	-1.79 (-3.07, -0.51)
Kaplan et al, 2006	-2.12 (-2.81, -1.42)
Kupelian et al, 2006	-2.80 (-3.90, -1.70)
Maggio et al, 2006	-1.18 (-2.37, 0.01)
Robeva et al, 2006	-9.43 (-14.59, -4.27)
Kalme et al, 2005	-6.80 (-9.08, -4.53)
Muller et al, 2005	-3.78 (-4.86, -2.70)
Nuver et al, 2005	-1.75 (-4.64, 1.14)
Tong et al, 2005 (1)	-2.60 (-4.47, -0.73)
Tong et al, 2005 (2)	-2.30 (-3.67, -0.93)
Laaksonen et al, 2003	-4.00 (-4.81, -3.19)
Overall (I-squared = 89.1%, p = 0.000)	-2.64 (-2.95, -2.32)
Women	
Healy et al, 2010	0.07 (-0.14, 0.28)
Ni et al, 2009	-0.10 (-0.28, 0.08)
de Soussa et al, 2009	0.30 (0.06, 0.54)
Janssen et al, 2008	0.17 (0.06, 0.28)
Maggio et al, 2007	0.17 (-0.03, 0.37)
Park et al, 2007	-0.06 (-0.55, 0.43)
Coviello et al, 2006	0.28 (-0.20, 0.76)
Ehrmann et al, 2006	-0.03 (-0.28, 0.21)
Pasanisi et al, 2006	0.28 (0.00, 0.55)
Weinberg et al, 2006	0.24 (0.10, 0.37)
Apridonidze et al, 2005	0.42 (0.02, 0.81)
Dokras et al, 2005	-0.03 (-0.43, 0.36)
Golden et al, 2004 (1)	0.25 (0.03, 0.47)
Golden et al, 2004 (2)	0.08 (-0.08, 0.24)
Korhonen et al, 2003	0.10 (-0.07, 0.27)
Overall (I-squared = 28.5%, p = 0.143)	0.14 (0.07, 0.20)

NOTE: Weights are from random effects analysis

-8 -7 -6 -5 -4 -3 -2 -1 0 1 2

Abbreviations: CI, confidence interval; MetS, metabolic syndrome; TT, total testosterone. Negative values indicate lower TT levels in subjects with MetS; positive values indicate higher TT levels in subjects with MetS. Sizes of squares represent the weight of each study.
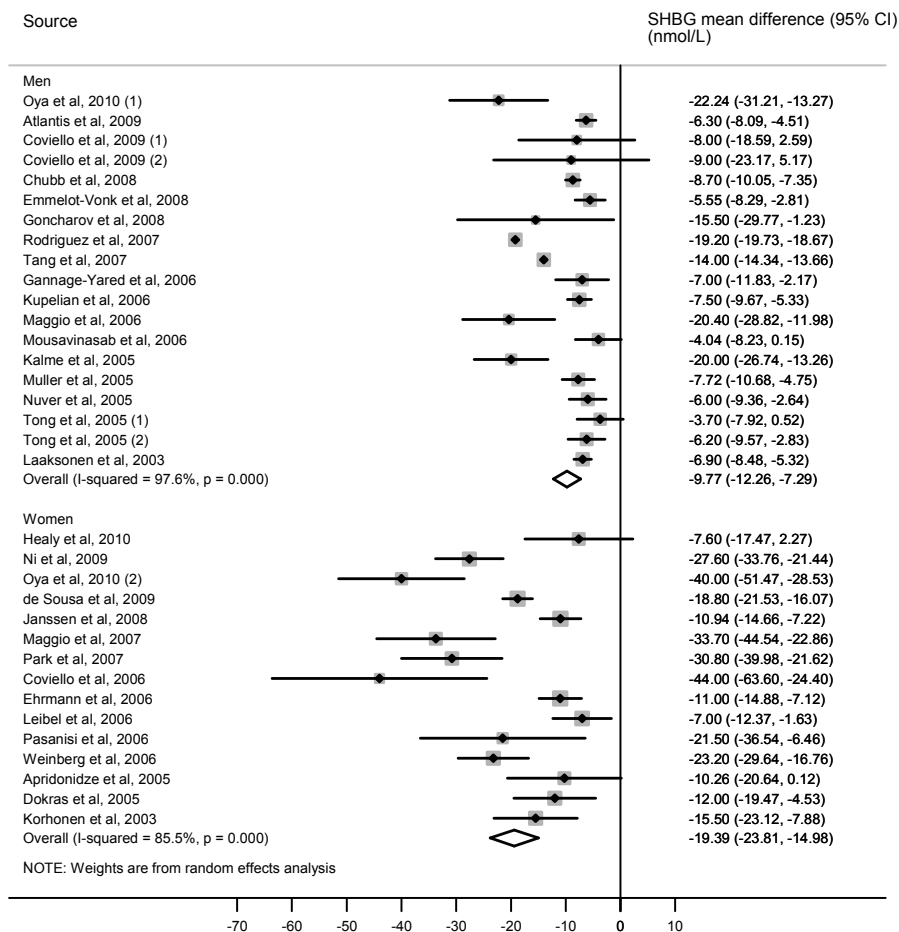
Sex hormone-binding globulin

Studies reporting SHBG levels in subjects with and without MetS comprised 10 537 men and 4006 women. In both sexes, SHBG levels were lower in subjects with MetS (men: mean difference = -9.77 nmol/L; 95% CI, -12.26 to -7.29; women: mean difference = -19.39 nmol/L; 95% CI, -23.81 to -14.98) than in those without (Figure 2B). Overall, the inverse association between SHBG and MetS was stronger in women than men (P = 0.003). In multivariable metaregression analyses this sex difference remained significant after adjusting for study level differences in age, BMI, diabetes status and control for age.

Substantial between-study heterogeneity was observed in both men (I^2 = 97.6%) and women (I^2 = 85.5%). In men, this heterogeneity was partly explained by differences in age. Univariable metaregression analyses including age as a dichotomous term showed that the association between SHBG and MetS tended to be more pronounced in men aged 55 years and older (P = 0.08). This effect of age, however, disappeared when age was entered as a continuous term. In women, the association appeared to be stronger in those with a BMI < 25 kg/m^2 (Table 5). This effect of BMI was also observed in metaregression analyses including BMI as a continuous term (P = 0.04) Sensitivity analyses showed no effect of study quality on the associations between SHBG and MetS in both men and women.

Studies reporting RR estimates for SHBG comprised 10 057 men and 3868 women. Analysis of RR estimates showed similar inverse associations between SHBG and MetS risk in men (RR estimate highest versus lowest SHBG tertile = 0.29; 95% CI, 0.21 - 0.41) and women (RR estimate for highest versus lowest SHBG tertile = 0.30; 95% CI, 0.21 - 0.42) (Figure 3B), without evidence of a sex difference (P = 0.74). There was heterogeneity among RR estimates in men (I^2 = 80.7%) which remained unexplained in stratified and metaregression analyses. In women, no substantial heterogeneity was observed (I^2 = 37.1%).

There were indications for publication bias among studies reporting mean differences in men (Egger's test = 3.73; 95% CI, 0.18-7.27). Funnel plots showed asymmetry and pointed to missing studies in the lower left-handed corner, indicating a lack of studies reporting large SHBG differences with high precision (Supplementary Figure 1A). In women, no publication bias was observed (Egger's test = -2.03; 95% CI, -4.92 to 0.86). Egger's test did not detect publication bias among studies reporting RR estimates (men: Egger's test = -1.87; 95% CI, -6.50 to 2.88, women: Egger's test = -1.57; 95% CI, -3.35 to 0.19), but in women the funnel plot showed some asymmetry (Supplementary Figure 1B).

Figure 2B. Random effects pooled mean difference of SHBG levels between subjects with and without MetS, stratified by sex.

Source	SHBG mean difference (95% CI) (nmol/L)
Men	
Oya et al, 2010 (1)	-22.24 (-31.21, -13.27)
Atlantis et al, 2009	-6.30 (-8.09, -4.51)
Coviello et al, 2009 (1)	-8.00 (-18.59, 2.59)
Coviello et al, 2009 (2)	-9.00 (-23.17, 5.17)
Chubb et al, 2008	-8.70 (-10.05, -7.35)
Emmelot-Vonk et al, 2008	-5.55 (-8.29, -2.81)
Goncharov et al, 2008	-15.50 (-29.77, -1.23)
Rodriguez et al, 2007	-19.20 (-19.73, -18.67)
Tang et al, 2007	-14.00 (-14.34, -13.66)
Gannage-Yared et al, 2006	-7.00 (-11.83, -2.17)
Kupelian et al, 2006	-7.50 (-9.67, -5.33)
Maggio et al, 2006	-20.40 (-28.82, -11.98)
Mousavinasab al, 2006	-4.04 (-8.23, 0.15)
Kalme et al, 2005	-20.00 (-26.74, -13.26)
Muller et al, 2005	-7.72 (-10.68, -4.75)
Nuver et al, 2005	-6.00 (-9.36, -2.64)
Tong et al, 2005 (1)	-3.70 (-7.92, 0.52)
Tong et al, 2005 (2)	-6.20 (-9.57, -2.83)
Laaksonen et al, 2003	-6.90 (-8.48, -5.32)
Overall (I-squared = 97.6%, p = 0.000)	-9.77 (-12.26, -7.29)
Women	
Healy et al, 2010	-7.60 (-17.47, 2.27)
Ni et al, 2009	-27.60 (-33.76, -21.44)
Oya et al, 2010 (2)	-40.00 (-51.47, -28.53)
de Sousa et al, 2009	-18.80 (-21.53, -16.07)
Janssen et al, 2008	-10.94 (-14.66, -7.22)
Maggio et al, 2007	-33.70 (-44.54, -22.86)
Park et al, 2007	-30.80 (-39.98, -21.62)
Coviello et al, 2006	-44.00 (-63.60, -24.40)
Ehrmann et al, 2006	-11.00 (-14.88, -7.12)
Leibel et al, 2006	-7.00 (-12.37, -1.63)
Pasanisi et al, 2006	-21.50 (-36.54, -6.46)
Weinberg et al, 2006	-23.20 (-29.64, -16.76)
Apridonidze et al, 2005	-10.26 (-20.64, 0.12)
Dokras et al, 2005	-12.00 (-19.47, -4.53)
Korhonen et al, 2003	-15.50 (-23.12, -7.88)
Overall (I-squared = 85.5%, p = 0.000)	-19.39 (-23.81, -14.98)

NOTE: Weights are from random effects analysis

Abbreviations: CI, confidence interval; MetS, metabolic syndrome; SHBG, sex hormone-binding globulin. Negative values indicate lower SHBG levels in subjects with MetS; positive values indicate higher SHBG levels in subjects with MetS. Sizes of squares represent the weight of each study.

Figure 2C. Random effects pooled mean difference of FT levels between subjects with and without MetS, stratified by sex.

Source | FT standardized mean difference (95% CI) (pmol/L)

Men
Kabatami et al, 2010 — -0.67 (-0.95, -0.40)
Chubb et al, 2008 — -0.18 (-0.28, -0.09)
Emmelot-Vonk et al, 2008 — 0.25 (-0.04, 0.55)
Goncharov et al, 2008 — -0.41 (-0.92, 0.11)
Suetomi et al, 2008 — -0.20 (-0.64, 0.23)
Corona et al, 2007 — -0.43 (-0.56, -0.31)
Guay et al, 2007 — -0.31 (-0.63, 0.01)
Tang et al, 2007 — -0.14 (-0.37, 0.08)
Kupelian et al, 2006 — -0.22 (-0.40, -0.05)
Maggio et al, 2006 — 0.25 (-0.00, 0.50)
Muller et al, 2005 — -0.45 (-0.68, -0.22)
Nuver et al, 2005 — -0.37 (-0.86, 0.12)
Laaksonen et al, 2003 — -0.45 (-0.57, -0.33)
Overall (I-squared = 79.9%, p = 0.000) — -0.26 (-0.39, -0.13)

Women
Alemzadeh et al, 2010 — 0.61 (0.19, 1.03)
Ni et al, 2009 — 0.54 (0.32, 0.76)
de Sousa et al, 2009 — 0.68 (0.34, 1.03)
Park et al, 2007 — 1.01 (0.47, 1.56)
Ehrmann et al, 2006 — 0.20 (-0.01, 0.42)
Leibel et al, 2006 — 0.84 (0.09, 1.60)
Apridonidze et al, 2005 — 0.69 (0.29, 1.08)
Dokras et al, 2005 — 0.02 (-0.35, 0.38)
Korhonen et al, 2003 — 0.59 (0.26, 0.92)
Overall (I-squared = 61.1%, p = 0.009) — 0.52 (0.33, 0.71)

NOTE: Weights are from random effects analysis

-1   -.5   0   .5   1   1.5   2

Abbreviations: CI, confidence interval; MetS, metabolic syndrome; FT, free testosterone. Negative values indicate lower FT levels in subjects with MetS; positive values indicate higher FT levels in subjects with MetS. Sizes of squares represent the weight of each study.
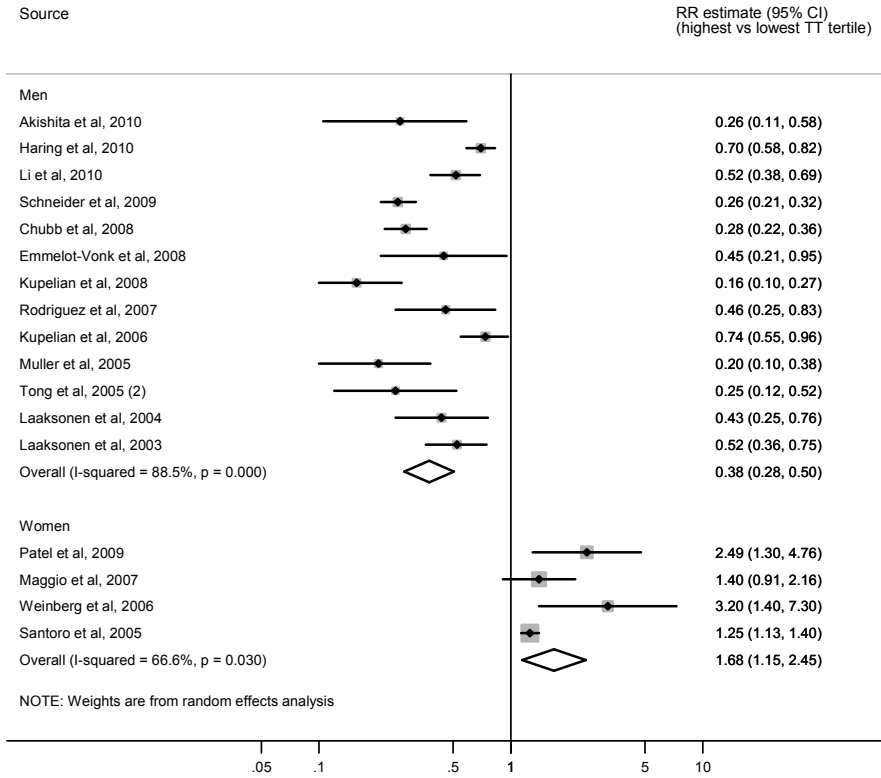
Free testosterone

Studies presenting FT levels in subjects with and without MetS included 8750 men and 1744 women in total. A sex difference was found (P = 0.004), such that women with MetS had higher FT levels (mean difference = 0.52; 95% CI, 0.33 - 0.71), while men with the metabolic syndrome had lower levels of FT than those without (mean difference = -0.26; 95% CI, -0.39 to -0.13) (Figure 2C). This sex-dependent association remained significant in multivariable analyses.

Substantial between-study heterogeneity was observed in both men (I^2 = 79.9%) and women (I^2 = 61.1%). In men, heterogeneity was partly explained by the different MetS criteria used across studies. As for TT, the inverse association with FT tended to be weaker among studies using NCEP ATP III criteria (P = 0.08) (Table 5). Furthermore, the association between MetS and FT differed according to the mean age of the study population (P = 0.01), with a stronger association being observed in younger men (Table 5). In women, no sources of heterogeneity were identified. In sensitivity analyses, exclusion of studies using RIA did not change the observed associations materially. Associations were also not affected by differences in study quality.

Studies reporting RR estimates for FT comprised 7281 men. Consistent with the findings for TT, high FT levels were associated with a reduced MetS risk, albeit not statistically significant (RR estimate highest versus lowest FT tertile = 0.64; 95% CI, 0.41 - 1.01) (Figure 3C). There was evidence of substantial between-study heterogeneity (I^2 = 86.4%), of which no sources could be identified. One study in women reported a RR estimate for FT (RR estimate highest versus lowest FT tertile = 1.24; 95% CI, 0.67 - 2.31).
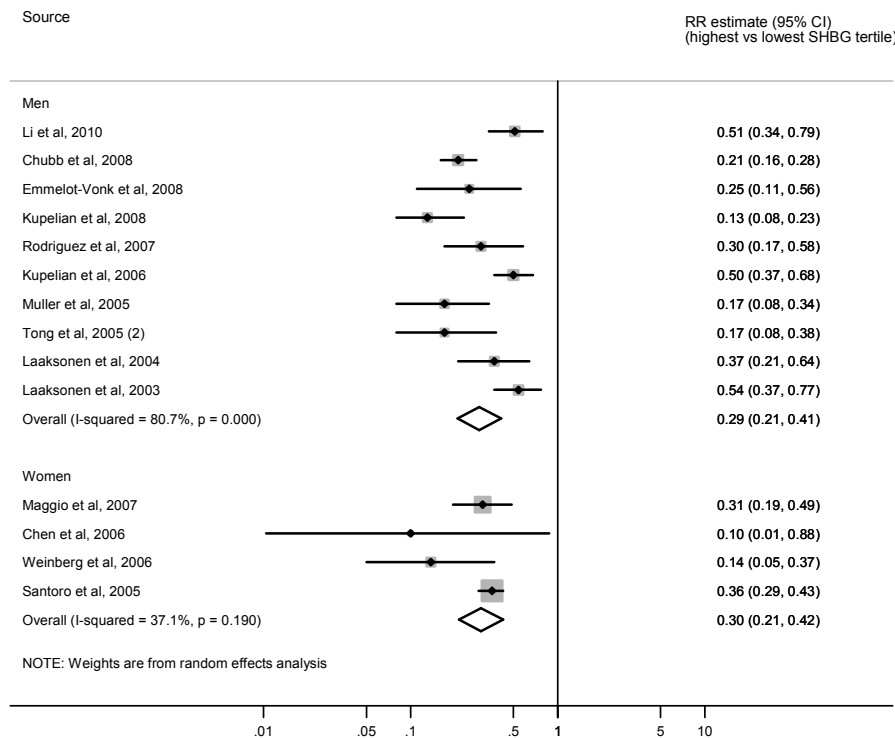
No publication bias was detected among studies providing FT mean differences in men (Egger's test = -1.19; 95% CI, -3.25 to 0.88) and RR estimates in men (Egger's test = -2.69; 95% CI, -10.55 to 5.16). In women, funnel plots disclosed publication bias among studies reporting mean differences (Egger's test = 2.36; 95% CI, 0.51 - 4.21), indicating a lack of small studies reporting small FT differences (Supplementary Figure 1A).

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Figure 3A. Random effects pooled relative risk estimate for MetS comparing highest versus lowest TT tertile, stratified by sex.


Source		RR estimate (95% CI) (highest vs lowest TT tertile)
Men		
Akishita et al, 2010		0.26 (0.11, 0.58)
Haring et al, 2010		0.70 (0.58, 0.82)
Li et al, 2010		0.52 (0.38, 0.69)
Schneider et al, 2009		0.26 (0.21, 0.32)
Chubb et al, 2008		0.28 (0.22, 0.36)
Emmelot-Vonk et al, 2008		0.45 (0.21, 0.95)
Kupelian et al, 2008		0.16 (0.10, 0.27)
Rodriguez et al, 2007		0.46 (0.25, 0.83)
Kupelian et al, 2006		0.74 (0.55, 0.96)
Muller et al, 2005		0.20 (0.10, 0.38)
Tong et al, 2005 (2)		0.25 (0.12, 0.52)
Laaksonen et al, 2004		0.43 (0.25, 0.76)
Laaksonen et al, 2003		0.52 (0.36, 0.75)
Overall (I-squared = 88.5%, p = 0.000)		0.38 (0.28, 0.50)
Women		
Patel et al, 2009		2.49 (1.30, 4.76)
Maggio et al, 2007		1.40 (0.91, 2.16)
Weinberg et al, 2006		3.20 (1.40, 7.30)
Santoro et al, 2005		1.25 (1.13, 1.40)
Overall (I-squared = 66.6%, p = 0.030)		1.68 (1.15, 2.45)

NOTE: Weights are from random effects analysis

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Abbreviations: CI, confidence interval; MetS, metabolic syndrome; TT, total testosterone. Sizes of squares represent the weight of each study.

Figure 3B. Random effects pooled relative risk estimate for MetS comparing highest versus lowest tertile of SHBG, stratified by sex.

Source		RR estimate (95% CI) (highest vs lowest SHBG tertile)
Men		
Li et al, 2010		0.51 (0.34, 0.79)
Chubb et al, 2008		0.21 (0.16, 0.28)
Emmelot-Vonk et al, 2008		0.25 (0.11, 0.56)
Kupelian et al, 2008		0.13 (0.08, 0.23)
Rodriguez et al, 2007		0.30 (0.17, 0.58)
Kupelian et al, 2006		0.50 (0.37, 0.68)
Muller et al, 2005		0.17 (0.08, 0.34)
Tong et al, 2005 (2)		0.17 (0.08, 0.38)
Laaksonen et al, 2004		0.37 (0.21, 0.64)
Laaksonen et al, 2003		0.54 (0.37, 0.77)
Overall (I-squared = 80.7%, p = 0.000)		0.29 (0.21, 0.41)
Women		
Maggio et al, 2007		0.31 (0.19, 0.49)
Chen et al, 2006		0.10 (0.01, 0.88)
Weinberg et al, 2006		0.14 (0.05, 0.37)
Santoro et al, 2005		0.36 (0.29, 0.43)
Overall (I-squared = 37.1%, p = 0.190)		0.30 (0.21, 0.42)

NOTE: Weights are from random effects analysis

Abbreviations: CI, confidence interval; MetS, metabolic syndrome; SHBG, sex hormone-binding globulin. Sizes of squares represent the weight of each study.

Figure 3C. Random effects pooled relative risk estimate for FT comparing highest versus lowest tertile of FT, stratified by sex.
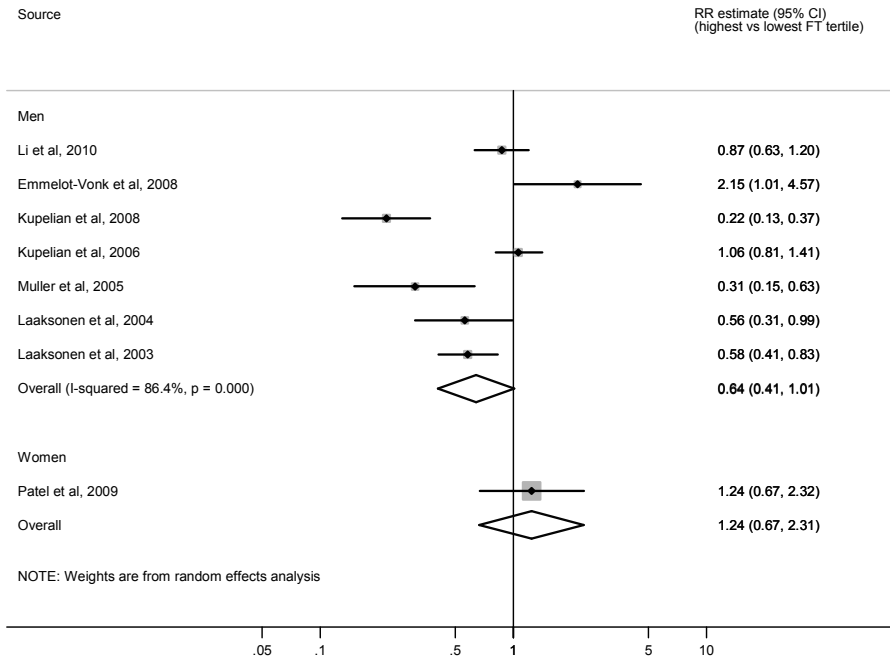
Abbreviations: CI, confidence interval; MetS, metabolic syndrome; FT, free testosterone. Sizes of squares represent the weight of each study.

Table 5. Mean differences of TT, SHBG and FT between subjects with and without the MetS.

	Men			Women		
	Studies No.	TT mean difference (95% CI) (nmol/L)	I² (%) and (P)	Studies No.	TT mean difference (95% CI) (nmol/L)	I² (%) and (P)
Overall random effects	26	-2.64 (-2.95; -2.32)	89.1 (<0.001)	15	0.14 (0.07-0.20)	28.5 (0.14)
Age						
- Age < 55 years	12	-3.03 (-3.60; -2.45)	65.7 (<0.001)	9	0.10 (0.00-0.21)	42.6 (0.08)
- Age ≥ 55 years	14	-2.38 (-2.78; -1.99)	92.6 (<0.001)	6	0.18 (0.10-0,25)	0.0 (0.55)
BMI						
- BMI < 25 kg/m²	4	-2.77 (-3.45; -2.08)	20.7 (0.29)	2	-0.10 (-0.26; 0.07)	0.0 (0.89)
- BMI ≥ 25 kg/m²	19	-2.42 (-2.78; -2.06)	73.7 (<0.001)	11	0.16 (0.11-0.22)	0.0 (0.47)
PCOS status (women)						
- PCOS (women)	-	-	-	6	0.03 (-0.13; 0.18)	28.2 (0.22)
- no PCOS (women)	-	-	-	9	0.17 (0.12-0.23)	0.0 (0.66)
MetS criteria †						
- NCEP ATP III	20	-2.49 (-2.81; -2.17)	89.8 (< 0.001)	12	0.16 (0.10-0.22)	0.0 (0.52)
- Other (WHO, IDF, EGIR)	10	-3.57 (-4.35; -2.80)	62.4 (0.004)	3	0.08 (-0.14; 0.30)	71.3 (0.03)
Control for age						
- adjusted for age	6	-2.87 (-3.68; -2.05)	79.5 (< 0.001)	2	0.18 (0.05-0.31)	36.3 (0.21)
- not adjusted for age	20	-2.62 (-3.00; -2.23)	76.0 (< 0.001)	13	0.13 (0.05-0.20)	30.2 (0.14)
Type 2 diabetes excluded						
- yes	7	-2,84 (-4.02; -1.66)	87.9 (< 0.001)	2	0.10 (-0.09; 0.29)	54.8 (0.14)
- no	19	-2,67 (-3.01; -2.32)	89.9 (< 0.001)	13	0.15 (0.07-0.22)	30.9 (0.14)
Study design						
- cross-sectional	24	-2.64 (-.2.97; -2.32)	89.7 (< 0.001)	14	0.14 (0.07-0.21)	32.7 (0.11)
- case-control	1	-9.43 (-14.59; -4.27)	NA	1	0.10 (-0.07; 0.27)	-
- longitudinal	1	-2.20 (-2.82; -1.58)	NA			

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Table 5. Continued.

	Studies No.	SHBG mean difference (95% CI) (nmol/L)	I^2 (%) and (P)	Studies No.	SHBG mean difference (95% CI) (nmol/L)	I^2 (%) and (P)
Overall random effects	19	-9.77 (-12.26; -7.29)	97.6 (< 0.001)	15	-19.39 (-23.81; -14.98)	85.5 (<0.001)
Age						
- Age < 55 years	10	-6.69 (-8.20; -5.19)	48.9 (0.04)	11	-18.73 (-23.73; -13.73)	87.3 (<0.001)
- Age ≥ 55 years	9	-12.00 (-15.13; -8.87)	98.2 (< 0.001)	4	-21.42 (-31.76; -11.09)	76.5 (0.01)
BMI						
- BMI < 25 kg/m^2	4	-10.36 (-17.50; 3.23)	93.7 (p<0.001)	3	-31.46 (-38.05; -24.86)	42.7 (0.17)
- BMI ≥ 25 kg/m^2	13	-9.52 (-13.96; -5.08)	98.0 (p<0.001)	11	-16.07 (-20.64; -11.51)	83.2 (< 0.001)
PCOS status (women)						
- PCOS (women)	-	-	-	7	-18.57 (-26.33; -10.82)	88.0 (<0.001)
- no PCOS (women)	-	-	-	8	-20.41 (-26.15; -14.67)	83.9 (< 0.001)
MetS criteria †						
- NCEP ATP III	13	-10.00 (-12.86; -7.13)	98.0 (<0.001)	11	-17.94 (-23.01; -12.88)	82.0 (<0.001)
- Other (WHO, IDF, EGIR)	9	-7.85 (-10.50; -5.21)	74.2 (<0.001)	4	-23.05 (-32.46; -13.63)	87.6 (<0.001)
Control for age						
- adjusted for age	4	-12.19 (-21.34; -3.05)	97.1 (<0.001)	2	-19.63 (-27.16; -12.11)	56.3 (0.13)
- not adjusted for age	15	-9.02 (-11.70; -6.33)	95.2 (<0.001)	13	-19.48 (-24.45; -14.51)	87.0 (<0.001)
Type 2 diabetes excluded						
- yes	6	-7.04 (-8.59; -5.49)	46.7 (0.10)	2	-10.97 (-13.65; -8.28)	0.0 (0.98)
- no	13	-11.03 (-13.89; -8.17)	97.7 (<0.001)	13	-21.19 (-26.32; -16.06)	83.6 (<0.001)
Study design						
- cross-sectional	18	-10.11 (-12.65; -7.57)	97.7 (<0.001)	14	-19.75 (-24.45; -15.05)	86.6 (< 0.001)
- case-control				1	-15.50 (-23.12; -7.88)	-
- longitudinal	1	-4.04 (-8.23; 0.15)	-			

Table 5. Continued.

	Studies No.	FT standardized mean difference (95% CI) (pmol/L)	I^2 (%) and (P)	Studies No.	FT standardized mean difference (95% CI) (pmol/L)	I^2 (%) and (P)
Overall random effects	13	-0.26 (-0.39; -0.13)	79.9 (<0.001)	9	0.52 (0.33-0.71)	61.1 (0.01)
Age						
- Age < 55 years	7	-0.41 (-0.51; -0.31)	32.9 (0.18)	9	0.52 (0.33-0.71)	61.1 (0.01)
- Age ≥ 55 years	6	-0.09 (-0.29; 0.11)	79.3 (<0.001)			
BMI						
- BMI < 25	3	-0.35 (-0.71; 0.02)	77.2 (0.01)	2	0.71 (0.27-1.15)	59.8 (0.12)
- BMI ≥ 25 kg/m²	9	-0.20 (-0.36; -0.04)	81.5 (<0.001)	6	0.54 (0.33-0.76)	51.2 (0.07)
PCOS status (women)						
- PCOS (women)	-	-	-	7	0.49 (0.26-0.73)	66.7 (0.01)
- no PCOS (women)	-	-	-	2	0.64 (0.40-0.88)	0 (0.71)
MetS criteria †						
- NCEP ATP III	11	-0.24 (-0.38; -0,09)	80.0 (<0.001)	7	0.51 (0.25-0.76)	66.5 (0.01)
- Other (WHO, IDF)	6	-0.46 (-0.54; -0.38)	0.0 (0.69)	2	0.58 (0.40-0.77)	0.0 (0.49)
Control for age						
- adjusted for age				1	0.59 (0.26-0.92)	NA
- not adjusted for age	13	-0.26 (-0.39; -0.13)	79.9 (< 0.001)	8	0.52 (0.31-0.73)	64.9 (0.01)
Type 2 diabetes excluded						
- yes	5	-0.29 (-0.53; -0.05)	87.8 (<0.001)	1	0.20 (-0.01; 0.42)	NA
- no	8	-0.23 (-0.40; -0.07)	74.1 (<0.001)	8	0.58 (0.39-0.76)	45.8 (0.07)
Study design						
- cross-sectional	13	-0.26 (-0.39; -0.13)	79.9 (<0.001)	8	0.52 (0.31-0.73)	64.9 (0.01)
- case-control				1	0.59 (0.26-0.92)	NA
- longitudinal						
Method of FT assessment						
- Direct measurement	3	-0.47 (-0.64; -0.30)	38.0 (0.20)	6	0.57 (0.34-0.80)	59.9 (0.03)
- Algorithms	9	-0.18 (-0.34; -0.03)	81.7 (< 0.001)	3	0.44 (0.04-0.84)	75.1 (0.02)

Abbreviations: CI, confidence interval; BMI, body mass index; FT, free testosterone; MetS, metabolic syndrome; No., number; PCOS, polycystic ovary syndrome; SHBG, sex hormone-binding globulin; TT, total testosterone; -, not applicable.

Discussion

Results of this meta-analysis support the presence of a sex-dependent association between endogenous testosterone and MetS. TT levels were lower in men MetS, whilst they were higher in women with MetS. There was also some evidence for a sex-specific association between free testosterone and MetS with FT levels being lower in men with MetS, while being higher in women with MetS. Interestingly no sex specific association was observed for SHBG. In both sexes, MetS was associated with a decrease in SHBG levels. Although the mean difference in SHBG levels between those with and without MetS was larger in women, this sex difference was lost after taking potential confounders into account in pooled analyses of RR estimates.

Some limitations of our meta-analysis need to be considered while interpreting the findings. First of all, we could only partly explain between-study heterogeneity. In metaregression analyses we found that at least some of the heterogeneity in men was explained by differences in age, MetS criteria and study design. In older men the associations of TT and FT with MetS tended to be less pronounced. This effect of age has been reported previously [43] and may be attributed to the age-related decline in testosterone and the higher imprecision of hormone assays toward the lower end of the distribution. Associations of TT and FT with MetS were also weaker when NCEP ATP II criteria were used. These criteria differ from other criteria in degree of emphasis of the individual MetS components. While the NCEP ATP III criteria put equal emphasis on the five MetS components, other criteria assign greater value to a particular component: impaired glucose metabolism (WHO and EGIR) and presence of abdominal obesity (IDF). Therefore, this differential effect of MetS criteria suggests that abdominal obesity and impaired glucose metabolism are important mediators of the observed associations between testosterone and MetS in men. Furthermore, analyses stratified for study design showed stronger associations in cross-sectional studies. In women, the association between TT and MetS was weaker in PCOS patients. High baseline levels of testosterone in this specific patient population may result in lower interindividual variation and low power to detect an association. Metaregression analyses further showed that the association between SHBG and MetS was more pronounced in leaner women, suggesting that in obesity SHBG is only one of the contributing factors. Another potential source of between-heterogeneity in both men and women is the variety of methods used for measuring free testosterone levels [81,82]. FT values vary between different algorithms and FT measurements by RIA have been criticized due to a lack of accuracy [36]. However, sensitivity analyses showed that the use of RIA did not have a major impact on the association between FT

and MetS. In spite of material heterogeneity, we decided to pool the data from all studies. While pooling of heterogeneous studies may affect the validity of the pooled estimates, the results of individual studies were largely compatible with the pooled estimates and pointed in the same direction as the overall estimate.

Another concern is the presence of potential publication bias among studies reporting SHBG mean differences in men and FT mean differences in women. However, evaluation of this publication bias by the "trim and fill" method showed that imputation of missing studies did not significantly alter the observed associations of SHBG and FT with MetS. It is important to recognize that asymmetry is not necessarily the result of publication bias, but can also be caused by between-study heterogeneity. A final limitation is the major contribution of cross-sectional studies to our meta-analysis, which precludes us from drawing conclusions about the directionality of the observed associations. In men, findings from four longitudinal studies [18,25,43,48] support a causal role for testosterone in the MetS etiology. Experimental studies have demonstrated that testosterone has beneficial effect on glucose and fat metabolism in male rats [83-86]. Moreover, intervention studies in hypogonadal men show improvements in individual components [87,88] and even reversal of MetS following testosterone therapy [89,90]. However, associations in the opposite direction have been reported as well. In obese men, weight loss and maintenance cause an increase in testosterone and SHBG levels [91,92] and experimental data show suppressive effects of adiposity and insulin on testosterone production in men [93-95]. Furthermore, MetS has been associated with an increased risk of hypogonadism in middle aged men [96]. Hence, complex, bidirectional relationships between testosterone and MetS seem to be plausible. In women, evidence for a causal role of testosterone in MetS is limited. This is reflected by the lack of longitudinal studies in this meta-analysis. Nevertheless, some recent findings suggest that testosterone may be a risk factor in women as well. In a prospective study [97], low SHBG and high testosterone levels at baseline were found to be associated with an increased MetS risk. Furthermore, high testosterone levels have been associated with an increased risk of diabetes in postmenopausal women [98] and a decrease in insulin sensitivity in female rats [99]. On the other hand, metformin therapy and weight loss reduce androgen excess in women [100,101], while insulin stimulates the ovarian production of testosterone [102].

Since TT and SHBG are correlated, it is also unclear whether the observed associations between SHBG and MetS reflect an independent effect of SHBG. However, increasing evidence from epidemiological studies support the involvement of SHBG in MetS [10,25,97] and diabetes etiology [98,103,104]. Moreover, polymorphisms in the SHBG gene have recently been shown to affect not only SHBG levels but also type 2 diabetes

risks in men as well as in women [98,105], suggesting a potential causal role for SHBG in pathophysiological mechanisms.

Pooled estimates of our meta-analysis are comparable (regarding strength and direction) with those previously reported for type 2 diabetes by Ding et al. [106]. This once more suggests a predominant role for glucose metabolism in the associations of testosterone with MetS and further indicates that the sex-dependent role of testosterone is not restricted to type 2 diabetes, but also exist in preceding conditions such as MetS, and may even be found in earlier stages of disease. Although the exact mechanisms underlying the sex specific associations between testosterone and MetS are not completely understood, similar sex-specific effects of testosterone have been observed in animal models. Low testosterone levels following castration in male rats, for instance, have been linked to obesity, insulin resistance and dyslipidemia [84,107,108]. Whereas prenatal and postnatal administration of testosterone has adverse effects on various MetS components in female rats [109-111].

The lack of a sex specific association between SHBG and MetS is not fully understood. Nevertheless, recent findings from genetic studies [112,113] provide some explanation. In these studies, one particular SHBG SNP, rs1799941, was found to have no effect on total testosterone levels in women while raising testosterone levels in men. Based on these data, it has been hypothesized that women with genetically lower SHBG levels are exposed to proportionally more of the adverse effects of the biologically active unbound testosterone, such as increasing risk of MetS and diabetes in women. On the other hand, in men there is recent evidence that bound testosterone may be biologically active. If this is the case, then men with lower total testosterone due to lower SHBG will be exposed to less of the protective metabolic effects of androgens, despite similar levels of unbound or free testosterone and also experience higher risk of MetS and diabetes [105]. Thus similar 'genetic' levels of SHBG may affect MetS risk in men and women differently, by altering the levels of testosterone in a sex-specific manner. Further research is necessary to elucidate the role of SHBG in the pathophysiology of MetS and diabetes.

In conclusion, findings of this meta-analysis support the presence of a sex-dependent association between TT and MetS, with high endogenous TT lowering MetS risk in men, while increasing MetS risk in women. There are also indications for a sex difference in the association between FT and MetS. Higher SHBG levels are associated with a lower MetS risk in both men and women. Differences in age, BMI, MetS criteria, PCOS status and study design account for some of the variability observed. The comparability of our pooled estimates with those available for type 2 diabetes

suggests a major contribution of impaired glucose metabolism to the observed associations. To further clarify the causal nature of the observed associations, more large-scale longitudinal studies are required, in women in particular. However, longitudinal studies are not perfect as early disease processes before the actual diagnosis of MetS may influence the level of testosterone and SHBG as well. Therefore, additional tools, such as Mendelian randomization studies and intervention studies, are needed to establish causation.

6

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Supplementary Table 1A. Definitions of the metabolic syndrome used across studies including men.

Source	NCEP ATP III	NCEP ATP III modified	Modification	WHO	WHO modified	Modification	IDF	IDF modified	Modification	EGIR
Akishita et al, 2010 [27]								X	- waist circumference ≥ 85 cm	
Haring et al, 2010 [43]		X	- waist circumference ≥ 94 cm; - triglycerides ≥ 2.0 mmol/L; - fasting glucose ≥ 8.0 mmol/L							
Li et al, 2010 [68]		X	- fasting glucose ≥ 5.6 mmol/L							
Katabami et al, 2010 [39]		X	- waist circumference > 90 cm							
de Oya et al, 2010 (1) [28]								X	- waist circumference ≥ 90th percentile or adult cut-off if lower; - HDL cholesterol < 1.04 mmol/L (Criteria Zimmet et al, 2007)	
Atlantis et al, 2009 [40]		X	- fasting glucose ≥ 5.6 mmol/L					X		
Coviello et al, 2009 [41]	X									
Demir et al, 2009 [42]	X									
Chubb et al, 2008 [10]		X	- fasting glucose ≥ 5.6 mmol/L							
Emmelot-Vonk et al, 2008 [44]	X									
Goncharov et al, 2008 [45]				X						
Kupelian, 2008 [69]		X								
Laughlin et al, 2008 [31]		X								
Suetomi et al, 2008 [46]							X			
Yeh et al, 2008 [47]	X									
Corona et al, 2007 [17]	X									
Guay et al, 2007 [33]		X	- BMI > 28.8 kg/m²		X	- no microalbuminuria				
Rodriguez et al, 2007 [48]		X								
Tang et al, 2007 [49]		X	- waist circumference > 90 cm							
Chen et al, 2006 [29]	X									
Gannage-Yared et al, 2006 [50]		X	- fasting glucose ≥ 5.6 mmol/L							
Kaplan et al, 2006 [51]		X	- BMI ≥ 30 kg/m²							

Study				Definition
Kupelian et al, 2006 [18]	X			- self reported diabetes
Maggio et al, 2006 [52]	X			
Mousavinasab et al, 2006 [53]			X	
Robeva et al, 2006 [54]		X		- impaired glucose metabolism: fasting insulin in top of the 25% of the nondiabetic men or impaired glucose tolerance - no microalbuminuria
Kalme et al, 2005 [55]			X	
Muller et al, 2005 [14]	X			
Nuver et al, 2005 [56]	X			
Tong et al, 2005 [30]		X		- waist-to-hip ratio > 0.9 and/or BMI ≥ 25 kg/m^2 - impaired glucose metabolism: HOMA-IR - microalbuminuria: ACR ≥ 3.5 mg/mmol
Laaksonen et al, 2004 [25]	X			
Laaksonen et al, 2003 [16]		X		- impaired glucose metabolism: fasting insulin in top of the 25% of the nondiabetic men, impaired fasting glucose or diabetes - no microalbuminuria

Abbreviations: ACR, albumin: creatinine ratio; BMI, body mass index; EGIR, European Group for the study of Insulin Resistance [75]; HOMA-IR, homeostasis model assessment of insulin resistance; IDF, International Diabetes Federation [5]; NCEP ATP III, National Cholesterol Education Program (NCEP) Adult Treatment Panel III [3]; WHO, World Health Organization [4].

Supplementary Table 1B. Definitions of the metabolic syndrome used across studies including women.

Source	NCEP ATP III	NCEP ATP III modified	Modification	IDF	IDF modified	Modification
Alemzadeh et al, 2010 [57]		X	- age adjusted BMI > 95th percentile - age adjusted SBP or DBP > 90th percentile - age adjusted triglycerides > 90 th percentile - age adjusted HDL cholesterol < 5th percentile - fasting glucose > 5.6 mmol/L (Criteria Weiss et al, 2004)			
Healy et al, 2010 [58]				X		
Ni et al, 2009 [59]				X		
de Oya et al, 2010 (2) [28]					X	- waist circumference ≥ 90th percentile or adult cut-off if lower - HDL cholesterol < 1.04 mmol/L (Criteria Zimmet et al, 2007)
de Sousa et al, 2010 [60]					X	- waist circumference > 90th percentile - HDL cholesterol < 1.04 mmol/L if age < 16 years and < 1.30 in age ≥ 16 years. (Criteria Zimmet et al, 2007)
Patel et al, 2009 [70]		X	- fasting glucose ≥ 5.6 mmol/L			
Janssen et al, 2008 [61]		X	- fasting glucose ≥ 5.6 mmol/L			
Maggio et al, 2007 [15]	X					
Park et al, 2007 [62]		X	- waist circumference > 80 cm			
Chen et al, 2006 [71]		X	- waist circumference > 80 cm - fasting glucose ≥ 5.6 mmol/L			
Coviello et al, 2006 [63]		X	- age adjusted waist circumference ≥ 90th percentile - age and height adjusted SBP or DBP ≥ 90th percentile - age adjusted triglycerides ≥ 1.2 mmol/L - age adjusted HDL cholesterol ≤ 1.0 mmol/L - fasting glucose ≥ 6.1 mmol/L (Criteria Cook et al, 2003)			
Ehrmann et al, 2006 [12]	X					

Study			Criteria
Leibel et al, 2006 [19]		X	- waist circumference > 88 cm - age and height adjusted SBP or DBP > 90th percentile - triglycerides ≥ 1.2 mmol/L - HDL cholesterol ≤ 1.0 mmol/L - fasting glucose ≥ 5.6 mmol/L
Pasanisi et al, 2006 [64]	X		
Weinberg et al, 2006 [32]		X	- BMI > 26.7 kg/m² - self reported diabetes at baseline or follow-up
Apridonidze et al, 2005 [65]		X	- BMI > 32 kg/m²
Dokras et al, 2005 [66]		X	- BMI > 30 kg/m²
Santoro et al, 2005 [72]	X		
Golden et al, 2004 [67]		X	- HDL cholesterol < 1.0 mmol/L - fasting insulin ≥ 100 pmol/L or fasting glucose ≥ 6.1 mmol/L or type 2 diabetes
Korhonen et al, 2003 [20]	X		

Abbreviations: BMI, body mass index; Cook, Cook criteria (adapted NCEP ATP III criteria for adolescents) [76]; DBP, diastolic blood pressure; HDL, high-density lipoprotein; IDF, International Diabetes Federation [5]; NCEP ATP III, National Cholesterol Education Program (NCEP) Adult Treatment Panel III [3]; SBP, systolic blood pressure; criteria Weiss et al. 2004 [77]; criteria of Zimmet et al. [78]

Supplementary Table 2. Methods of FT assessment in studies relating free testosterone to the metabolic syndrome, men and women.

Source	Sex	Direct measurement			Algorithms		
		ELISA	Equilibrium dialysis	Radioimmunoassay	Vermeulen [a]	Nanjee-Wheeler [a]	Anderson [a]
Li et al, 2010 [68]	Men				X		
Katabami et al, 2010 [39]	Men			X			
Chubb et al, 2008 [10]	Men				X		
Emmelot-Vonk et al, 2008 [44]	Men				X		
Goncharov et al, 2008 [45]	Men				X		
Kupelian et al, 2008 [69]	Men				X		
Corona et al, 2007 [17]	Men			X			
Guay et al, 2007 [33]	Men			X			
Tang et al, 2007 [49]	Men					X	
Kupelian et al, 2006 [18]	Men				X		
Maggio et al, 2006 [52]	Men				X		
Muller et al, 2005 [14]	Men				X		
Nuver et al, 2005 [56]	Men				X		
Laaksonen et al, 2004 [25]	Men						X
Laaksonen et al, 2003 [16]	Men						X
Alemzadeh et al, 2010 [57]	Women		X				
de Sousa et al, 2010 [60]	Women				X		
Ni et al, 2009 [59]	Women	X					
Patel et al, 2009 [70]	Women		X				
Park et al, 2007 [62]	Women			X			
Ehrmann et al, 2006 [64]	Women		X				
Leibel et al, 2006 [19]	Women		X				
Apridonidze et al, 2005 [65]	Women		X				
Dokras et al, 2005 [66] b	Women						
Korhonen et al, 2003 [20]	Women						X

[a] Algorithms for free testosterone assessment: Vermeulen [36], Nanjee-Wheeler [79] and Anderson [80].
b Algorithm used for calculation of FT levels not reported.

Supplementary Table 3A. Quality assessment of studies including men.

Source	Population-based sample	Exclusion of subjects taking hormonal therapy	Use of fasting blood samples for assessment of MetS components	Blood sample collection in the morning for hormone assessment	Covariates assessed
MD studies					
Katabami et al, 2010 [39]	Yes	Unclear	Yes	Yes	None
de Oya et al, 2010 [28]	Yes	Unclear	Yes	Unclear	None
Atlantis et al, 2009 [40]	Yes	Unclear	Yes	Yes	None
Coviello et al, 2009 [41]	No	Unclear	Yes	Yes	None
Demir et al, 2009 [42]	No	Unclear	Yes	Yes	None
Chubb et al, 2008 [10]	Yes	Yes	Yes	Yes	None
Emmelot-Vonk et al, 2008 [44]	Yes	Yes	Yes	Yes	None
Goncharov et al, 2008 [45]	No	Yes	Yes	Yes	None
Laughlin et al, 2008 [31]	Yes	Unclear	Yes	Yes	Age
Suetomi et al, 2008 [46]	No	Unclear	Yes	Yes	None
Yeh et al, 2008 [47]	No	Unclear	Yes	Yes	None
Corona et al, 2007 [17]	No	Unclear	Yes	Yes	None
Guay et al, 2007 [33]	No	Unclear	Yes	Yes	None
Rodriguez et al, 2007 [48]	Yes	Unclear	Yes	Yes	Age
Tang et al, 2007 [49]	No	No	Yes	Unclear	None
Chen et al, 2006 [29]	Yes	Unclear	Yes	Unclear	None
Gannage-Yared et al, 2006 [50]	Unclear	Yes	Yes	Unclear	None
Kaplan et al, 2006 [51]	No	Unclear	Yes	Yes	None
Kupelian et al, 2006 [18]	Yes	Unclear	No	Yes	None
Maggio et al, 2006 [52]	Yes	Unclear	Yes	Yes	Age
Mousavinasab et al, 2006 [53]	No	Yes	Yes	Unclear	None
Robeva et al, 2006 [54]	No	Unclear	Yes	Yes	None
Kalme et al, 2005 [55]	Yes	Unclear	Yes	Yes	Age
Muller et al, 2005 [14]	Yes	No	Yes	Yes	None
Nuver et al, 2005 [56]	No	Yes	Yes	Yes	None
Tong et al, 2005 [30]	No	Yes	Yes	Yes	Age
Laaksonen et al, 2003 [16]	Yes	Unclear	Yes	Yes	None
RR estimate studies					
Akishita et al, 2010 [27]	Yes	Yes	Yes	Yes	Age
Haring et al, 2009 [43]	Yes	Yes	No	None	None

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Supplementary Table 3A. Continued.

Source	Population-based sample	Exclusion of subjects taking hormonal therapy	Use of fasting blood samples for assessment of MetS components	Blood sample collection in the morning for hormone assessment	Covariates assessed
Li et al, 2010 [68]	Yes	Unclear	Yes	Yes	Age, smoking, alcohol consumption, physical activity, race, LDL-cholesterol, CRP and HOMA-IR
Chubb et al, 2008 [10]	Yes	Yes	Yes	Yes	None
Emmelot-Vonk et al, 2008 [44]	Yes	Yes	Yes	Yes	Age, smoking, alcohol consumption
Kupelian et al, 2008 [69]	Yes	Unclear	No	Yes	Age, smoking, alcohol consumption, physical activity, ethnicity
Rodriguez et al, 2007 [48]	Yes	Unclear	Yes	Yes	Age, BMI
Kupelian et al, 2006 [18]	Yes	Unclear	No	Yes	None
Muller et al, 2005 [14]	Yes	No	Yes	Yes	Age, smoking, alcohol consumption, physical activity
Tong et al, 2005 [30]	No	Yes	Yes	Yes	Age, smoking, family history of diabetes, CRP, IFG-1
Laaksonen et al, 2004 [25]	Yes	Unclear	Yes	Yes	Age, BMI, smoking, alcohol consumption, presence of CVD, socioeconomic status
Laaksonen et al, 2003 [16]	Yes	Unclear	Yes	Yes	Age, BMI, smoking, alcohol consumption, presence of CVD, socioeconomic status

Abbreviations: MD, mean difference; RR, relative risk; BMI, body mass index; CVD, cardiovascular disease; CRP, C-reactive protein; HOMA-IR, homeostasis model assessment for insulin resistance; IFG-1, insulin-like growth factor-1; LDL, low-density lipoprotein.

Supplementary Table 3B. Quality assessment of studies including women.

Source	Population-based sample	Exclusion of subjects taking hormonal therapy reported	Use of fasting blood samples for assessment of MetS components	Covariates assessed
MD studies				
Alemzadeh et al, 2010 [57]	No	Yes	Yes	None
Healy et al, 2010 [58]	No	No	Yes	None
Ni et al, 2009 [59]	No	Unclear	Yes	None
de Oya et al, 2010 [28]	Yes	Unclear	Yes	None
de Sousa et al, 2010 [60]	No	Yes	Yes	None
Janssen et all, 2008 [61]	Yes	Yes	Yes	None
Maggio et al, 2007 [15]	Yes	Yes	Yes	Age
Park et al, 2007 [62]	No	Unclear	Yes	None
Coviello et al, 2006 [63]	No	Yes	Yes	None
Ehrmann et al, 2006 [12]	No	Yes	Yes	None
Leibel et al, 2006 [19]	No	Yes	Yes	None
Pasanisi et al, 2006 [64]	No	No	Yes	None
Weinberg et al, 2006 [32]	Yes	Yes	No	Age
Apridonidze et al, 2005 [65]	No	Yes	Yes	None
Dokras et al, 2005 [66]	No	No	Yes	None
Golden et al, 2004 (1) [67]	No	Yes	Yes	None
Golden et al, 2004 (2) [67]	No	Yes	Yes	None
Korhonen et al, 2003 [20]	Yes	Unclear	Yes	None
RR estimate studies				
Patel et al, 2009 [70]	Yes	No	Yes	Age, race, estrogen use, bilateral ovarian removal
Maggio et al, 2007 [15]	Yes	Yes	Yes	None
Chen et al, 2006 [71]	No	Yes	Yes	Age
Weinberg et al, 2006 [32]	Yes	Yes	No	Age, BMI, smoking, alcohol consumption, physical activity and presence of CVD at follow-up
Santoro et al, 2005 [72]	Yes	Yes	Yes	Age, smoking, ethnicity, site

Abbreviations: MD, mean difference; RR, relative risk; BMI, body mass index; CVD, cardiovascular disease; MetS, metabolic syndrome.

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Supplementary Figure 1A. Funnel plots of studies reporting TT, SHBG and FT mean differences.
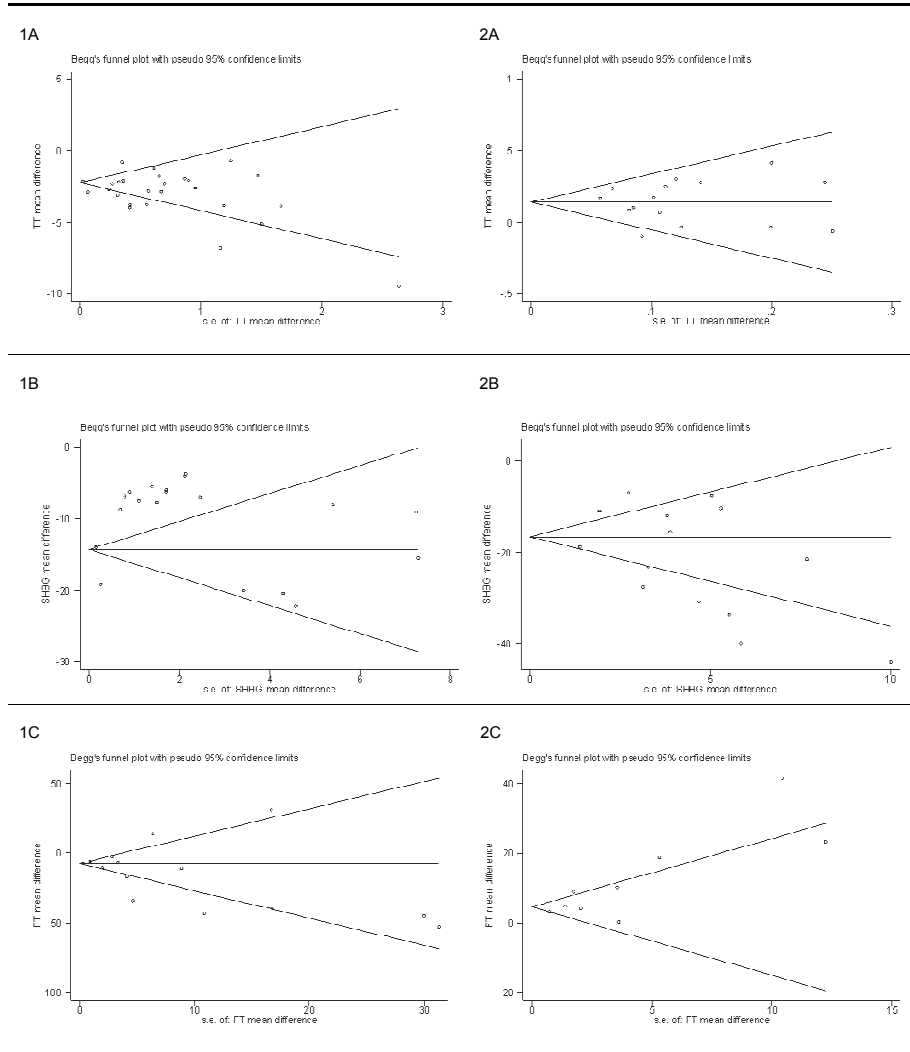
1 = men; 2 = women; A = TT mean difference, B = SHBG mean difference, C = FT mean difference.

Supplementary Figure 1B. Funnel plots of studies reporting TT, SHBG and FT relative risk estimates.
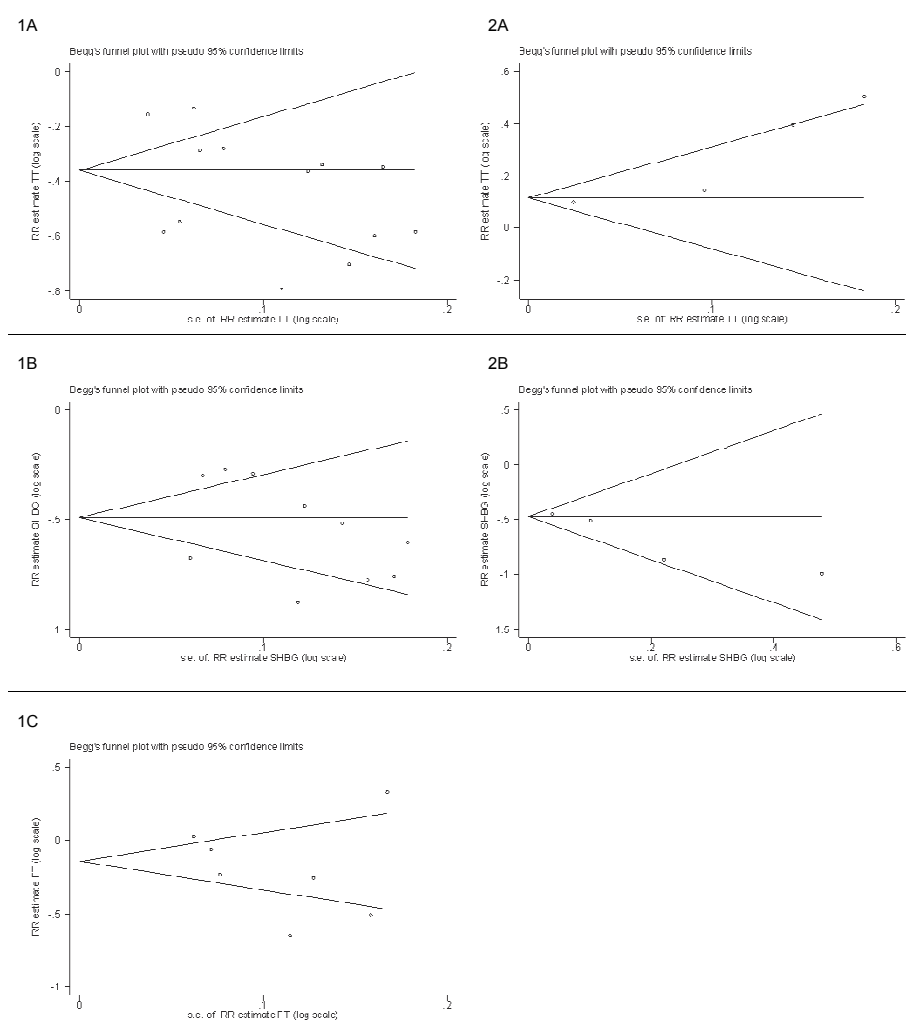
1 = men; 2 = women; A = relative risk estimate TT, B = relative risk estimate SHBG, C = relative risk estimate FT

Chapter 7

**Testosterone, SHBG and the metabolic syndrome in men:
an individual participant data meta-analysis**

Abstract

Background: In men, low testosterone and sex hormone binding globulin (SHBG) concentrations been associated with the metabolic syndrome (MetS), but the reported strength of association varies considerably. We aimed to investigate whether associations of testosterone and SHBG with MetS differ across specific subgroups (according to age and BMI) and individual MetS components.

Methods: Individual participant data meta-analysis of 20 observational studies conducted up to January 2011. Mixed effects models were used to analyse the cross-sectional and prospective associations of total testosterone (TT), SHBG and free testosterone (free T) with MetS and its individual components. Multivariable adjusted odds ratios (ORs) and hazard ratios (HRs) were calculated and effect modification by age and BMI was explored.

Results: Men with low concentrations of TT, SHBG or FT were more likely to have prevalent MetS (ORs per quartile decrease were 1.69 (95% CI 1.60-1.77), 1.73 (95% CI 1.62-1.85) and 1.46 (95% CI 1.36-1.57) for TT, SHBG and FT respectively) and incident MetS (HRs per quartile decrease were 1.25 (95% CI 1.16-1.36), 1.44 (95% 1.30-1.60) and 1.14 (95% 1.01-1.28) for TT, SHBG and FT respectively). Overall, the magnitude of associations was largest in non-overweight men and varied across individual components: stronger associations were observed with hypertriglyceridemia, abdominal obesity and hyperglycaemia and associations were weakest for hypertension.

Conclusions: Associations of testosterone and SHBG with MetS appear to depend on BMI and individual MetS components. These findings provide more insight into the pathophysiological mechanisms linking low testosterone and SHBG concentrations to MetS.

Introduction

The metabolic syndrome (MetS) affects approximately 25% of the adult population [1] and its prevalence is increasing worldwide [2-4]. MetS is associated with a twofold increased risk of cardiovascular disease (CVD) and a nearly fivefold increased risk of type 2 diabetes [5,6]. Given its major public health impact, there is an urgent need for a better understanding of the underlying mechanisms of MetS, in particular factors driving and influencing its pathophysiology.

A large number of epidemiological studies have linked low concentrations of total testosterone (TT) and its carrier protein, sex hormone-binding globulin (SHBG), to MetS in men, both cross-sectionally [7-11] and prospectively [12-15]. Despite the clear link between testosterone, SHBG and MetS, the exact nature of the observed associations remains uncertain, given the high variability in the strength of associations reported. This between-study heterogeneity can be partially explained by incomparability in study design (i.e. with regard to MetS criteria, hormone assays and sample size), but also by differences in population structure. Recent evidence suggests that associations may differ according to age and BMI, as weaker associations have been reported in older [12] and obese [14] men. Strength of associations may also vary across individual MetS components. Cross-sectionally, stronger associations have been reported for abdominal obesity and hypertriglyceridemia [7-9,16], but conflicting data exist for the other MetS components [7-9,16] and no studies so far have examined these associations prospectively.

We previously re-examined the observational data on testosterone, SHBG and MetS in a literature-based meta-analysis [17], but analyses for specific subgroups and MetS components were hampered by the absence of individual data. In addition, individual studies were largely heterogeneous with regard to MetS criteria and methods used for free testosterone (FT) estimation and confounder adjustment. To conduct a more comprehensive and powerful assessment of the associations of testosterone and SHBG with MetS, we pooled the original raw data of observational studies. Such a meta-analysis of individual participant data provides a unique opportunity to 1) examine associations of testosterone and SHBG with MetS in a uniform way; 2) produce estimates for specific subgroups according to age and body mass index (BMI) and 3) determine the MetS components through which associations with testosterone and SHBG are primarily mediated. In this article, we present the findings of this collaborative project.

Methods

Identification of studies
Studies were eligible for inclusion if they had data on TT and/or SHBG in combination with MetS in men using a cross-sectional or prospective design. Most studies were identified in previously published meta-analyses [17,18]; additional studies were identified following an updated systematic search in PubMed and EMBASE (using the key words 'metabolic syndrome', 'insulin resistance syndrome' and 'syndrome X' combined with 'testosterone', 'sex hormone-binding globulin', 'SHBG', 'androgens', 'sex hormones' and 'sex steroids'), hand searching of relevant journals and correspondence with collaborating investigators. Thirty-three eligible studies were identified, and communication was established with the authors of 24 studies. From these studies, four declined and 20 agreed to participate. All studies used a cross-sectional design and four studies also collected outcome data prospectively. All studies were published previously and had each received local institutional review board approvals and consent from participants.

Data collection
Collaborators were asked to provide data on the following variables for each individual: waist circumference, systolic and diastolic blood pressure, high density lipoprotein (HDL) cholesterol, triglycerides, glucose, TT and SHBG concentrations, age at recruitment, use of hormonal therapy, timing of blood sample collection and details of any overnight fast, assay methods and length of follow-up for prospective data. If available, data were also collected on ethnicity, smoking status, alcohol consumption, physical activity, BMI, insulin concentration, history of CVD, type 2 diabetes and hypertension.

The original data were checked for completeness and possible inconsistencies using the original publications. For most studies, the data provided were identical to those analysed and published previously. In the TARF (Turkish Adult Risk Factor) [19], SHIP (Study of Health in Pomerania) [12] and DETECT (Diabetes Cardiovascular Risk-Evaluation: Targets and Essential Data for Commitment of Treatment) [20,21] cohorts additional prospective data were available that were not included in their previously published reports.

Data processing & measures

Blood samples were mostly collected in the morning after an overnight fast. In SHIP [12] samples were collected in a non-fasting state throughout the day. In DETECT [20,21] ~40% of the samples were non-fasting. Not all studies performed SHBG measurements, and various assays were used for the measurement of TT and SHBG (for a full description of the assay methods and samples used for the hormone analyses, see eTable 1). When both TT and SHBG were provided, FT concentrations were calculated using the equation of Vermeulen et al. [36] assuming a fixed albumin concentration of 43 g/L. We recoded categorical variables on alcohol consumption (drinker vs. non-drinker), cigarette smoking (smoker vs. non-smoker) and physical activity (active vs. inactive) to maximize comparability among studies. When both glucose and insulin concentrations were provided, the homeostasis model assessment for insulin resistance (HOMA-IR) was calculated using the formula HOMA-IR = (fasting insulin in mIU/L x fasting glucose in mmol/L)/22.5. Values of HOMA-IR were not normally distributed and transformed logarithmically prior to analysis.

MetS was defined according to the most recent harmonized definition presented in the 2009 Joint Scientific Statement [37], using ethnic-specific cut-offs for abdominal obesity. Men were considered to have MetS if they had ≥ 3 of the following components: 1. abdominal obesity (waist circumference ≥ 102 cm for Caucasian men and waist circumference ≥ 90 cm for Asian men); 2. hypertriglyceridemia (triglycerides ≥ 1.7 mmol/L); 3. low HDL-cholesterol (HDL-cholesterol < 1.03 mmol/L), 4. hyperglycaemia (fasting blood glucose ≥ 5.6 mmol/L); 5. hypertension (systolic blood pressure ≥ 130 mm Hg or diastolic blood pressure ≥ 85 mm Hg). Men taking antihypertensive medication were considered having high blood pressure and those with type 2 diabetes were counted as having hyperglycaemia. We slightly modified the criteria for men having non-fasting blood samples (using a blood glucose cut-off of ≥ 8.0 mmol/L and triglyceride cut-off of ≥ 2.3 mmol/L) [38].

Statistical analyses

Analyses were restricted to men aged 18 years and older not using hormonal therapy (N = 14,025). We excluded men with missing data on individual MetS components (N = 1186). We further removed extreme outliers > 4 standard deviations (SD) from the mean for measured TT, SHBG, and calculated FT concentrations (N = 28), leaving 12,811 men with complete data on TT and 9525 men with complete data on SHBG and FT, respectively. Sex hormone concentrations were categorized into quartiles using cut-off points determined separately for cross-sectional and prospective data.

We first examined the cross-sectional associations of sex hormones with MetS. To account for between-study heterogeneity and within study correlation, we used generalized linear mixed-effects models with a random intercept for study. In these models, odds ratios (ORs) and 95% confidence intervals (95% CIs) were estimated using the Laplace approximation [39]. We calculated a linear trend by entering the quartiles as a continuous term into the analyses and we estimated ORs per quartile decrease of TT, SHBG and FT to provide a summary measure of association.

Next, we examined the prospective associations between sex hormones and MetS. For these analyses, we excluded all individuals with MetS at baseline. We used Cox proportional hazards analyses with random effects at the study level (i.e. shared frailty model) to estimate hazard ratios (HRs) and 95% CIs.

To investigate the influence of potential confounders, we calculated age-adjusted and multivariable-adjusted ORs and HRs including age and lifestyle factors (smoking status, alcohol consumption and physical activity). In a next step, we adjusted the multivariable adjusted analyses for BMI and HOMA-IR to examine whether associations between sex hormones and MetS were independent of body composition and insulin resistance. To investigate whether associations of TT with MetS were influenced by SHBG, we adjusted the multivariable adjusted models for SHBG in a separate analysis. We tested for effect modification by age and BMI by including interaction terms using the Wald-test. If a significant interaction was found, we stratified the analyses for age (< 40 years, 40-60 years, > 60 years) and BMI (< 25 kg/m^2, 25-30 kg/m^2, $\geq$ 30 kg/m^2). We also performed a series of sensitivity analyses. First, we excluded men with prevalent type 2 diabetes (diagnosed diabetes or fasting blood glucose $\geq$ 7 mmol/L) and CVD at baseline. Next, we excluded men with non-fasting blood samples to examine a potential influence of fasting state. To investigate the impact of methodological differences between studies, we also repeated the analyses using study-specific quartiles of TT, SHBG and FT.

Finally, we examined associations with each MetS component separately, both cross-sectionally and prospectively. For the latter, we studied incidence of individual components after excluding men with the respective component at baseline. We used linear mixed effects models to estimate multivariable-adjusted means of TT, SHBG and FT across categories of MetS components (0, 1, 2, and $\geq$ 3).

All statistical analyses were performed using STATA version 11.1 (Stata Corp., College Station, TX, USA).

Table 1. Participant characteristics of the included studies.

Study	Country	No. of men	Follow-up (years) Median (range)	Age (years) Mean (SD)	BMI (kg/m²) Mean (SD)	Insulin (mIU/L) Mean (SD)	Smoking (yes) % (n)	Alcohol drinking (yes) % (n)	Physically active (yes) % (n)	History of diabetes (yes) % (n)	History of CVD (yes) % (n)
Akishita et al, 2010 [22]	Japan	192	NA	48.8 (9.4)	25.2 (4.0)	6.7 (4.1)	44.1 (52)	NA	NA	0.0 (0)	NA
Chen et al, 2010 [23]	Singapore	206	NA	55.1 (7.1)	25.2 (3.8)	NA	22.1 (45)	29.4 (60)	51.6 (98)	15.1 (31)	NA
Haring et al, 2009 [12]	Germany	2000	5.0 (4.4-8.5)	50.8 (16.5)	27.6 (4.0)	NA	33.7 (671)	76.5 (1521)	69.9 (1390)	8.6 (172)	NA
Schneider et al, 2009 [20]	Germany	2448	4.0 (0.9-4.6)	58.7 (13.3)	27.8 (4.3)	NA	18.6 (387)	84.9 (1804)	27.1 (623)	22.9 (538)	20.3 (496)
Chubb et al, 2008 [8]	Australia	2489	NA	77.0 (3.6)	26.2 (3.5)	NA	8.4 (136)	NA	NA	0.0 (0)	37.6 (936)
Corona et al, 2008 [24]	Italy	587	NA	54.1 (12.6)	NA	NA	NA	NA	NA	27.6 (162)	15.2 (89)
Emmelot-Vonk et al, 2008 [25]	Netherlands	200	NA	67.3 (4.9)	27.3 (3.9)	9.1 (8.3)	15.0 (30)	80.5 (161)	NA	0.0 (0)	NA
Goncharov et al, 2008 [26]	Russia	60	NA	30.2 (6.4)	32.1 (2.9)	15.7 (14.5)	26.7 (16)	NA	23.3 (14)	0.0 (0)	NA
Onat et al, 2007 [19]	Turkey	564	2.0 (2.0-2.0)	53.5 (11.0)	27.7 (4.3)	10.1 (8.3)	36.9 (208)	17.4 (98)	48.1 (270)	0.0 (0)	11.7 (66)
Chen et al, 2006 [27]	Australia	60	NA	76.4 (5.2)	26.7 (3.2)	NA	NA	NA	NA	6.7 (4)	NA
Gannagé-Yared et al, 2006 [28]	Lebanon	152	NA	59.3 (7.0)	27.3 (3.7)	9.2 (4.6)	NA	NA	77.0 (117)	0.0 (0)	14.5 (22)
Maggio et al, 2006 [29]	Italy	421	NA	73.8 (6.7)	27.1 (3.3)	11.2 (6.1)	21.4 (90)	88.8 (371)	52.3 (219)	15.0 (63)	19.5 (82)
Robeva et al, 2006 [30]	Bulgaria	18	NA	31.9 (9.3)	30.0 (6.6)	14.3 (9.2)	44.4 (8)	55.6 (10)	NA	5.6 (1)	5.6 (1)
Muller et al, 2005 [10]	Netherlands	376	NA	60.0 (11.3)	26.2 (3.5)	8.6 (5.8)	24.7 (93)	84.1 (313)	66.7 (248)	5.1 (19)	16.0 (60)
Nuver et al, 2005 [31]	Netherlands	161	NA	37.9 (9.3)	25.0 (3.3)	9.3 (5.5)	36.0 (58)	NA	NA	0.6 (1)	NA
Undén et al, 2005 [32]	Sweden	137	NA	47.0 (16.2)	25.6 (3.9)	11.1 (6.2)	16.8 (23)	92.0 (126)	29.6 (40)	5.1 (7)	9.5 (13)
Tong et al, 2005 [33]	China	295	NA	41.0 (8.7)	25.3 (3.8)	NA	22.7 (67)	26.2 (77)	NA	0.0 (0)	0.0 (0)
Laaksonen et al, 2004 [15]	Finland	2028	11.2 (9.7-14.4)	52.7 (5.7)	27.0 (3.5)	11.7 (7.5)	31.4 (637)	86.6 (1753)	54.1 (1094)	5.0 (102)	36.0 (730)
Ukkola et al, 2001 [34]	Canada	321	NA	36.2 (13.7)	27.1 (5.1)	10.5 (7.2)	14.8 (47)	57.6 (183)	0.0 (0)	0.0 (0)	0.0 (0)
Hautanen et al, 2000 [35]	Finland	96	NA	45.0 (4.8)	26.3 (4.0)	9.0 (7.3)	36.5 (35)	95.0 (91)	30.2 (29)	0.0 (0)	0.0 (0)

Abbreviations: BMI = body mass index; CVD = cardiovascular disease; SD = standard deviation; NA = not available.

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Results

Table 1 summarizes the participant characteristics for each individual study. All men had complete data on age and history of type 2 diabetes. Nineteen studies had recorded data on BMI, 13 studies had data on insulin concentrations and CVD history and 9 studies collected data on all lifestyle factors. Absolute sex hormone concentrations varied across individual studies: variations for TT, SHBG, and FT were 1.6-fold, 2.0-fold and 2.2-fold respectively (Supplementary Table 1).

Cross-sectional associations between sex hormones and MetS
The overall prevalence of MetS was 27.9% (N = 3574). An inverse relation was observed between TT, SHBG, FT, and MetS (Table 2). Men with low TT concentrations were more likely to have MetS compared to men with high TT concentrations (OR per quartile decrease = 1.70 (95% CI 1.63-1.77)). Associations were similar for SHBG (OR per quartile decrease = 1.75 (95% CI 1.66-1.84)), but weaker for FT (OR per quartile decrease = 1.40 (95% CI 1.32-1.47)). Adjustment for lifestyle factors did not materially change the ORs. Associations were attenuated after adjustment for BMI and HOMA-IR, but remained statistically significant (Table 2). The association between TT and MetS weakened, but persisted after adjusting for SHBG (OR per quartile decrease of TT = 1.48 (95% CI 1.37-1.59)).
Results from models including interaction terms are shown in Table 3. The association between SHBG and MetS was modified by BMI. The association with SHBG was stronger in men with a lower BMI (*P* for interaction = 0.03). Associations with TT and FT were not modified by BMI. We also observed a significant interaction with age. Associations of TT and FT with MetS were stronger in men aged < 60 years (*P* for interaction = 0.004 and 0.01 respectively).

Table 2. Odds ratios for the metabolic syndrome according to quartiles of total testosterone, SHBG and free testosterone – results from cross-sectional studies.

	Total dataset	Subset data (lifestyle factors)		Subset data (BMI)		Subset data (HOMA-IR)	
	Model 1	Model 1	Model 2	Model 1	Model 3	Model 1	Model 4
Total testosterone	N = 12811	N = 8094	N = 8094	N = 8066	N = 8066	N = 3724	N = 3724
Q1	5.07 (4.45-5.80)	4.84 (4.13-5.68)	4.93 (4.20-5.79)	4.84 (2.13-5.68)	2.79 (2.34-3.33)	4.69 (3.65-6.02)	1.93 (1.44-2.59)
Q2	2.84 (2.49-3.25)	2.70 (2.30-3.18)	2.75 (2.33-3.24)	2.72 (2.31-3.20)	1.83 (1.53-2.19)	2.92 (2.27-3.76)	1.56 (1.17-2.09)
Q3	1.83 (1.59-2.10)	1.75 (1.49-2.06)	1.77 (1.50-2.09)	1.75 (1.49-2.07)	1.37 (1.14-1.64)	1.70 (1.33-2.19)	1.20 (0.90-1.59)
Q4	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)
P (trend)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
per quartile decrease	1.70 (1.63-1.77)	1.68 (1.60-1.76)	1.69 (1.60-1.77)	1.68 (1.60-1.76)	1.41 (1.33-1.49)	1.68 (1.55-1.81)	1.25 (1.14-1.37)
SHBG	N = 9525	N = 5552	N = 5552	N = 5547	N = 5547	N = 3304	N = 3304
Q1	5.26 (4.46-6.20)	5.03 (4.08-6.21)	5.18 (4.19-6.40)	5.04 (4.09-6.22)	2.96 (2.34-3.75)	5.77 (4.27-7.79)	2.61 (1.84-3.71)
Q2	2.93 (2.50-3.45)	3.08 (2.51-3.79)	3.13 (2.54-3.84)	3.08 (2.51-3.79)	2.31 (1.84-2.90)	3.61 (2.66-4.88)	2.35 (1.66-3.33)
Q3	1.67 (1.42-1.97)	1.74 (1.42-2.13)	1.75 (1.43-2.15)	1.73 (1.41-2.12)	1.44 (1.15-1.80)	1.60 (1.16-2.20)	1.21 (0.84-1.74)
Q4	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)
P (trend)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
per quartile decrease	1.75 (1.66-1.84)	1.72 (1.61-1.83)	1.73 (1.62-1.85)	1.72 (1.61-1.84)	1.45 (1.34-1.56)	1.83 (1.67-2.01)	1.41 (1.27-1.58)
Free testosterone	N = 9525	N = 5552	N = 5552	N = 5547	N = 5547	N = 3304	M = 3304
Q1	2.75 (2.32-3.27)	3.16 (2.53-3.96)	3.13 (2.50-3.92)	3.16 (2.53-3.95)	1.99 (1.55-2.56)	2.88 (2.16-3.84)	1.23 (0.87-1.75)
Q2	2.08 (1.76-2.45)	2.37 (1.91-2.93)	2.35 (1.89-2.91)	2.36 (1.91-2.93)	1.68 (1.32-2.13)	2.31 (1.78-3.00)	1.26 (0.92-1.72)
Q3	1.47 (1.25-1.73)	1.53 (1.25-1.89)	1.53 (1.24-1.88)	1.54 (1.25-1.89)	1.27 (1.01-1.60)	1.57 (1.24-1.99)	1.09 (0.83-1.43)
Q4	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)
P (trend)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.15
per quartile decrease	1.40 (1.32-1.47)	1.47 (1.37-1.58)	1.46 (1.36-1.57)	1.47 (1.37-1.58)	1.26 (1.16-1.37)	1.44 (1.31-1.57)	1.08 (0.97-1.21)

OR (95% CI)

Abbreviations: BMI = body mass index; HOMA-IR = homeostasis model assessment for insulin resistance; SHBG = sex hormone-binding globulin; OR = odds ratio; CI = confidence interval. Lifestyle factors: smoking, alcohol consumption and physical activity.

Model 1: adjusted for age
Model 2: Model 1 plus lifestyle factors
Model 3: Model 2 plus BMI
Model 4: Model 3 HOMA-IR

Table 3. Odds ratios for the metabolic syndrome per quartile decrease of total testosterone, SHBG and free testosterone, stratified by age and BMI – results from cross-sectional studies.

| | OR (95% CI) | | |
	Total testosterone	SHBG	Free testosterone
Body mass index			
< 25 kg/m^2	N = 2377	N = 1688	N = 1688
	1.43 (1.24-1.65)	2.20 (1.77-2.72)	1.22 (0.97-1.53)
25-30 kg/m^2	N = 3969	N = 2714	N = 2714
	1.51 (1.40-1.62)	1.50 (1.35-1.66)	1.29 (1.16-1.44)
> 30 kg/m^2	N = 1720	N = 1145	N = 1145
	1.37 (1.24-1.51)	1.33 (1.18-1.50)	1.31 (1.15-1.50)
P interaction	0.40	0.003	0.67
Age			
< 40 years	N = 1080	N = 875	N = 875
	1.75 (1.47-2.09)	1.56 (1.27-1.92)	1.41 (1.15-1.72)
40-60 years	N = 3985	N = 3185	N = 3185
	1.79 (1.66-1.93)	1.76 (1.61-1.92)	1.51 (1.37-1.65)
> 60 years	N = 3029	N = 1492	N = 1492
	1.55 (1.44-1.67)	1.63 (1.44-1.83)	1.35 (1.18-1.53)
P interaction	0.004	0.11	0.01

Odds ratios are adjusted for age, smoking, alcohol consumption and physical activity. Abbreviations: SHBG = sex hormone-binding globulin; OR = odds ratio; CI = confidence interval.

Prospective associations between sex hormones and MetS
In total, 584 incident MetS cases were recorded during 17,625 person years of follow-up. Men with low sex hormone concentrations at baseline had an increased risk of incident MetS at follow-up (Table 4). HRs per quartile decrease were 1.24 (95% CI 1.16-1.35), 1.43, (95% CI 1.29-1.59) and 1.14 (95% CI 1.01-1.29) for TT, SHBG and FT respectively. Again, adjustment for lifestyle factors had little effect, but associations weakened after adjustment for BMI and HOMA-IR and the HR for FT was no longer significant after adjusting for BMI (Table 4). The association with TT was attenuated, but remained significant after adjustment for SHBG (HR per quartile decrease of TT = 1.13 (95% CI 1.01-1.27)).

Table 4. Hazard ratios for the metabolic syndrome according to quartiles of total testosterone, SHBG and free testosterone – results from prospective studies.

	Total dataset	Subset data (lifestyle factors)		Subset data (BMI)		Subset data (HOMA-IR)	
	Model 1	Model 1	Model 2	Model 1	Model 3	Model 1	Model 4
Total testosterone	N = 3022	N = 2941	N = 2941	N = 2933	N = 2933	N = 792	N = 792
Q1	2.01 (1.56-2.59)	2.00 (1.55-2.59)	2.02 (1.56-2.62)	2.01 (1.55-2.59)	1.48 (1.13-1.92)	2.56 (1.60-4.11)	1.66 (1.01-2.72)
Q2	1.84 (1.42-2.38)	1.81 (1.40-2.35)	1.83 (1.41-2.38)	1.80 (1.39-2.33)	1.52 (1.17-1.99)	2.68 (1.71-4.19)	1.75 (1.10-2.80)
Q3	1.42 (1.09-1.86)	1.40 (1.07-1.83)	1.40 (1.07-1.83)	1.39 (1.07-1.82)	1.23 (0.94-1.61)	1.73 (1.09-2.75)	1.24 (0.77-1.99)
Q4	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)
P (trend)	< 0.001	< 0.001	< 0.001	< 0.001	0.002	< 0.001	0.02
per quartile decrease	1.24 (1.16-1.35)	1.25 (1.16-1.35)	1.25 (1.16-1.36)	1.25 (1.16-1.35)	1.14 (1.05-1.23)	1.39 (1.20-1.59)	1.20 (1.03-1.40)
SHBG	N = 1899	N = 1894	N = 1894	N = 1892	N = 1892	N = 788	N = 788
Q1	2.98 (2.15-4.12)	2.96 (2.14-2.09)	3.02 (2.18-4.19)	2.96 (2.14-4.09)	2.06 (1.46-2.89)	3.45 (1.82-6.53)	1.95 (1.00-3.81)
Q2	1.89 (1.37-2.61)	1.88 (1.36-2.59)	1.90 (1.38-2.63)	1.88 (1.36-2.59)	1.51 (1.09-2.10)	2.03 (1.05-3.92)	1.31 (0.67-2.55)
Q3	1.45 (1.05-1.98)	1.45 (1.05-1.98)	1.45 (1.05-1.98)	1.44 (1.05-1.98)	1.22 (0.89-1.68)	1.44 (0.69-2.98)	1.08 (0.52-2.24)
Q4	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)
P (trend)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.01
per quartile decrease	1.43 (1.29-1.59)	1.43 (1.29-1.58)	1.44 (1.30-1.60)	1.43 (1.29-1.59)	1.27 (1.14-1.42)	1.59 (1.29-1.84)	1.30 (1.07-1.58)
Free testosterone	N = 1899	N = 1894	N = 1894	N = 1892	N = 1892	N = 788	N = 788
Q1	1.46 (1.00-2.12)	1.45 (0.99-2.11)	1.44 (0.99-2.10)	1.45 (0.99-2.11)	1.27 (0.87-1.85)	2.06 (0.97-4.36)	1.66 (0.76-3.63)
Q2	1.54 (1.11-2.13)	1.53 (1.11-2.12)	1.53 (1.10-2.11)	1.53 (1.11-2.12)	1.43 (1.03-1.98)	1.64 (1.05-2.56)	1.56 (0.99-2.48)
Q3	1.24 (0.91-1.68)	1.24 (0.91-1.69)	1.24 (0.91-1.69)	1.24 (0.92-1.69)	1.28 (0.95-1.74)	1.41 (0.98-2.05)	1.22 (0.84-1.78)
Q4	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)	1.00 (REF)
P (trend)	0.03	0.03	0.04	0.03	0.19	0.01	0.04
per quartile decrease	1.14 (1.01-1.29)	1.14 (1.01-1.28)	1.14 (1.01-1.28)	1.14 (1.01-1.28)	1.08 (0.96-1.22)	1.28 (1.06-1.54)	1.22 (1.01-1.48)

Abbreviations: BMI = body mass index; HOMA-IR = homeostasis model assessment for insulin resistance; SHBG = sex hormone-binding globulin; HR = hazard ratio; CI = confidence interval. Lifestyle factors: smoking, alcohol consumption and physical activity.

Model 1: adjusted for age
Model 2: Model 1 plus lifestyle factors
Model 3: Model 2 plus BMI
Model 4: Model 3 HOMA-IR

Interaction analyses showed that the association between TT and MetS was strongest in men with a BMI < 25 kg/m^2 (Table 5, *P* for interaction = 0.02). Although no signification interaction between SHBG and BMI was observed, there was some evidence of a U-shaped relation with associations being strongest in men < 25 kg/m^2 (Table 5). In contrast to the cross-sectional data, no effect modification by age was observed in prospective analyses.

We repeated all analyses first, using non-fasting blood samples and second, after excluding men with a history of type 2 diabetes and CVD. Estimates were not materially different in these sensitivity analyses. Results remained also unchanged in analyses using studyspecific quartiles (data not shown).

Table 5. Hazard ratios for the metabolic syndrome per quartile decrease of total testosterone, SHBG and free testosterone, stratified by age and BMI – results from prospective studies.

	HR (95% CI)		
	Total testosterone	SHBG	Free testosterone
Body mass index			
< 25 kg/m^2	N = 1045	N = 625	N = 625
	1.58 (1.25-2.00)	1.59 (1.15-2.21)	1.16 (0.79-1.69)
25-30 kg/m^2	N = 1546	N = 1028	N = 1028
	1.08 (0.98-1.19)	1.17 (1.03-1.33)	1.09 (0.94-1.26)
> 30 kg/m^2	N = 342	N = 239	N = 239
	1.13 (0.95-1.36)	1.49 (1.16-1.93)	1.12 (0.85-1.48)
P interaction	0.02	0.65	0.62
Age			
< 40 years	N = 487	N = 372	N = 372
	1.21 (0.98-1.50)	1.41 (1.11-1.80)	0.92 (0.70-1.22)
40-60 years	N = 1449	N = 1027	N = 1027
	1.26 (1.13-1.41)	1.41 (1.23-1.62)	1.17 (1.00-1.37)
> 60 years	N = 1005	N = 495	N = 495
	1.19 (1.04-1.35)	1.30 (1.06-1.61)	1.14 (0.89-1.47)
P interaction	0.31	0.53	0.45

Hazard ratios are adjusted for age, smoking, alcohol consumption and physical activity. Abbreviations: SHBG = sex hormone-binding globulin; OR = odds ratio; CI = confidence interval.

Associations between sex hormones and number of MetS components
Figure 1 shows the mean concentrations of TT, SHBG, and FT according to the number of MetS components. In cross-sectional analyses, TT, SHBG, and FT concentrations decreased gradually with increasing number of MetS components (*P* trend < 0.001). Although differences in sex hormone concentrations were smaller in prospective analyses, a gradual linear decrease of TT, SHBG, and FT was observed as the number of MetS components increased (Figure 1).

Figure 1. Sex hormone concentrations by number of prevalence and incident metabolic syndrome components – results from cross-sectional and prospective studies.
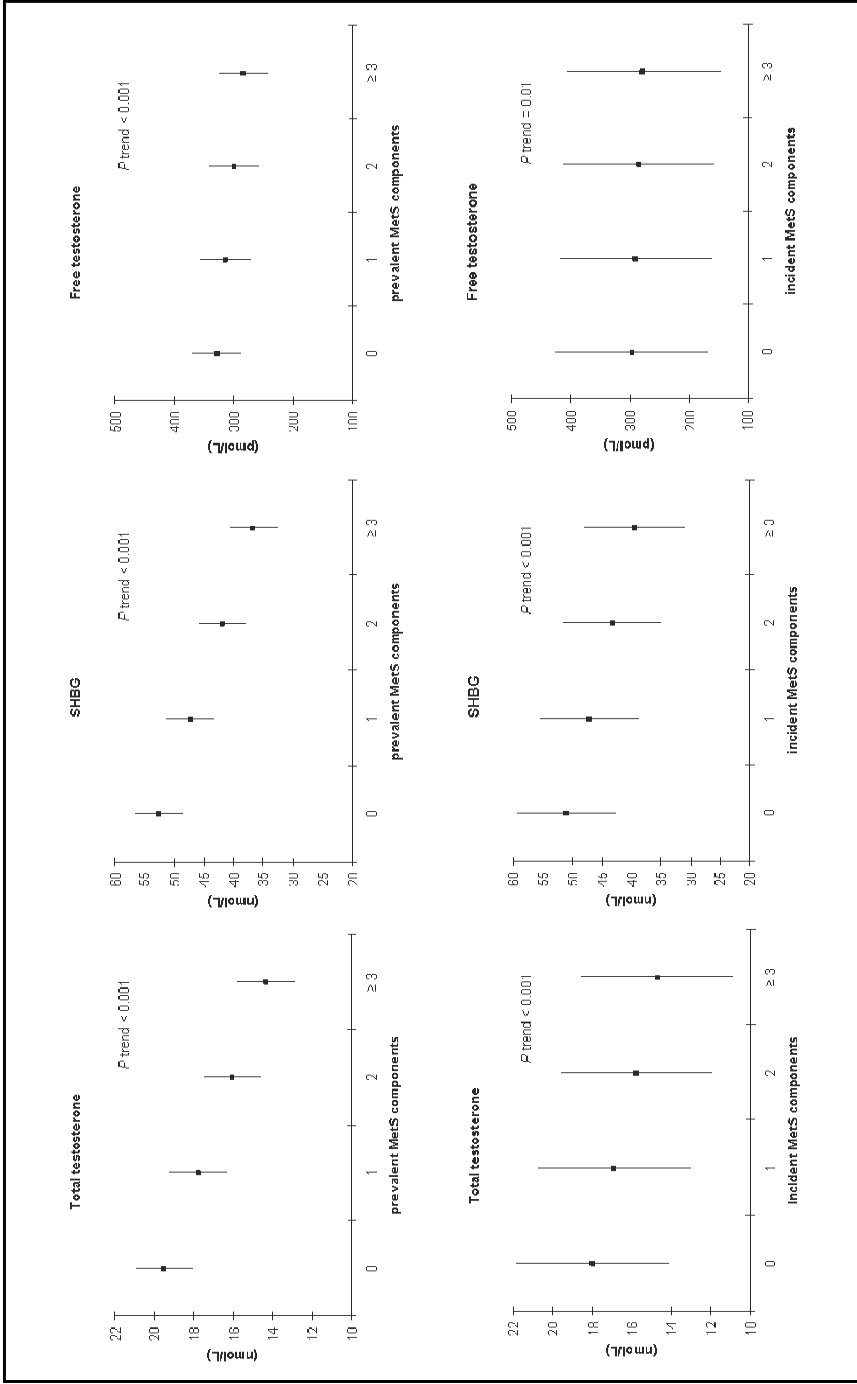
Multivariable adjusted means and 95% confidence intervals for sex hormone concentrations by number of prevalent and incident metabolic syndrome components and the P value for linear trend. Abbreviations: SHBG = sex hormone-binding globulin. Means are adjusted for age, smoking, alcohol consumption and physical activity.

Associations between sex hormones and individual MetS components
Figure 2 shows the multivariable-adjusted ORs of TT, SHBG, and FT for each component in the cross-sectional analysis. Associations with TT were strongest for abdominal obesity (OR per quartile decrease = 1.58 (95% CI 1.51-1.66)) and hypertriglyceridemia (OR per quartile decrease = 1.57 (95% CI 1.50-1.65)), and weakest for hypertension (OR per quartile decrease = 1.24 (95% CI 1.18-1.31)). A similar pattern was observed for SHBG and FT, with the exception that low FT and SHBG concentrations were also strongly linked to hyperglycaemia (Figure 2).

In prospective analyses, differences in strength were less marked across the individual MetS components, although a similar pattern for TT was observed as in the cross-sectional analysis. Low TT concentrations at baseline were most strongly associated with incident abdominal obesity (HR per quartile decrease = 1.19 (95% CI 1.09-1.29)) and hypertriglyceridemia (HR per quartile decrease = 1.21 (95% CI 1.10-1.34)). Low baseline SHBG concentrations increased the risk of all MetS components. Associations were strongest for hyperglycaemia (HR per quartile decrease = 1.46 (95% CI 1.20-1.77)) and hypertriglyceridemia (HR per quartile decrease = 1.40 (95% CI 1.23-1.61)). Low FT concentrations were associated with incident hypertriglyceridemia (HR = 1.18 (95% CI 1.01-1.38)) and abdominal obesity (HR = 1.13 (95% CI 0.98-1.29)), although the latter was not statistically significant.

Figure 2. Odds ratios and hazard ratios for individual metabolic syndrome components per quartile decrease of total testosterone, SHBG and free testosterone.

Hormone	MetS component	N	OR (95% CI)
Total testosterone	Abdominal obesity	8094	1.58 (1.51, 1.66)
	Hypertriglyceridemia	8094	1.57 (1.50, 1.65)
	Hyperglycaemia	8094	1.38 (1.30, 1.46)
	Low HDL cholesterol	8094	1.33 (1.26, 1.39)
	Hypertension	8094	1.24 (1.18, 1.31)
SHBG	Abdominal obesity	5552	1.50 (1.40, 1.60)
	Hypertriglyceridemia	5552	1.71 (1.61, 1.82)
	Hyperglycaemia	5552	1.58 (1.45, 1.72)
	Low HDL cholesterol	5552	1.31 (1.23, 1.40)
	Hypertension	5552	1.33 (1.25, 1.41)
Free testosterone	Abdominal obesity	5552	1.50 (1.40, 1.61)
	Hypertriglyceridemia	5552	1.32 (1.24, 1.41)
	Hyperglycaemia	5552	1.33 (1.21, 1.47)
	Low HDL cholesterol	5552	1.21 (1.13, 1.29)
	Hypertension	5552	1.09 (1.02, 1.17)

.75   1   1.25   1.5   1.75   2

Hormone	MetS component	N	HR (95% CI)
Total testosterone	Abdominal obesity	2528	1.19 (1.09, 1.29)
	Hypertriglyceridemia	2458	1.21 (1.10, 1.34)
	Hyperglycaemia	2722	1.12 (0.98, 1.27)
	Low HDL cholesterol	2575	1.09 (1.02, 1.17)
	Hypertension	858	1.08 (0.97, 1.21)
SHBG	Abdominal obesity	1705	1.24 (1.10, 1.39)
	Hypertriglyceridemia	1565	1.40 (1.23, 1.61)
	Hyperglycemia	1830	1.46 (1.20, 1.77)
	Low HDL cholesterol	1669	1.27 (1.17, 1.37)
	Hypertension	548	1.14 (1.00, 1.37)
Free testosterone	Abdominal obesity	1705	1.13 (0.98, 1.29)
	Hypertriglyceridemia	1565	1.18 (1.01, 1.38)
	Hyperglycaemia	1830	0.97 (0.78, 1.20)
	Low HDL cholesterol	1669	0.97 (0.88, 1.06)
	Hypertension	548	0.97 (0.80, 1.16)

.75   1   1.25   1.5   1.75   2

Models are adjusted for age, smoking, alcohol consumption and physical activity. Abbreviations: SHBG = sex hormone-binding globulin; OR = odds ratio; HR = hazard ratio; CI = confidence interval.

Discussion

In this unique meta-analysis of individual participant data, we found that men with lower concentrations of TT, SHBG and FT have an increased risk of prevalent and incident MetS. For all sex hormones, associations were independent of age and lifestyle factors, and were weaker prospectively than cross-sectionally. SHBG was the main determinant of incident MetS risk, but adjustment for SHBG did not fully explain associations of TT with MetS. Associations of testosterone and SHBG with MetS were strongest in non-overweight men and abdominal obesity, hypertriglyceridemia and hyperglycaemia were the main driving components.

The major strength of our study was that by re-analysing the individual data from 20 observational studies, we were able to study relevant subgroups and individual MetS components with sufficient statistical power. Furthermore, the use of raw data enabled us to apply consistent methods for MetS assessment and FT estimation, and to adjust for potential confounders in a uniform way. Nevertheless, some potential limitations should also be discussed. First, not all eligible studies participated in this collaborative meta-analysis, which may have introduced 'collaboration bias', a term equivalent to publication bias in literature-based meta-analyses. However, we think that reasons to participate are pragmatic, not related to either determinant or outcome status, therefore minimizing the likelihood of presence of this bias. Second, individual studies were methodologically heterogeneous; different hormone assays were used, and confounder and outcome data were not collected in a standardised way. Our statistical approach accounted for these methodological differences between studies by incorporating random effects at the study level. Moreover, when we repeated the analysis using studyspecific hormone quartiles, results did not change substantially, indicating that methodological differences in hormone measurement did not have a major impact on our findings. Twenty-four percent of all participants had non-fasting blood samples. In our analysis, we adjusted for fasting state by using sample-specific cut-offs. Since results were not materially different in analyses excluding non-fasting samples, we consider differential misclassification due to fasting state negligible. Fourth, sex hormones were measured only once at baseline in each individual study, precluding us from studying time-related changes in sex hormone concentrations and MetS risk. Also, we could not assess the role of aromatization because estradiol concentrations were not measured in all studies. Another limitation is that free testosterone concentrations were not measured but calculated using fixed albumin concentrations. However, the algorithm of Vermeulen used to calculate FT has been shown to be a reasonable approximation of actual FT concentrations [36].

Notwithstanding the prospective design, we cannot draw definitive conclusions on the causal directionality of the observed associations. Stronger associations of sex hormones with prevalent than incident MetS suggest that low testosterone and SHBG are merely a result rather than cause of MetS. Indeed, weight loss and maintenance have been associated with an increase in testosterone and SHBG concentrations in obese men with MetS [40,41]. Likewise, experimental studies show suppressive effects of adiposity and insulin resistance on testosterone production in men [36,42,43]. On the other hand, testosterone and SHBG may also influence MetS etiology. Polymorphisms in the SHBG gene have been associated with risk of type 2 diabetes, suggesting a causal role for SHBG in metabolic disease risk [44,45]. Moreover, a recent meta-analysis of the few available testosterone supplementation studies shows that testosterone therapy is associated with a significant reduction of fasting glucose, HOMA-IR, triglycerides and waist circumference as well as an increase of HDL-cholesterol [18]. Thus, observational and experimental data point to bidirectional relationships between sex hormones and MetS.

Adjustment for lifestyle factors had little effect on the observed associations of TT, SHBG and FT with MetS, but the strength of associations was nearly halved after adjustment for BMI and HOMA-IR. The major impact of body composition and insulin resistance was expected, as both factors represent the core abnormality of MetS [46]. Hence, adjusting for BMI and HOMA-IR may represent overadjustment. Consistent with our literature-based meta-analysis [17], we found an increased MetS risk with lower FT concentrations. Associations with TT and MetS remained also significant after adjusting for SHBG. These findings are important because they show that the association between testosterone and MetS cannot solely be attributed to SHBG. The fact that previous studies have reported conflicting results for FT [7-9,14,15,47], might be due to differences in sample size and handling of potential confounders as described above. The large sample size of the present pooled meta-analysis enhanced the statistical power to detect small to moderate associations between FT and MetS.

Apart from being associated with MetS as an entity, sex hormones also show an inverse association with the number of MetS components. Previous data regarding this association are limited. In the BACH study, [9] the largest difference in sex hormones was found between men having one vs. two MetS components, suggesting a decline in sex hormone concentrations before the actual onset of MetS. Our results do not support such a threshold effect, as all sex hormones decreased gradually with increasing number of MetS components. Among the five components, total testosterone was most strongly associated with hypertriglyceridemia and abdominal obesity. A similar pattern was found previously in cross-sectional studies [7-9,16], but this

is the first study showing such a relationship with incident MetS components. Apart from hypertriglyceridemia and abdominal obesity, SHBG and free testosterone were also strongly associated with hyperglycaemia.

Interestingly, we found that the prospective association between TT and MetS was strongest in men with a BMI < 25 kg/m^2. The reason for this interaction is not clear, but the weaker association in overweight men suggests a dominant role for non-androgenic risk factors in this specific subgroup. This finding may also indicate the emergence of relative androgen insensitivity with increasing BMI. In children an inverse association between BMI and androgen receptor sensitivity has been reported [48], but no studies so far have explored this association in middle-aged and older men. Cross-sectionally, we found that SHBG was more strongly associated with MetS in men with a lower BMI. However, a clear interaction with BMI could not be confirmed in prospective analysis, although the association between SHBG and MetS was strongest in non-overweight men. Previously, a subgroup effect of BMI has been demonstrated in relation to leptin [49], with associations of SHBG and FT being absent in obese men. Leptin resistance becomes more prevalent with increasing BMI [50], providing an alternative explanation for the weaker associations in overweight men. Finally, the observed interactions with BMI may also reflect the higher imprecision of hormone assays toward the lower end of the hormone distribution. Testosterone and SHBG concentrations decrease with increasing BMI. As a consequence, associations may be more difficult to detect in subgroups of overweight and obese men. In cross-sectional analysis of testosterone data, we also found some evidence of effect modification by age, but this interaction could not be replicated in prospective analysis.

In conclusion, we observed a robust, dose-response relationship of low testosterone and SHBG concentrations with prevalent and incident MetS in men, with associations being primarily mediated through abdominal obesity, hypertriglyceridemia and hyperglycaemia. The weaker associations observed in overweight men warrant further investigation as this specific subgroup may represent a target for future prevention and intervention. Altogether, these findings provide more insight into the biological mechanisms linking low testosterone and SHBG to MetS.

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Supplementary Table 1. Assays and samples used for hormone analyses per study.

Study	Testosterone assay	SHBG assay	Fasting, % (n)	Timing blood sample collection	Total testosterone (nmol/L) Mean (SD)	SHBG (nmol/L) Mean (SD)	Free testosterone (pmol/L) Mean (SD)
Akishita et al, 2010 [22]	RIA commercial kit - radioimmunoassay	NA	100 (192)	7.00 a.m. - 9.00 a.m.	19.1 (6.2)	NA	NA
Chen et al, 2010 [23]	Roche Elecsys - electrochemiluminescent immunoassay	Roche Elecsys - electrochemiluminescent immunoassay	100 (206)	9.00 a.m.	15.8 (5.3)	37.0 (15.3)	303.2 (77.5)
Haring et al, 2009 [12]	Immulite - chemiluminescent immunoassay	NA	0 (0)	7.00 a.m. - 4.00 p.m.	16.6 (5.8)	49.7 (20.6)	273.1 (99.6)
Schneider et al, 2009 [20]	Roche Elecsys - electrochemiluminescent immunoassay	NA	58.5 (1433)	4.00 a.m. – 21.00 p.m.	15.3 (6.5)	NA	NA
Chubb et al, 2008 [8]	Immulite - chemiluminescent immunoassay	Immulite - chemiluminescent immunoassay	100 (2490)	8.00 a.m. – 10.30 a.m.	16.0 (5.4)	43.0 (15.6)	283.7 (84.7)
Corona et al, 2008 [24]	Roche Elecsys - electrochemiluminescent immunoassay	Roche Elecsys - electrochemiluminescent immunoassay	100 (558)	NA	15.8 (6.6)	35.4 (16.6)	321.0 (132.9)
Emmelot-Vonk et al, 2008 [25]	Immulite - chemiluminescent immunoassay	Immulite - chemiluminescent immunoassay	100 (200)	8.00 a.m. - 11.00 a.m.	13.2 (2.4)	33.1 (10.3)	272.4 (59.4)
Goncharov et al, 2008 [26]	Vitros Eci - electrochemiluminescent immunoassay	AutoDelfia - fluoroimmunoassay	100 (60)	8.30 a.m. – 10.30 a.m.	13.4 (5.9)	34.4 (22.8)	276.6 (112.0)
Onat et al, 2007 [19]	Roche Elecsys - electrochemiluminescent immunoassay	Roche Elecsys - electrochemiluminescent immunoassay	95.7 (536)	8.00 a.m. – 10.00 a.m.	12.9 (7.7)	44.6 (20.4)	226.4 (137.4)
Chen et al, 2006 [27]	Roche Elecsys - electrochemiluminescent immunoassay	NA	100 (60)	NA	13.1 (4.6)	NA	NA
Gannagé-Yared et al, 2006 [28]	Immulite - chemiluminescent immunoassay	Immulite - chemiluminescent immunoassay	100 (152)	8.00 a.m. – 9.00 a.m.	13.3 (4.1)	36.7 (14.8)	253.7 (69.9)
Maggio et al, 2006 [29]	Diagnostic Systems Laboratories - radioimmunoassay	NA	100 (421)	7.00 a.m. - 8.00 a.m.	15.0 (4.5)	NA	NA
Robeva et al, 2006 [30]	RIA commercial kit - radioimmunoassay	NA	100 (18)	8.00 a.m. – 9.00 a.m.	16.9 (7.7)	NA	NA
Muller et al, 2005 [10]	In-house competitive RIA - radioimmunoassay	Immulite - immunoradiometric assay	99.5 (374)	8.00 a.m. - 10.00 a.m.	18.6 (5.4)	40.6 (14.4)	352.1 (98.5)
Nuver et al, 2005 [31]	RIA commercial kit - radioimmunoassay	Binding assay	100 (161)	8.00 a.m. - 10.00 a.m.	20.5 (5.9)	24.9 (10.1)	507.4 (138.1)
Undén et al, 2005 [31]	RIA commercial kit - radioimmunoassay	AutoDelfia - fluoroimmunoassay	100 (137)	8.00 a.m. - 12.00 a.m.	19.1 (6.8)	44.5 (21.9)	349.1 (139.7)
Tong et al, 2005 [33]	Immulite - chemiluminescent immunoassay	Immulite - chemiluminescent immunoassay	100 (295)	8.00 a.m. - 10.00 a.m.	18.1 (5.6)	29.2 (13.2)	413.8 (112.7)
Laaksonen et al, 2004 [15]	AutoDelfia - fluoroimmunoassay	AutoDelfia - fluoroimmunoassay	100 (2028)	8.00 a.m. - 10.00 a.m.	20.4 (7.2)	38.7 (16.6)	399.7 (113.0)
Ukkola et al, 2001 [34]	In house-RIA with hexane ethyl acetate extraction - radioimmunoassay	Diagnostics Systems Laboratories - immunoradiometric assay	100 (321)	NA	15.1 (5.9)	38.4 (16.5)	292.0 (115.8)
Hautanen et al, 2000 [35]	RIA commercial kit - radioimmunoassay	Delfia - immunoradiometric assay	100 (96)	7.30 a.m.	17.3 (5.3)	38.1 (16.0)	335.3 (97.7)

Abbreviations: RIA; radioimmunoassay; SHBG = Sex hormone-binding globulin; SD = standard deviation; NA = not available.

Chapter 8

Associations of endogenous testosterone and SHBG with glycated haemoglobin in middle-aged and older men.

Abstract

Objective: In men, low circulating levels of testosterone and sex hormone-binding globulin (SHBG) have been associated with an increased cardiovascular risk. This association may be partially mediated through changes in glucose metabolism, but relatively few data are available on the relationship between sex hormones and markers of long-term glycemia. We assessed the associations of endogenous testosterone and SHBG with glycated haemoglobin (HbA_{1c}).

Design and subjects: Cross-sectional study of 1292 men from the Norfolk population of European Prospective Investigation into Cancer (EPIC-Norfolk).

Measurements: HbA_{1c}, total testosterone (TT) and SHBG levels were measured and free testosterone (FT) levels were calculated. Multiple linear regression models were used to assess the associations of TT, SHBG and FT with HbA_{1c}.

Results: Men with self-reported diabetes or undiagnosed diabetes had lower testosterone and SHBG levels. In non-diabetic men, HbA_{1c} levels were inversely associated with TT and calculated FT independently of age, body mass index, smoking, alcohol consumption and physical activity. The adjusted change in HbA_{1c} was 0.055 (95% CI 0.025; 0.085) per standard deviation (sd) decrease in TT and 0.041 (95% CI 0.010; 0.073) per sd decrease in calculated FT respectively. SHBG levels were inversely associated with HbA_{1c} after multivariable adjustment (beta = 0.038 per sd decrease (95% CI 0.004; 0.071)).

Conclusions: In middle-aged and older men, low endogenous testosterone and SHBG levels are associated with glycemia, even below the threshold for diabetes. Further studies are needed to determine the effects of interventions that raise testosterone levels in men having increased HbA_{1c} and subnormal testosterone levels.

Introduction

Increasing observational evidence suggests a protective role of endogenous testosterone in men's cardiovascular health. Low circulating levels of testosterone in men have been associated with an unfavourable cardiovascular risk profile [1] and an increased risk of carotid and aortic atherosclerosis [2, 3]. Recent findings from large scale longitudinal studies suggest that low endogenous testosterone levels are also associated with an increase in cardiovascular morbidity and mortality [4, 5].

The cardiovascular effects of endogenous testosterone may be partially mediated through changes in glucose metabolism. Low circulating levels of total testosterone (TT) and sex hormone-binding globulin (SHBG) have been associated with high fasting glucose levels, insulin resistance and type 2 diabetes in men [6, 7]. These associations are at least partly direct and not fully explained by differences in body composition (total and abdominal obesity) [6, 7]. An independent role of testosterone in glucose metabolism is also supported by findings from experimental studies. In healthy men with idiopathic hypogonadotropic hypogonadism, acute sex steroid withdrawal reduces insulin sensitivity without concomitant changes in body composition [8]. Similarly, a rapid onset of insulin resistance has been reported in male rats following castration [9].

Glycated haemoglobin (HbA_{1c}) is a marker of long-term glycemia as it reflects the average glucose concentration over the preceding three months. HbA_{1c} has been reported to be an independent predictor of cardiovascular disease, even across the normal range [10, 11]. Testosterone and SHBG have been associated with various measures of glucose metabolism (e.g. high fasting glucose levels, insulin resistance and type 2 diabetes), but little is known regarding their relation with HbA_{1c} and it is unclear whether an association exists below the threshold for diabetes. We therefore examined this relation in the baseline data of a large cohort of middle-aged and older men.

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Methods

Study population
We studied men of the Norfolk cohort of the European Prospective Investigation of Cancer (EPIC-Norfolk), a prospective population-based study of ~ 25 000 men and women aged 40 - 79 years living in Norfolk, United Kingdom. Details on the design and recruitment of the cohort have been described previously [12].

Briefly, participants were recruited between 1993 and 1997 using general practice age sex registers. At baseline, participants completed the Health and Lifestyle questionnaire and attended a health examination at which anthropometric variables and blood samples were collected. The Norwich local research ethics committee granted ethical approval for the study and all participants gave written informed consent. For the present study, we included a subset of men who had baseline data on testosterone, SHBG and HbA$_{1c}$ levels available.

Sex hormone measurements
At the baseline Health Check (1993-1997) non-fasting blood samples were collected (between 9 AM and 4 PM) from all participants. Blood samples were stored frozen in liquid nitrogen in separate aliquots of serum and plasma. Serum samples of a subset of men (who were selected for a series of nested case-control studies) were thawed in 2003 for measurement of total testosterone and sex hormone-binding globulin. Total testosterone (TT) and sex hormone binding globulin (SHBG) were measured in a research laboratory using an Immulite Chemiluminescent Immunoassay system (Siemens Healthcare Diagnostics Inc. Frimley, Surrey, United Kingdom, formally known as DPC). The intra-assay and inter-assay coefficients of variation were 7.5% and 6.6% for testosterone and 6.1% and 7.5% for SHBG respectively. The lower limit of detection was 0.3 nmol/L for TT and 0.2 nmol/L for SHBG. Free testosterone (FT) levels were calculated using the algorithm of Vermeulen et al. [13] based on TT and SHBG concentrations and assuming a fixed albumin concentration of 43 g/L. We followed the recommendations from the European and American expert panel [14] and considered men with TT < 12 nmol/L and calculated FT levels < 250 pmol/L as having subnormal testosterone levels.

HbA1c measurements

From November 1995, when funding became available, an additional EDTA anticoagulated blood sample was collected for HbA_{1c} measurement. Half of the cohort population had HbA_{1c} measured. Blood samples were stored in a refrigerator (4-7 ^0C) until transport to the Department of Clinical Biochemistry, Cambridge University. HbA_{1c} was assayed by high-performance liquid chromatography on a BioRad Diamat Automated Glycosylated Haemoglobin Analyser (Hemel Hempstead, United Kingdom). The coefficient of variation was 3.6%.

Other variables

At baseline participants completed a Health and Lifestyle Questionnaire which included questions on age, smoking, physical activity and medical history. In this questionnaire, smoking history was derived from questions asking whether they had ever smoked a cigarette a day for as long as a year and whether they were current smokers. Based on this information, smoking was categorized as current, former, or never. Alcohol consumption was derived from the question "How many alcohol drinks do you have each week?" with four separate categories of drinks. Total alcohol consumption was estimated as the total units consumed per week. Alcohol intake was categorised as non drinker, 1-7 units/week, 8-14 units/week and > 14 units/week. Combined work and leisure physical activity was assessed using two questions referring to work and leisure time activity during the past year and coded using the following four-level index: inactive, moderately inactive, moderately active and active [15]. Prevalent diabetes, cancer, heart disease and stroke were recorded as a positive response to the question: "Has a doctor ever told you that you have any of the following?" followed by a list of conditions including diabetes, cancer, heart disease and stroke.

At the baseline health examination anthropometric measures were taken in standing position in light indoor clothing without shoes. Height was measured using a stadiometer and weight was measured using Salter scales. Body mass index (BMI) was calculated as weight (kg)/height (m^2). Waist circumference was measured at the smallest circumference between the ribs and iliac crest with participants standing with abdomen relaxed, or at the level of the umbilicus if there was no natural waistline.

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Data analysis

Data were analysed using the SPSS statistical package for Windows version 16.0 (SPSS Inc., Chicago, IL, USA). For the analyses, we included men who completed the baseline Health Check, the Health and Lifestyle Questionnaire and had TT, SHBG and HbA_{1c} measured (N = 1523). We excluded men with a history of cardiovascular disease or cancer (N = 226) to exclude potential confounding by pre-existing disease. Extreme outliers > 4 SD from the mean were removed for TT and calculated FT (N = 5), leaving 1292 men with complete data on TT, SHBG, FT and HbA_{1c}. Although the distributions of testosterone, SHBG and HbA_{1c} were slightly skewed, results were similar when log-transformed. Hence, the untransformed data are shown for ease of interpretation. Alcohol consumption, smoking and physical activity were entered as dummy variables into analyses.

First, men were divided into four HbA_{1c} categories: those with self-reported or undiagnosed diabetes ($HbA_{1c} \geq 6.5$ %), those with high risk of future diabetes (HbA_{1c} 5.7 - 6.4) [16], the remainder was divided into two categories using a clinically convenient cut-off point. Differences across HbA_{1c} categories were tested using univariate analysis of variance for continuous variables and Chi-Square tests for categorical variables. After excluding men with diabetes, quartiles of TT, SHBG and calculated FT were created based on their distribution in the study population. Analysis of covariance was used to calculate adjusted means of HbA_{1c} across quartiles of TT, SHBG and calculated FT. The covariates included age (in years), smoking (current, former and never), alcohol consumption (non drinker, 1-7 units/week, 8-14 units/week and > 14 units/week) and physical activity (inactive, moderately inactive, moderately active, active). Tests of linear trend were conducted by entering the quartiles as continuous terms. To determine whether and to what extent the observed associations might be explained by intermediate factors, analyses were also adjusted for two measures of obesity (BMI and waist circumference). Finally, multiple linear regression models were conducted and unstandardized beta coefficients were calculated to assess the linear association of testosterone and SHBG with HbA_{1c}. We adjusted analyses for the covariates listed above and for blood sampling time to investigate the impact of diurnal variations in testosterone and SHBG. To explore the relative contribution of testosterone and SHBG, we also included both variables in the same multivariable model.

Results

The mean age of the study population was 64 ± 8.4 years and the prevalence of diabetes (self-reported and undiagnosed) was 12.1%. Table 1 presents the characteristics of the participants according to HbA_{1c} category. Men who had self-reported or undiagnosed diabetes were older, had a higher BMI and waist circumference, and were less physically active than men with HbA_{1c} levels in the normal range. Levels of TT, SHBG and calculated FT decreased with increasing HbA_{1c} category. Of the men with diabetes 33.3% had subnormal TT levels. Corresponding percentages in the lower three HbA_{1c} categories ($\leq 5.0\%$, $5.1–5.6\%$ and $5.7–6.4\%$) were 12.5%, 20.2% and 23.1% respectively. The prevalence of subnormal FT levels also increased across increasing HbA_{1c} categories.

After excluding men with diabetes, TT and calculated FT remained inversely associated with HbA_{1c}. Table 2 shows the age-adjusted, age- and BMI adjusted and multivariable adjusted means of HbA_{1c} across quartiles of TT, SHBG and calculated FT in non-diabetic men. HbA_{1c} increased with decreasing quartiles of TT and calculated FT (P for linear trend < 0.001). The associations of TT and calculated FT with HbA_{1c} were unchanged after adjusting for age and BMI. Further adjustment for smoking, alcohol consumption and physical activity did not substantially affect these associations. After multivariable adjustment, mean HbA_{1c} was 0.15% higher in the lowest compared to the highest TT quartile, and 0.14% higher in the lowest compared to the highest quartile of calculated FT. Although crude HbA_{1c} levels did not significantly differ across SHBG quartiles, an increasing trend was observed in multivariable models (P for linear trend $= 0.03$). Mean HbA_{1c} levels were 0.09% higher in the lowest versus the highest SHBG quartile. When we adjusted for waist circumference instead of BMI, results were similar.

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Table 1. Study population characteristics (N = 1292) by categories of glycated haemoglobin.

	HbA$_{1c}$ %				P value
	≤ 5 (N = 345)	5.1 - 5.6 (N = 531)	5.7 - 6.4 (N = 260)	≥ 6.5 or self reported diabetes (N = 156)	
Age, years, mean (SD)	61.6 (8.8)	63.3 (8.6)	65.0 (7.3)	66.0 (7.2)	< 0.001
Body mass index, kg/m^2, mean (SD)	26.5 (3.0)	26.4 (3.1)	27.1 (3.8)	27.9 (3.6)	< 0.001
Waist circumference, cm, mean (SD)	96.7 (9.6)	96.1 (9.3)	98.1 (10.5)	101.5 (11.1)	< 0.001
HbA$_{1c}$, %, mean (SD)	4.67 (0.33)	5.36 (0.16)	5.92 (0.21)	7.73 (1.60)	< 0.001
SHBG, nmol/L, mean (SD)	42.4 (15.7)	42.1 (15.2)	42.1 (14.8)	37.3 (17.8)	0.003
Total testosterone, nmol/L, mean (SD)	17.6 (5.6)	16.5 (5.7)	16.1 (5.1)	15.2 (5.9)	< 0.001
Free testosterone, pmol/L, mean (SD)	325.0 (106.0)	301.4 (106.4)	293.4 (95.6)	295.9 (98.2)	< 0.001
Total testosterone ≤ 12 nmol/L, % (n)	12.5 (43)	20.2 (107)	23.1 (60)	33.3 (52)	< 0.001
Free testosterone ≤ 250 pmol/L, % (n)	24.6 (85)	34.1 (181)	37.3 (97)	39.1 (61)	0.001
Smoking, % (n)					< 0.001
Current	9.6 (33)	8.3 (44)	17.2 (44)	11.6 (18)	
Former	50.7 (174)	55.2 (291)	58.2 (149)	66.5 (103)	
Never	39.7 (136)	36.4 (192)	24.6 (63)	21.9 (34)	
Alcohol consumption, % (n)					0.72
0 units/week	10.8 (37)	8.3 (44)	10.5 (27)	12.2 (19)	
1 - 7 units/week	42.2 (145)	44.9 (237)	48.0 (123)	45.5 (71)	
8 - 14 units/week	23.0 (79)	23.7 (125)	18.8 (48)	19.9 (31)	
> 14 units/week	24.1 (83)	23.1 (122)	22.7 (58)	22.4 (35)	
Physical activity, % (n)					<0.001
Inactive	35.7 (123)	36.7 (195)	37.7 (98)	56.4 (88)	
Moderately inactive	24.1 (83)	21.5 (114)	27.3 (71)	19.9 (31)	
Moderately active	20.6 (71)	20.5 (109)	21.5 (56)	13.5 (21)	
Active	19.7 (68)	21.3 (113)	13.5 (35)	10.3 (16)	

Abbreviations: HbA$_{1c}$, glycated haemoglobin; SHBG, sex hormone-binding globulin.

Table 2. Mean glycated haemoglobin levels by quartiles of total testosterone, sex hormone-binding globulin and free testosterone in men without diabetes (N = 1136), crude and adjusted for covariates.

Hormone quartiles	HbA$_{1c}$ %, mean (SE)				
	Crude	Model 1	Model 2	Model 3	Model 4
Total testosterone (nmol/L)					
Q1 (≤ 12.6)	5.38 (0.03)	5.37 (0.03)	5.37 (0.03)	5.36 (0.03)	5.37 (0.03)
Q2 (12.7 - 15.9)	5.28 (0.03)	5.29 (0.03)	5.28 (0.03)	5.28 (0.03)	5.29 (0.03)
Q3 (16.0 - 19.8)	5.23 (0.03)	5.23 (0.03)	5.23 (0.03)	5.24 (0.03)	5.24 (0.03)
Q4 (≥ 19.9)	5.22 (0.03)	5.22 (0.03)	5.23 (0.03)	5.21 (0.03)	5.21 (0.03)
P value (F test)	0.001	0.002	0.003	0.004	0.003
P value (trend)	< 0.001	< 0.001	0.001	< 0.001	< 0.001
SHBG (nmol/L)					
Q1 (≤ 30.6)	5.29 (0.03)	5.33 (0.03)	5.32 (0.03)	5.33 (0.03)	5.33 (0.03)
Q2 (30.7 - 39.5)	5.28 (0.03)	5.29 (0.03)	5.28 (0.03)	5.29 (0.03)	5.29 (0.03)
Q3 (39.6 - 50.2)	5.27 (0.03)	5.25 (0.03)	5.25 (0.03)	5.25 (0.03)	5.25 (0.03)
Q4 (≥ 50.3)	5.28 (0.03)	5.25 (0.03)	5.26 (0.03)	5.24 (0.03)	5.23 (0.03)
P value (F test)	0.99	0.31	0.46	0.19	0.14
P value (trend)	0.91	0.08	0.15	0.03	0.02
Free testosterone (pmol/L)					
Q1 (≤ 234.1)	5.38 (0.03)	5.36 (0.03)	5.36 (0.03)	5.35 (0.03)	5.35 (0.03)
Q2 (234.2 - 294.3)	5.31 (0.03)	5.30 (0.03)	5.30 (0.03)	5.29 (0.03)	5.29 (0.03)
Q3 (294.4 - 364.7)	5.24 (0.03)	5.24 (0.03)	5.24 (0.03)	5.25 (0.03)	5.25 (0.03)
Q4 (≥ 364.8)	5.19 (0.03)	5.22 (0.03)	5.22 (0.03)	5.21 (0.03)	5.21 (0.03)
P value (F test)	< 0.001	0.005	0.007	0.01	0.01
P value (trend)	< 0.001	< 0.001	0.001	0.001	0.001

Abbreviations: HbA$_{1c}$, glycated haemoglobin; SHBG, sex hormone-binding globulin; SE, standard error.

Model 1: adjusted for age.
Model 2: adjusted for age and body mass index.
Model 3: adjusted for age, body mass index, smoking, alcohol consumption and physical activity.
Model 4: adjusted for age, waist circumference, smoking, alcohol consumption and physical activity.

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Linear regression analyses showed that lower TT and calculated FT levels were associated with higher HbA$_{1c}$ levels. The unadjusted change in HbA$_{1c}$ was 0.057 (95% CI 0.027; 0.087) per standard deviation (sd) decrease in TT and 0.060 (95% CI 0.030; 0.090) per sd decrease in calculated FT (Table 3). TT and calculated FT remained significantly associated with HbA$_{1c}$ after adjusting for age, BMI, smoking, alcohol consumption and physical activity. These associations also persisted after adding SHBG to the multivariable model. Although for SHBG the crude linear regression coefficient was not significant, this was significant (beta = 0.038 per sd decrease; 95% CI 0.004; 0.071) after adjusting for multiple covariates. SHBG remained significantly associated with HbA$_{1c}$ after adjusting for calculated FT, but not when TT was included in the multivariable model. Adjusting for sampling time did not alter the results (data not shown).

Table 3. Linear regression models representing change in glycated haemoglobin (%) per standard deviation increase in total testosterone, sex hormone-binding globulin and free testosterone in men without diabetes (N = 1136).

	HbA$_{1c}$ (%)	
	Regression coefficient	95 % CI
Total testosterone (per 5.6 nmol/L) decrease		
Crude	0.057	(0.027; 0.087)
Model 1	0.055	(0.025; 0.084)
Model 2	0.052	(0.022; 0.082)
Model 3	0.055	(0.025; 0.085)
Model 4	0.057	(0.027; 0.088)
Model 5	0.050	(0.016; 0.084)
SHBG (per 15.7 nmol/L) decrease		
Crude	0.005	(-0.025; 0.034)
Model 1	0.032	(0.000; 0.064)
Model 2	0.028	(-0.005; 0.061)
Model 3	0.038	(0.004; 0.071)
Model 4	0.040	(0.007; 0.074)
Model 5	0.012	(-0.026; 0.05)
Model 6	0.041	(0.008; 0.075)
Free testosterone (per 103.8 pmol/L) decrease		
Crude	0.060	(0.030; 0.090)
Model 1	0.045	(0.014; 0.076)
Model 2	0.043	(0.012; 0.074)
Model 3	0.041	(0.010; 0.073)
Model 4	0.042	(0.010; 0.073)
Model 6	0.044	(0.013; 0.076)

Abbreviations: HbA$_{1c}$, glycated haemoglobin; SHBG, sex hormone-binding globulin.

Model 1: adjusted for age.
Model 2: adjusted for age and body mass index.
Model 3: adjusted for age, body mass index, smoking, alcohol consumption and physical activity.
Model 4: adjusted for age, waist circumference, smoking, alcohol consumption and physical activity.

Discussion

Consistent with previous work [17], we found that circulating testosterone and SHBG levels were lowered in men with diabetes. The prevalence of subnormal TT levels was nearly doubled in men with self-reported or undiagnosed diabetes compared with the rest of the study sample. The prevalence of subnormal FT levels also increased across increasing HbA_{1c} categories. After excluding men with diabetes, TT and calculated FT levels remained inversely associated with $HbA_{1c,}$ independent of potential confounding factors including age, smoking, alcohol consumption and physical activity. Although crude SHBG levels were not significantly associated with HbA_{1c} in non-diabetic men, a significant linear trend was found after adjusting for multiple covariates.

In this study we observed a difference of approximately 0.15% HbA_{1c} between extreme quartiles of TT and calculated FT. Although these differences are relatively small compared with the reported contribution of HbA_{1c} to diabetes (HR = 1.8 per 1-percentage point increase in HbA_{1c}) [11] and cardiovascular disease risk (RR = 1.2 per 1-percentage point increase in HbA_{1c}) [10] in non-diabetic men, they may explain some of the association between endogenous testosterone and cardiometabolic risk in men. They also provide insights into physiological relationships between hormones and glucose metabolism. Our findings suggest that testosterone and SHBG are markers of pathological processes resulting in elevated glucose levels, even among men without diabetes. The presence of subnormal testosterone levels in non-diabetic men with raised HbA_{1c} levels may further provide useful directions for intervention and prevention.

Few studies have examined the associations between androgens and HbA_{1c} in men. In 391 elderly men a negative correlation between TT and HbA_{1c} was found [18], although this association did not persist after adjustment for BMI. Fernandez-Real et al [19] reported a negative correlation between SHBG and HbA_{1c} in 39 men and women with various degrees of obesity and glucose tolerance, but did not adjust this correlation for potential confounders. In men with type 2 diabetes, low circulating levels of TT have also been associated with poor glycemic control [20]. Only one study examined associations within the normal range of HbA_{1c}. In the Tromsø study [21] an independent association of TT and SHBG with HbA_{1c} was found in all participants, including men with diabetes. However, in contrast to our findings, this study failed to demonstrate an association in non-diabetic men. Our results are consistent with previous studies showing an inverse relationship of TT and SHBG with fasting glucose

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levels in non-diabetic men [6, 22]. The observed associations between FT and HbA$_{1c}$ have not been described previously in men without diabetes, but do agree with previous observations for fasting glucose levels [6, 22].

As expected, men in the highest HbA$_{1c}$ categories were more obese than the rest of the study sample. In men, low levels of testosterone and SHBG are associated with various measures of obesity [23]. Obesity could be a confounder or an intermediate in the causal pathway between testosterone and HbA$_{1c}$. In the current study, associations between testosterone, SHBG and HbA$_{1c}$ were not explained by differences in BMI and waist circumference, suggesting that testosterone and SHBG are associated with long-term glycemia independently of obesity. Previous studies have also reported independent associations of testosterone and SHBG with fasting glucose levels, HbA$_{1c}$ and insulin resistance [6, 7, 21].

Increasing evidence suggests a major role of SHBG in diabetes etiology. Several studies have demonstrated a strong independent association between SHBG and measures of glucose metabolism (diabetes [24] and insulin resistance [25]). Recently, polymorphisms in the SHBG gene have been shown to affect not only SHBG levels but also diabetes risk in men [24, 26], further supporting a role for SHBG in pathophysiological mechanisms. Since SHBG and testosterone are correlated, it is important to ascertain whether the observed association with SHBG represents an independent effect of SHBG or simply reflects the effect of testosterone. In our study, the associations of TT and calculated FT with HbA$_{1c}$ persisted after adjusting for SHBG. On the other hand, SHBG remained associated with HbA$_{1c}$ after adjusting for calculated FT, but not when TT was included in the multivariable model. These results suggest that the observed associations with HbA$_{1c}$ are primarily mediated through testosterone. Previous studies investigating the independent role of testosterone and SHBG have yielded inconsistent results. Yeap et al. [7] studied 2470 non-diabetic men and found that TT and FT were associated with insulin resistance independently of SHBG, whereas no such independent association was observed for SHBG. Our findings contrast with those from the Massachusetts Male Aging Study (MMAS). In this study low SHBG levels were independently associated with incident diabetes in 1218 men aged 40-70 years, even after adjusting for TT and FT [27], while no independent associations were found for TT and FT. Similar results have been reported for insulin resistance [28]. It remains unclear what causes this inconsistency, although differences in sample size may partly explain the lack of association with testosterone found in the latter study. Therefore further studies are needed to explore the exact role of testosterone and SHBG in glucose metabolism.

The present study had limitations. The study population was not a representative

sample of the EPIC Norfolk cohort as testosterone and SHBG measurements were only available for a subset of men who represented cases or controls in prior studies relating baseline testosterone and SHBG levels to incident prostate cancer and mortality. Although this specific subset of men has an overrepresentation of men with increased risk of future morbidity and mortality, all men were considered to be apparently healthy at baseline. The use of blood samples collected throughout the day may have resulted in additional errors because of diurnal variation in hormone levels. However, adjusting for sampling time in the analyses did not alter the results. Furthermore, testosterone, SHBG and HbA_{1c} were measured only once and we did not have information on medications that may affect testosterone and SHBG levels or glucose metabolism. Nevertheless, all these measurement errors are expected to be random and may therefore only have attenuated any underlying associations. We considered age, smoking, alcohol intake and physical activity to be potential confounders. Although associations remained significant after adjusting for these confounders, residual confounding (by known and unknown factors) cannot be ruled out. Potential confounding effects of insulin and body fat, for instance, could not be assessed, because these variables were not measured in this study.

Another limitation is the cross-sectional design, which prevents us from drawing conclusions about the causal direction of the observed associations. There is, however, evidence to support causal associations in both directions. Hyperglycemia may lower testosterone production through impaired testicular and adrenal function [29, 30]. On the other hand, testosterone replacement therapy reduces insulin resistance and improves glycemic control in hypogonadal men with type 2 diabetes [31] and the metabolic syndrome [32]. Similar effects of testosterone replacement have been observed in healthy elderly men, including a decrease in fasting glucose levels and insulin resistance [33]. There are also indications for a bidirectional relationship between SHBG and glycemia. Experimental studies in transgenic mice and HepG2 hepatoblastoma cell models show that monosaccharides, including glucose and fructose, reduce hepatic SHBG production [34]. Simultaneously, low SHBG has been reported to be an independent predictor of diabetes and recently SHBG polymorphisms have been associated with diabetes risk [24, 26].

In summary, low circulating levels of TT, SHBG and FT are independently associated with an increase in HbA_{1c} in middle-aged and older men, even below the threshold for diabetes. Clarifying the nature of this relationship may provide new insights into determinants of glucose and sex hormone metabolism and how these may contribute to risk of cardiovascular disease.

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Chapter 9

**Testosterone, SHBG and differential white blood cell count
in middle-aged and older men**

Abstract

Objective: Low-grade chronic inflammation is increasingly being implicated in cardiovascular disease (CVD) etiology and may represent an alternative pathway through which testosterone and sex hormone-binding globulin (SHBG) influence CVD risk. We examined the associations between endogenous testosterone, SHBG and total and differential white blood cell (WBC) count in men.

Methods: Cross-sectional study of 2418 men aged 40-78 years from the Norfolk population of European Prospective Investigation into Cancer (EPIC-Norfolk) who had no history of CVD or cancer and complete data on sex hormones (total testosterone (TT), SHBG and free testosterone (FT)) and WBC counts. Associations between sex hormones and WBC count were assessed using linear regression models.

Results: Higher SHBG and TT levels were associated with lower WBC counts. After adjustment for age, BMI, smoking, physical activity and diabetes status, total WBC count decreased by 0.163 (95% CI -0.236; -0.091) and 0.102 (-0.170; -0.034) per standard deviation (sd) increase in SHBG and TT respectively. Associations of SHBG and TT with total WBC count were mainly accounted for by a lower granulocyte count (β coefficient = -0.132 (-0.194; -0.070) per sd increase in SHBG and β coefficient = -0.104 (-0.161; -0.046) per sd increase in TT). No associations between FT and total and differential WBC count were found.

Conclusions: Endogenous TT and SHBG levels are inversely associated with total WBC and granulocyte count in middle-aged and older men. Even though the underlying mechanism and causal directionality requires further exploration, these results support a link between hormonal status and low-grade inflammation.

Introduction

Cardiovascular disease (CVD) is the leading cause of death and a major determinant of chronic disability worldwide. Even though men have a higher incidence and earlier onset of CVD than women [1] testosterone does not appear to have a detrimental effect on the cardiovascular system. On the contrary, high circulating levels of testosterone have been associated with a more favourable cardiovascular risk profile [2] and a reduced risk of atherosclerosis [3]. High endogenous testosterone levels have also been associated with a reduction in cardiovascular morbidity and mortality [4, 5]. Remarkably, associations between endogenous testosterone and CVD remain unchanged after adjustments for traditional cardiovascular risk factors (e.g. age, hypertension, obesity and diabetes) [4, 5], suggesting the involvement of alternative pathways through which testosterone is linked to CVD risk.

Low grade inflammation is increasingly being implicated in the pathogenesis of cardiovascular disease and there is some evidence suggesting a link with testosterone. In men, the age-related decrease in testosterone levels is paralleled by an increase in inflammatory markers [6, 7] and low testosterone levels are common in patients with chronic inflammatory conditions such as rheumatoid arthritis [8]. Furthermore, acute withdrawal of testosterone in normal elderly men seems to increase the inflammatory response [9].

White blood cell (WBC) count is a stable and well-standardized inflammatory marker and several epidemiological studies have reported an association between a higher total WBC count and cardiovascular morbidity and mortality [10, 11]. The contribution of individual WBC subsets varies with neutrophils or granulocytes having the highest predictive value [10, 11]. The relation between testosterone and WBC and its subfractions is not well established. We therefore examined this association cross-sectionally in a population-based sample of middle-aged and older men.

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Methods

Study population
The European Prospective Investigation into Cancer in Norfolk (EPIC-Norfolk) is a population-based prospective study of ~ 25 000 men and women living in Norfolk, United Kingdom. Between 1993 and 1997 ~ 30 000 men and women aged 40 - 79 years were recruited through general practice age-sex registries. Participants who consented completed a baseline questionnaire survey that included questions

on cigarette smoking habit, physical activity (work and leisure activity) and questions whether a doctor ever informed them of having any medical condition, including diabetes, heart disease and stroke. Participants were then invited for a baseline Health Check (1993 – 1997) at which anthropometric variables (BMI and waist circumference) and non-fasting blood samples were collected. Blood samples were processed at the EPIC Norfolk research laboratory and stored in liquid nitrogen in separate aliquots of serum and plasma. From 1994, when funding became available, an additional EDTA anticoagulated blood sample was collected. The present study was based on a subset of men who were selected for a series of case control studies on incident prostate cancer [12] and mortality [4].

Biochemical analyses
WBC count was measured in approximately 75% of the baseline cohort. Absolute blood cell counts were determined on fresh EDTA samples using the HMD18 haematology analyser (Coulter Corporation, Miami, FL, USA). The WBC coefficient of variation was $\leq$ 3.0%. The standard deviations for the differential lymphocyte, monocyte and granulocyte counts were less than or equal to 1.5, 1.5 and 3.0 respectively [13]. Total testosterone (TT) and SHBG levels were measured in a subset of men who were selected for a series of nested case-control studies. In 2003 frozen serum samples (taken at the baseline visit) were thawed and TT and SHBG levels were measured using an Immulite Chemiluminescent Immunoassay system (Siemens Healthcare Diagnostics Inc. Frimley, Surrey, United Kingdom). The intra-assay and inter-assay coefficients of variation were 7.5% and 6.6% for TT and 6.1% and 7.5% for SHBG respectively. The lower limit of detection was 0.3 nmol/L for TT and 0.2 nmol/L for SHBG. Free testosterone (FT) levels were calculated using the algorithm of Vermeulen et al. [14] based on TT and SHBG concentrations and assuming a fixed albumin concentration of 43 g/L.

Data analyses
Of the participants who completed the baseline questionnaire and attended the Health Check, 2856 men had data on sex hormones and WBC available. To prevent confounding by pre-existing disease, 407 men with prevalent disease conditions (heart disease, stroke and cancer) were excluded. We also excluded participants (N = 31) with clinically abnormal WBC values (> 15.0 x 10^3 cells/μL) or sex hormone levels greater than four standard deviations from the mean, leaving 2418 individuals for the analyses.

Distributions of baseline characteristics were examined across quartiles of TT using univariate analysis of variance for continuous variables and Chi-Square tests for categorical variables.

Next, sex hormones were divided into quartiles based on their distribution in the study population. Analysis of covariance was used to calculate adjusted means of total WBC count across quartiles of TT, SHBG and FT. The covariates included age (years), BMI (kg/m^2), waist circumference (cm), physical activity (inactive, moderately inactive, moderately active, active), smoking habit (current, former, never) and self reported diabetes (yes vs no). To assess the linearity of the associations, tests of linear trend were performed by entering the quartiles as continuous terms. We further explored the associations of TT, SHBG and FT with total and differential leukocyte counts using multiple linear regression models.

All statistical analyses were performed using SPSS statistical package for Windows version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

The mean age of the population was 63.9 ± 8.0 years. The mean WBC count was 6.7 x 10^3 cells/µL and 11.2% of the participants were current smokers. Characteristics of the study population are summarized by quartiles of TT in Table 1. Men with low TT levels were older and had a higher BMI and waist circumference. Men with low TT levels were also less likely to be smokers and more often reported having diabetes.

Table 2 shows the adjusted means of total WBC count across TT, SHBG and FT quartiles. SHBG levels were inversely associated with total WBC count. In the multivariable adjusted model, mean WBC count was 0.45 x 10^3 cell/µL higher in the lowest quartile as compared with the highest quartile (*P* trend < 0.001). A similar linear association was observed for TT (*P* trend = 0.01), with total WBC count being highest in the lowest TT quartile. Mean WBC count did not vary with FT quartiles, neither in crude nor in adjusted analyses.

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Table 1. Study characteristics by quartiles of total testosterone.

	Total testosterone quartiles				
	< 12.7 (N = 597)	12.7 – 15.8 (N = 604)	15.9 – 19.5 (N = 610)	> 19.5 (N = 607)	P value
Age, years, mean (SD)	64.5 (8.2)	64.2 (7.8)	63.8 (7.9)	63.2 (8.1)	0.03
Body mass index, kg/m², mean (SD)	27.9 (3.8)	26.8 (3.2)	26.5 (3.2)	25.9 (3.0)	< 0.001
Waist circumference, cm, mean (SD)	101.2 (10.7)	97.2 (9.4)	96.3 (9.6)	94.2 (9.5)	< 0.001
White blood cell count x 10^3 cells/L, mean (SD)	6.81 (1.80)	6.67 (1.70)	6.67 (1.70)	6.59 (1.62)	0.15
Total testosterone, nmol/L, mean (SD)	10.0 (2.1)	14.3 (0.9)	17.5 (1.0)	23.6 (3.7)	< 0.001
SHBG, nmol/L, mean (SD)	33.2 (11.6)	39.2 (12.7)	43.2 (12.7)	51.61 (15.9)	< 0.001
Free testosterone, pmol/L, mean (SD)	203.4 (55.0)	271.7 (54.6)	319.8 (63.3)	403.1 (93.9)	< 0.001
Smoking, % (n)					< 0.001
Current	8.0 (47)	9.5 (57)	10.7 (65)	17.0 (102)	
Former	63.8 (375)	59.2 (354)	55.1 (335)	54.4 (327)	
Never	28.2 (166)	31.3 (187)	34.2 (208)	28.6 (172)	
Physical activity, % (n)					0.24
Inactive	40.5 (242)	36.3 (219)	36.2 (221)	36.9 (224)	
Moderately inactive	24.1 (144)	25.3 (153)	22.8 (139)	21.6 (131)	
Moderately active	17.4 (104)	19.5 (118)	23.6 (144)	22.1 (134)	
Active	17.9 (107)	18.9 (114)	17.4 (106)	19.4 (118)	
Self reported diabetes, % (n)	7.2 (43)	5.3 (32)	4.1 (25)	2.8 (17)	0.003

Abbreviations: SD = standard deviation, SHBG = sex hormone-binding globulin.

Table 2. Mean white blood cell count by quartiles of total testosterone, sex hormone-binding globulin and free testosterone, crude and adjusted for covariates.

Hormone quartiles	White blood cell count 10^3 cells/μL, mean (95% CI)				
	Crude	Model 1	Model 2	Model 3	Model 4
SHBG (nmol/L)					
Q1 < 31.6	6.84 (6.70-6.97)	6.85 (6.70-6.99)	6.84 (6.70-6.98)	6.91 (6.77-7.05)	6.91 (6.78-7.05)
Q2 31.6 – 40.0	6.70 (6.57-6.84)	6.71 (6.57-6.84)	6.71 (6.58-6.85)	6.74 (6.61-6.87)	6.74 (6.61-6.87)
Q3 40.1 – 50.0	6.64 (6.51-6.78)	6.64 (6.50-6.77)	6.64 (6.51-6.78)	6.63 (6.49-6.76)	6.62 (6.49-6.76)
Q4 > 50.0	6.56 (6.42-6.70)	6.55 (6.41-6.69)	6.54 (6.40-6.68)	6.46 (6.33-6.60)	6.46 (6.32-6.60)
P trend	0.004	0.004	0.004	< 0.001	< 0.001
Total testosterone (nmol/L)					
Q1 < 12.7	6.81 (6.68-6.95)	6.78 (6.64-6.92)	6.78 (6.64-6.92)	6.81 (6.67-6.94)	6.81 (6.68-6.95)
Q2 12.7 – 15.8	6.67 (6.53-6.80)	6.67 (6.53-6.80)	6.67 (6.53-6.81)	6.69 (6.56-6.82)	6.69 (6.56-6.82)
Q3 15.9 – 19.5	6.67 (6.54-6.81)	6.68 (6.54-6.81)	6.68 (6.55-6.82)	6.70 (6.57-6.83)	6.70 (6.57-6.83)
Q4 > 19.5	6.59 (6.45-6.73)	6.61 (6.48-6.75)	6.61 (6.48-6.75)	6.55 (6.41-6.68)	6.55 (6.41-6.68)
P trend	0.03	0.12	0.13	0.01	0.01
Free testosterone (pmol/L)					
Q1 < 230.9	6.78 (6.65-6.92)	6.76 (6.62-6.90)	6.75 (6.61-6.89)	6.75 (6.61-6.88)	6.75 (6.61-6.88)
Q2 230.9 – 288.2	6.65 (6.51-6.78)	6.64 (6.50-6.78)	6.65 (6.51-6.78)	6.66 (6.53-6.79)	6.66 (6.53-6.79)
Q3 288.3 – 356.3	6.61 (6.48-6.75)	6.62 (6.48-6.76)	6.62 (6.49-6.76)	6.65 (6.52-6.78)	6.65 (6.52-6.78)
Q4 > 356.3	6.70 (6.56-6.83)	6.72 (6.58-6.86)	6.72 (6.49-6.76)	6.69 (6.55-6.82)	6.69 (6.55-6.82)
P trend	0.35	0.70	0.72	0.53	0.53

Abbreviations: CI = confidence interval; SHBG = sex hormone-binding globulin.

Model 1: adjusted for age and BMI.
Model 2: adjusted for age, BMI and physical activity.
Model 3: adjusted for age, BMI, physical activity and smoking.
Model 4: adjusted for age, BMI, physical activity, smoking and diabetes.

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Table 3 shows the results from the linear regression analyses. High SHBG levels were associated with a lower total WBC count in the entire population, crudely and after adjustment for age, BMI, physical activity, smoking and diabetes status (β coefficient = -0.163; 95% CI -0.236; -0.091). Among the WBC components, SHBG levels were primarily associated with a lower granulocyte count (β coefficient = -0.132; 95% CI -0.194; -0.070) and weakly associated with a lower lymphocyte count (β coefficient = -0.044; 95% CI -0.071; -0.017). TT levels were also associated with a lower total WBC count after adjusting for smoking habit (β coefficient = -0.102; 95% CI -0.170; -0.034). As for SHBG, TT levels were inversely associated with a lower granulocyte count (β coefficient = -0.104; 95% CI -0.161; -0.046). No significant associations between FT and total and differential leukocyte counts were found. Results were not materially different when we adjusted for waist circumference in stead of BMI (data not shown).

Table 3. Linear regression models presenting change in total and differential white blood cell counts per standard deviation increase in total testosterone, sex hormone-binding globulin and free testosterone

	β coefficient (95% CI)			
	White blood cell count (x 10³ cells/ μL)	Lymphocyte count (x 10³ cells/ μL)	Monocyte count (x 10³ cells/ μL)	Granulocyte count (x 10³ cells/ μL)
SHBG (per sd increase)				
Crude	-0.101 (-0.169; -0.033)	-0.063 (-0.089; -0.038)	0.022 (0.007; 0.038)	-0.061 (-0.118; -0.004)
Model 1	-0.110 (-0.185; -0.035)	-0.029 (-0.057; -0.002)	0.018 (0.002; 0.035)	-0.098 (-0.160; -0.036)
Model 2	-0.110 (-0.185; -0.036)	-0.029 (-0.056; -0.001)	0.019 (0.003; 0.035)	-0.098 (-0.160; -0.036)
Model 3	-0.162 (-0.234; -0.090)	-0.045 (-0.072; -0.018)	0.016 (-0.001; 0.032)	-0.130 (-0.192; -0.068)
Model 4	-0.163 (-0.236; -0.091)	-0.044 (-0.071; -0.017)	0.016 (-0.001; 0.032)	-0.132 (-0.194; -0.070)
Total testosterone (per sd increase)				
Crude	-0.082 (-0.151; -0.014)	-0.005 (-0.031; 0.020)	0.007 (-0.008; 0.022)	-0.082 (-0.139; -0.025)
Model 1	-0.064 (-0.133; 0.006)	0.010 (-0.015; 0.036)	0.011 (-0.004; 0.027)	-0.080 (-0.138; -0.021)
Model 2	-0.061 (-0.131; 0.008)	0.011 (-0.015; 0.037)	0.012 (-0.004; 0.027)	-0.078 (-0.136; -0.020)
Model 3	-0.101 (-0.169; -0.033)	-0.002 (-0.027; 0.024)	0.009 (-0.007; 0.025)	-0.103 (-0.160; -0.045)
Model 4	-0.102 (-0.170; -0.034)	-0.001 (-0.026; 0.024)	0.009 (-0.007; 0.025)	-0.104 (-0.161; -0.046)
Free testosterone (per sd increase)				
Crude	-0.024 (-0.092; 0.045)	0.033 (0.007; 0.058)	-0.008 (-0.023; 0.007)	-0.044 (-0.101; 0.012)
Model 1	-0.005 (-0.076; 0.066)	0.025 (-0.001; 0.051)	0.001 (-0.014; 0.017)	-0.025 (-0.084; 0.034)
Model 2	-0.002 (-0.072; 0.069)	0.026 (0.000; 0.052)	0.002 (-0.014; 0.017)	-0.023 (-0.082; 0.036)
Model 3	-0.018 (-0.087; 0.051)	0.020 (-0.006; 0.045)	0.000 (-0.015; 0.016)	-0.032 (-0.091; 0.026)
Model 4	-0.018 (-0.087; 0.051)	0.020 (-0.006; 0.045)	0.000 (-0.015; 0.016)	-0.032 (-0.091; 0.026)

Abbreviations: CI = confidence interval; SHBG = sex hormone-binding globulin.

Model 1: adjusted for age and BMI.
Model 2: adjusted for age, BMI and physical activity.
Model 3: adjusted for age, BMI, physical activity and smoking.
Model 4: adjusted for age, BMI, physical activity, smoking and diabetes.

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Discussion

In this cross-sectional study SHBG and TT levels were inversely associated with total WBC count, independently of age, BMI, physical activity, smoking and diabetes status. No association between FT and total WBC count was found. Analyses for differential leukocyte counts showed that associations of TT and SHBG with total WBC count were mainly accounted for by the granulocyte subset.

Previous studies examining associations between sex hormones and inflammatory markers have yielded conflicting results. A strong inverse association of testosterone and SHBG with C-reactive protein (CRP) has been reported [15, 16], but this has not been confirmed by others [17, 18]. In the InChianti study [19] an inverse association between TT and soluble interleukin-6 receptor (sIL6r) was found but no association with pro-inflammatory cytokines or CRP levels. Results from experimental studies are also mixed with some studies showing a reduction in inflammatory markers after testosterone administration [20, 21] and others reporting no change [22-24]. This inconsistency may be partly due to differences in study population. Anti-inflammatory effects of testosterone replacement are primarily observed in men with comorbid conditions or abnormally low testosterone levels [20, 21]. To confirm a role of testosterone in inflammation, more large scale intervention studies are needed that allow subgroup analyses.

Data on the association with WBC count are limited, but seem to be more consistent. Tang et al. [25] found an inverse association between TT and WBC count in 381 elderly men. Similarly, Schneider et al. [26] reported an inverse association between TT and acute inflammatory reactions. A recent case report study also described a decrease in total leukocyte count in hypogonadal diabetic men following testosterone treatment [27]. To our knowledge, this is the first study to report associations with SHBG and differential WBC count. The strong association with granulocyte count is in line with the previously reported contribution of this WBC subset to CVD risk [10, 11]. Interestingly, the inverse association with SHBG appears to be stronger than that for testosterone. The mechanisms beyond the observed associations of TT and SHBG with total WBC and granulocyte counts are not completely understood. In gonadectomised male mice, a suppressive effect of testosterone on leukocyte production has been reported [28]. In young male rats, a similar reduction in leukocyte count was found following testosterone administration [29]. Recently, an effect of testosterone on neutrophil function and viability was also demonstrated in blood neutrophils isolated from healthy men [30]. So far, androgen receptors have been identified in various human leukocytes with a particularly high expression in neutrophils [31]. Whether the leukocyte

loss is the direct consequence of testosterone-binding to these cells, however, is not known.

Several limitations of this study need to be acknowledged. First, the study population was not a representative sample of the EPIC Norfolk cohort as testosterone and SHBG measurements were only available for a subset of men who represented cases or controls in prior studies relating baseline sex hormone levels to incident prostate cancer and mortality. Although this specific subset has an over-representation of men with increased risk of future morbidity and mortality, all men were considered to be healthy at baseline. Moreover, we excluded all men with a history of chronic disease and clinically abnormal WBC values. Hence, potential bias resulting from this selection is supposed to be negligible. Second, hormone measurements were based on a single sample collected throughout the day which may have introduced errors due to intra-individual and diurnal variation. Another limitation is that insulin levels and visceral fat were not measured in this study. Although associations remained significant after adjusting for age, BMI, physical activity, smoking and diabetes status, residual confounding by these and other unknown factors cannot be excluded. Finally, the cross-sectional design of the study precludes us from drawing conclusions about the causal directionality of the observed associations. Reverse causation cannot be neglected. Previous studies have demonstrated that pro-inflammatory cytokines inhibit testosterone secretion through their influence on the hypothalamic-pituitary-gonadotropic axis [32, 33]. Experimental data further suggest that kisspeptin, a modulator of GnRH function may repress the gonadotropic axis during acute inflammation resulting in a fall in testosterone levels [34]. Additionally, inflammatory molecules may influence liver metabolic function including the production of SHBG. We also cannot exclude the possibility that the fall in testosterone and SHBG and the increase in WBC result from distinct processes and are not linked in a direct manner. However, the observed increase in inflammatory cytokines in men with experimentally induced hypogonadism [9] and the immunosuppressive effect of androgen-stimulating therapy in hypogonadal men [35] seem to support a causal role for testosterone in inflammation.

In conclusion, TT and SHBG are inversely associated with total WBC and granulocyte counts. Although the underlying mechanism and causal directionality are not entirely understood, the present data support a link between testosterone and SHBG in low-grade inflammation and thereby provide an alternative mechanism by which sex hormones may influence cardiovascular risk in men. More experimental studies are needed to increase our understanding of the physiological basis of the observed associations.

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Chapter 10

Endogenous sex hormones and subclinical atherosclerosis in middle-aged and older men

Abstract

Background: Circulating sex hormone concentrations are associated with a wide range of cardiovascular risk factors in men, but data on cardiovascular events have been conflicting. We examined the cross-sectional and longitudinal associations between endogenous sex hormones and markers of subclinical atherosclerosis.

Methods: This was a population-based study of 400 middle-aged and older men, 270 of whom underwent a re-examination after a median follow-up of 8.8 years. Serum concentrations of sex hormones (total testosterone (TT), sex hormone-binding globulin (SHBG), estradiol (E2) and dihydroepiandrosterone sulfate (DHEAS) were measured at baseline and free testosterone (FT) and estradiol (FE2) concentrations were calculated. At baseline and follow-up, carotid intima-media thickness (cIMT) and pulse wave velocity (PWV) were assessed as measures of subclinical atherosclerosis. In addition, carotid artery plaque burden was evaluated at follow-up.

Results: There was no overall association between endogenous sex hormone concentrations and cIMT and PWV in either cross-sectional or longitudinal analyses. However, stratified analyses at baseline showed a significant interaction with age, such that low TT and SHBG concentrations were associated with higher PWV values in elderly men only. No association with carotid plaque burden was found. All associations remained unchanged after multivariable adjustment and in sensitivity analyses excluding prevent CVD cases and men using antihypertensive and lipid-lowering medication.

Conclusions: These data do not support a major role of endogenous sex hormones in subclinical atherosclerosis in middle-aged and older men. However, the presence of an age-specific association with arterial stiffness is suggestive of a threshold-relationship and merits further investigation.

Introduction

It is a universal finding that men have a higher risk of cardiovascular disease (CVD) than women, although the female advantage is apparently lost in postmenopausal women and women with hyperandrogenism [1-3]. These observations have led to the hypothesis that endogenous androgens and estrogens may play an important role in CVD etiology. Indeed, there is now substantial evidence suggesting that sex hormones may influence male CVD risk. Observational studies have linked endogenous sex hormone concentrations to a wide range of cardiovascular risk factors [4]. Experimental data also support a role for sex hormones in the pathogenesis of CVD. Androgen and estrogen receptors are present in both vascular smooth muscle and endothelial cells, and animal and *in vitro* studies have demonstrated direct and indirect effects of sex hormones on atherosclerosis progression [5-7].

However, prospective studies relating endogenous sex hormones to incident CVD in men have been inconclusive. For example, low testosterone concentrations have been associated with an increase in CVD morbidity and mortality [8-10], but others have reported no association between testosterone and cardiovascular events [11-13]. On a parallel note, low concentrations of dehydroepiandrosterone sulfate (DHEAS) have been associated with a greater CVD risk in some [14, 15] but not all studies [12, 16]. Data on the role of estradiol are even more conflicting, with studies reporting either a positive [17], inverse [12] or null association [13, 18].

Studies examining associations with markers of subclinical disease may help clarifying the role of sex hormones in male CVD risk. Pulse-wave velocity (PWV) is a reproducible and non-invasive marker of arterial stiffness [19], and has been shown to be predictive of cardiovascular events in various populations [20]. Carotid intima-media thickness (cIMT) as measured with B-mode ultrasound is an indicator of early atherosclerotic changes [21], whereas carotid plaques represent a later stage of the atherosclerotic process. Like PWV, cIMT and carotid plaques are good predictors of future CVD [22]. In the present study, we aimed to assess the cross-sectional and longitudinal associations between endogenous sex hormones and markers of subclinical atherosclerosis in a cohort of middle-aged and older men.

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Methods

Study population
Between March 2001 and April 2002, 400 independently living men aged 40 to 80 years were enrolled in a population-based cohort study. Details of the study design and recruitment procedures have been described elsewhere [23]. In brief, participants were recruited from two sources (convenience sampling and a random selection of the municipal register). All participants attended two baseline visits, at which a blood sample was taken and information on cardiovascular measurements, medical history and lifestyle factors was obtained.

Between 2010 and 2011, all participants who were still alive and not living abroad (N = 346), were invited for a re-examination. We were able to re-examine a total of 270 men (participation rate = 68%). Reasons for non-participation were physically or mentally unable to visit the study center (N = 40), not interested (N = 22) and non-response (N = 14). The Institutional Review Board of the University Medical Center Utrecht approved the baseline and follow-up studies and written informed consent was obtained from all participants.

Laboratory measurements
At baseline, blood samples were collected between 8 and 10 am after an overnight fast. Platelet-free serum was obtained by centrifugation and stored at -80 ^{0}C. Details of sex hormone measurements have been described elsewhere [23]. Total testosterone (TT) concentrations were measured in serum after diethylether extraction using an in-house competitive RIA. The lower limit of detection was 0.24 nmol/L and intra-assay variation was 6.0, 5.4, and 8.6% at 2.1, 5.6 and 23.0 nmol/L, respectively. SHBG was measured in serum using an immunometric technique on an IMMULITE analyzer (Diagnostic Products Corp., Los Angeles, CA, USA). The lower limit of detection was 5 nmol/L, and intra-assay variation was 6.1, 4.9, and 6.9% at 11.6, 36.0, and 93.0 nmol/L, respectively. Total estradiol (E2) was measured after diethylextraction and Sephadex chromatography using an in-house competitive RIA. The lower limit of detection was 20 pmol/L, and interassay variation was 10.0 and 3.1% at 81 and 660 pmol/L respectively. DHEAS was measured using an immunometric technique on an Advantage Chemiluminescence System (Nichols Institute Diagnostics, San Juan Capistrano, CA). The lower limit of detection was 0,1 umol/L, and interassay variation was 5.2, 5.6, and 4.2% at 1.0, 4.9, and 14.2 umol/L, respectively. Free testosterone (FT) and estradiol (FE2) concentrations were calculated using the algorithm of Vermeulen et al. [24].

Total cholesterol was measured using an automatic enzymatic procedure (Synchron LX Systems; Beckman Coulter, Mydrecht, The Netherlands) and fasting blood glucose was measured using a GlucoTouch reflectometer (LifeScan Inc., Benelux, Milpitas, CA, USA).

Measurement of subclinical atherosclerosis
Ultrasonography of both the left and right carotid artery was performed using a 7.5-MHz linear array transducer (Acuson Aspen). The media-adventitia of the near and the far wall of the distal common carotid artery were recorded at four predefined angles per side (180, 150, 120 and 90 ^{0} for the right carotid artery and 180, 210, 240 and 270 ^{0} for the left carotid artery) using Meijer's arc [25]. The average of the eight predefined angles was used for each subject as a measure of cIMT. PWV was determined using a SphygmoCor device (PWV Medical, Sydney, Australia), which allows an online pulse wave recording and automatic calculation of PWV. Two transducers (Millar SPT 301 pressure transducer; Milar Instruments, Sydney, Australia) were used; one positioned at the base or the neck over the common carotid artery and the other placed over the femoral artery. An average of 10 successive waveforms was used in the analyses to cover a complete respiratory cycle. The whole procedure was repeated three times per participant and the average PWV value was used for the analysis. In our laboratory, the intra-class correlation coefficients (ICC) for repeated cIMT and PWV measurements were 84% and 85% respectively.
At baseline, 399 men underwent cIMT ultrasonography and PWV was measured in 376 men. Two extreme values in PWV (2.75 and 30.51 m/s) were excluded, because they were considered biologically implausible. At follow-up, cIMT and PWV measurements were performed in 265 men and 243 men, respectively. Missing information at baseline and follow-up was entirely due to logistical reasons. At follow-up, we also visually inspected multiple arterial sites for the presence and severity of plaques (N = 249). For this purpose, ultrasound images were collected of the near and far walls of the right and left common carotid artery, the bifurcation and the internal carotid artery (2 x 2 x 3 = 12 arterial sites) and scored using a 4-level rating scale: 0 = no plaque, 1 = minimal plaque, 2 = moderate plaque and 3 = severe plaque [26]. For each participant, we calculated a mean plaque score (the average score of the 12 arterial sites) and a maximum plaque score which represents the most severe lesion at any of the 12 sites.

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Other measurements

At baseline, information on medical history and lifestyle factors was collected. Diabetes mellitus was defined as treatment with insulin or oral hypoglycaemic agents or a fasting plasma glucose ≥ 7.0 mmol/L. Cardiovascular disease was defined as present when participants reported a history of coronary heart disease, peripheral artery disease and/or stroke. Smoking status was based on self-report and categorized as current, former or never, and alcohol consumption was estimated from a validated food frequency questionnaire [27]. Participants were also asked about their current and past use of antihypertensive and lipid-lowering medication. To examine the impact of changes in medication use, we also collected information on antihypertensive and lipid-lowering medication at follow-up.

During the baseline examination, height and weight were measured in standing position without shoes. BMI was calculated as the weight in kilograms divided by the square of the height in meters. Waist circumference was measured midway between the lower rib margin and iliac crest with participants in standing position. Blood pressure was measured twice after a 10 min rest in the brachial artery (sitting position) with a semiautomated device (Dinamap 8100; Critikon Inc., Tampa, Finland). Mean systolic and diastolic blood pressure were based on two blood pressure measurements and the mean arterial pressure (MAP) was calculated as (2*DBP+SBP)/3.

Data analyses

We first examined the cross-sectional associations between sex hormones and cIMT and PWV at baseline. For this, sex hormones were divided into quartiles based on their distribution in the population. We used analysis of covariance to calculate adjusted means of cIMT and PWV across quartiles of TT, SHBG, FT, E2 and DHEAS. Covariates included in the analyses were age (years), BMI (kg/m^2), smoking (current, former, never), alcohol consumption (g/day) and antihypertensive and lipid-lowering medication (yes vs. no). Analyses for PWV were additionally adjusted for mean arterial pressure (MAP) and heart rate (beats per minute). To investigate whether associations were independent of potential intermediate factors, we further adjusted the analyses for systolic blood pressure (mm Hg), HDL cholesterol (mmol/L), triglycerides (mmol/L) and diabetes mellitus (yes vs. no). To assess the linearity of the associations, tests of linear trend were performed by entering the median of each quartile as a continuous term into the analyses. Sex hormones were also modelled per standard deviation (sd) decrease to obtain a summary measure of association.

Longitudinal associations between baseline sex hormone concentrations and cIMT and PWV change were assessed using generalized estimation equation (GEE) models [28], accounting for within-subject correlation and incorporating the following variables: sex hormones, time, interaction between sex hormones and time and all other covariates as described for the cross-sectional analysis. Time was defined as the interval between the date of recruitment and the date of re-examination (in years). The average annual rate of cIMT and PWV change was estimated from the regression coefficient of the main effect of time. Sex hormones were modelled per sd decrease, and the rate of change in cIMT and PWV per sd decrease in sex hormones was estimated from the regression coefficient of the interaction term. We used analysis of covariance to asses the association between baseline sex hormone concentrations and carotid plaques scores at follow-up.

We also conducted a series of sensitivity analyses. Recently, it has been suggested that associations between sex hormones and CVD risk may vary depending on age [29]. We therefore explored a possible interaction by age by adding a continuous interaction term to the multivariable adjusted model (Model 2). Second, we explored the impact of potential selection bias due to non-participation at follow-up using inverse probability weighting [30]. Finally, we repeated the analyses after excluding men with a history of cardiovascular disease and men using antihypertensive and lipid-lowering medication (at baseline and follow-up). All statistical analyses were performed using STATA, version 11.0 (Stata Corp., College Station, TX, USA).

Results

Table 1 shows the baseline characteristics of the study population. The mean (sd) age at entry was 60 (11) years and the mean (sd) BMI was 26.3 (3.5) kg/m^2. Antihypertensive drugs were used by 17.0% of the population and 12.5% used lipid-lowering medication. In total, 399 men had measurements of cIMT and 376 men had measurements of PWV: mean (sd) cIMT was 0.82 (0.15) mm and mean (sd) PWV was 9.41 (2.49) m/s at baseline.

Results of the cross-sectional analyses are presented in Table 2. After adjustment for age, an inverse relation between SHBG and PWV was observed (beta = 0.22 (95% CI: 0.01; 0.43) per sd decrease in SHBG), but this association was no longer significant after adjustment for potential confounders. Serum concentrations of TT, FT, E2 and DHEAS were not associated with either PWV or cIMT at baseline (Table 2). Results remained unchanged after excluding men with prevalent CVD and men using antihypertensive and lipid-lowering medication.

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Table 1. Baseline characteristics of the total cohort and stratified by participation at follow-up.

	Total population at baseline (N = 400)	Participation at follow-up (N = 270)	No participation at follow-up (N = 130)
Age (years), mean (SD)	60.2 (11.3)	57.7 (10.6)	63.5 (11.0)
Body mass index (kg/m^2), mean (SD)	26.3 (3.5)	26.2 (3.4)	26.5 (3.6)
Waist circumference (cm), mean (SD)	98.9 (9.4)	98.3 (9.4)	100.1 (9.2)
Total testosterone (nmol/L), mean (SD)	18.5 (5.3)	18.6 (5.3)	18.5 (5.4)
SHBG (nmol/L), mean (SD)	40.6 (14.5)	39.7 (14.9)	42.4 (13.4)
Free testosterone (pmol/L), mean (SD)	354.2 (98.1)	361.0 (100.7)	340.1 (91.2)
Estradiol (pmol/L), mean (SD)	91.2 (22.8)	90.7 (22.4)	94.3 (23.8)
Free estradiol (pmol/L), mean (SD)	2.3 (0.5)	2.3 (0.6)	2.3 (0.6)
DHEAS (pmol/L), mean (SD)	6.7 (3.3)	7.1 (3.2)	5.7 (3.2)
Systolic blood pressure (mm Hg), mean (SD)	143.4 (22.1)	140.8 (20.3)	148.9 (24.6)
Diastolic blood pressure (mm Hg), mean (SD)	81.5 (10.3)	80.2 (10.1)	84.1 (10.2)
Total cholesterol (mmol/L), mean (SD)	5.8 (1.1)	5.9 (1.1)	5.7 (1.0)
Triglycerides (mmol/L), mean (SD)	1.6 (1.4)	1.6 (1.3)	1.6 (1.5)
Carotid IMT (mm), mean (SD)	0.82 (0.15)	0.80 (0.15)	0.87 (0.15)
PWV (m/s), mean (SD)	9.41 (2.49)	8.93 (2.17)	10.43 (2.82)
Mean arterial pressure (mm Hg), mean (SD)	129.1 (16.6)	126.9 (15.2)	134.0 (18.4)
Heart rate (mbp), mean (SD)	64.2 (10.2)	63.5 (10.5)	65.5 (9.6)
Smoking, % (N)			
Current	24.3 (97)	25.6 (69)	21.5 (28)
Former	54.3 (217)	50.0 (135)	63.1 (82)
Never	21.5 (86)	24.4 (66)	15.4 (20)
Alcohol consumption (g/day), mean (SD)	20.2 (21.5)	21.9 (23.2)	16.7 (17.2)
Cardiovascular disease, % (N)	17.0 (68)	14.1 (38)	23.1 (30)
Diabetes, % (N)	5.3 (21)	2.6 (7)	10.8 (14)
Hypertension therapy, % (N)	17.0 (68)	14.1 (38)	23.1 (30)
Hyperlipidemia therapy, % (N)	12.5 (50)	11.1 (30)	15.4 (20)

Abbreviations: SHBG = sex hormone-binding globulin; DHEAS = dihydroepiandrosterone sulfate; IMT = intima-media thickness; PWV = pulse wave velocity.

Table 2. Cross-sectional associations between sex hormone concentrations and pulse wave velocity and carotid intima-media thickness at baseline.

	PWV (m/s), mean (95% CI) (N = 376)			Carotid IMT (mm), mean (95% CI) (N = 399)		
	Model 1	Model 2 *	Model 3	Model 1	Model 2	Model 3
Total testosterone						
Q1	9.71 (9.31-10.10)	9.34 (8.83-9.85)	9.28 (8.78-9.78)	0.81 (0.79-0.83)	0.81 (0.78-0.85)	0.82 (0.79-0.85)
Q2	9.54 (9.15-9.94)	9.33 (8.85-9.81)	9.35 (8.88-9.82)	0.84 (0.82-0.87)	0.85 (0.81-0.88)	0.85 (0.82-0.88)
Q3	9.32 (8.93-9.71)	9.34 (8.86-9.82)	9.31 (8.85-9.77)	0.83 (0.81-0.86)	0.85 (0.82-0.88)	0.85 (0.82-0.88)
Q4	9.23 (8.82-9.64)	9.35 (8.85-9.85)	9.32 (8.83-9.81)	0.81 (0.78-0.83)	0.82 (0.79-0.85)	0.82 (0.79-0.86)
P value F-test	0.34	0.99	0.99	0.12	0.11	0.10
P value trend	0.07	0.97	0.92	0.76	0.68	0.78
Per sd decrease	0.20 (-0.003; 0.40)	-0.004 (-0.20; 0.19)	-0.01 (-0.20; 0.18)	0.003 (-0.01; 0.01)	-0.002 (-0.01; 0.01)	-0.001 (-0.01; 0.01)
SHBG						
Q1	9.55 (9.14-9.95)	9.23 (9.70-9.75)	9.14 (8.63-9.65)	0.82 (0.80-0.85)	0.83 (0.80-0.87)	0.83 (0.80-0.87)
Q2	9.84 (9.46-10.21)	9.54 (9.05-10.03)	9.52 (9.04-10.00)	0.81 (0.79-0.84)	0.83 (0.79-0.86)	0.83 (0.80-0.86)
Q3	9.42 (9.02-9.82)	9.50 (9.00-10.00)	9.49 (9.00-9.97)	0.83 (0.81-086)	0.85 (0.81-0.88)	0.85 (0.82-0.88)
Q4	8.94 (8.53-9.36)	9.16 (8.69-9.64)	9.17 (8.71-9.63)	0.82 (0.80-0.84)	0.83 (0.80-0.87)	0.84 (0.81-0.87)
P value F-test	0.02	0.34	0.25	0.78	0.72	0.61
P value trend	0.02	0.80	0.94	0.95	0.62	0.59
Per sd decrease	0.22 (0.01; 0.43)	-0.02 (-0.22; 0.18)	-0.05 (-0.25; 0.15)	0.002 (-0.01; 0.01)	-0.001 (-0.01; 0.01)	-0.001 (-0.01; 0.01)
Free testosterone						
Q1	9.69 (9.28-10.11)	9.45 (8.94-9.96)	9.37 (8.87-9.86)	0.82 (0.80-0.85)	0.83 (0.80-0.86)	0.83 (0.80-0.86)
Q2	9.49 (9.09-9.90)	9.43 (8.95-9.92)	9.43 (8.96-9.91)	0.81 (0.79-0.84)	0.82 (0.79-0.85)	0.83 (0.80-0.86)
Q3	9.32 (8.92-9.72)	9.27 (8.77-9.77)	9.26 (8.78-9.74)	0.85 (0.82-0.87)	0.86 (0.83-0.89)	0.87 (0.83-0.90)
Q4	9.30 (8.87-9.73)	9.21 (8.70-9.71)	9.20 (8.71-9.69)	0.81 (0.79-0.84)	0.83 (0.79-0.86)	0.83 (0.80-0.86)
P value F-test	0.56	0.80	0.84	0.12	0.08	0.07
P value trend	0.18	0.34	0.46	0.77	0.48	0.49
Per sd decrease	0.08 (-0.13; 0.29)	0.01 (-0.18; 0.20)	0.02 (-0.16; 0.21)	0.003 (-0.01; 0.02)	-0.0002 (-0.01; 0.01)	0.0003 (-0.01; 0.01)

Abbreviations: SHBG = sex hormone-binding globulin; DHEAS = dihydroepiandrosterone sulfate; IMT = intima-media thickness; PWV = pulse wave velocity.

Model 1: age-adjusted
Model 2: Model 1 plus BMI, smoking, alcohol consumption, antihypertensive medication and lipid-lowering medication (Model 2*: additionally adjusted for mean arterial pressure and heart rate)
Model 3: Model 2 plus systolic blood pressure, HDL-cholesterol, triglycerides and history of diabetes

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Table 2. Continued.

	PWV (m/s), mean (95% CI) (N = 376)			Carotid IMT (mm), mean (95% CI) (N = 399)		
	Model 1	Model 2 *	Model 3	Model 1	Model 2	Model 3
Estradiol						
Q1	9.39 (9.01-9.77)	9.22 (8.73-9.70)	9.28 (8.80-9.75)	0.81 (0.79-0.83)	0.82 (0.79-0.86)	0.83 (0.80-0.86)
Q2	9.18 (8.77-9.58)	9.23 (8.71-9.75)	9.21 (8.71-9.72)	0.82 (0.79-0.84)	0.83 (0.80-0.86)	0.84 (0.80-0.87)
Q3	9.63 (9.27-9.99)	9.43 (8.97-9.88)	9.36 (8.91-9.80)	0.84 (0.82-0.86)	0.85 (0.82-0.88)	0.85 (0.82-0.88)
Q4	9.59 (9.14-10.04)	9.41 (8.89-9.93)	9.37 (8.87-9.87)	0.83 (0.80-0.85)	0.83 (0.80-0.86)	0.83 (0.80-0.87)
P value F-test	0.37	0.75	0.92	0.36	0.53	0.71
P value trend	0.26	0.33	0.60	0.15	0.39	0.64
Per sd decrease	-0.02 (-0.21; 0.18)	-0.01 (-0.19; 0.17)	0.03 (-0.14; 0.20)	-0.01 (-0.02; 0.002)	-0.01 (-0.02; 0.01)	-0.004 (-0.02; 0.01)
Free estradiol						
Q1	9.25 (8.85-9.64)	9.17 (8.68-9.66)	9.25 (8.77-9.72)	0.81 (0.79-0.84)	0.83 (0.80-0.86)	0.84 (0.80-0.87)
Q2	9.46 (9.07-9.86)	9.58 (9.08-10.09)	9.52 (9.03-10.01)	0.82 (0.80-0.84)	0.83 (0.80-0.87)	0.84 (0.80-0.87)
Q3	9.43 (9.04-9.83)	9.31 (8.82-9.80)	9.23 (8.74-9.71)	0.82 (0.80-0.85)	0.83 (0.80-0.86)	0.84 (0.80-0.87)
Q4	9.67 (9.27-10.07)	9.35 (8.87-9.82)	9.30 (8.84-9.77)	0.84 (0.81-0.86)	0.84 (0.81-0.87)	0.84 (0.81-0.87)
P value F-test	0.59	0.44	0.63	0.62	0.95	0.99
P value trend	0.17	0.73	0.89	0.21	0.62	0.85
Per sd decrease	-0.11 (-0.31; 0.09)	-0.002 (-0.18; 0.18)	0.04 (-0.13; 0.22)	-0.01 (-0.02; 0.001)	-0.01 (-0.02; 0.01)	-0.01 (-0.02; 0.01)
DHEAS						
Q1	9.44 (9.02-9.86)	9.28 (8.76-9.80)	9.27 (8.76-9.77)	0.84 (0.81-0.86)	0.84 (0.81-0.86)	0.85 (0.82-0.89)
Q2	9.18 (8.77-9.59)	9.09 (8.59-9.59)	9.09 (8.61-9.57)	0.83 (0.81-0.86)	0.83 (0.81-0.86)	0.85 (0.81-0.80)
Q3	9.46 (9.05-9.86)	9.35 (8.85-9.84)	9.31 (8.83-9.79)	0.82 (0.80-0.84)	0.82 (0.80-0.84)	0.84 (0.80-0.87)
Q4	9.72 (9.30-10.15)	9.57 (9.08-10.07)	9.54 (9.06-10.02)	0.80 (0.78-0.83)	0.80 (0.78-0.83)	0.82 (0.78-0.85)
P value F-test	0.36	0.40	0.45	0.26	0.37	0.32
P value trend	0.29	0.25	0.28	0.05	0.08	0.07
Per sd decrease	-0.13 (-0.36; 0.11)	-0.09 (-0.30; 0.12)	-0.07 (-0.28; 0.13)	0.01 (-0.01; 0.02)	0.01 (-0.01; 0.02)	0.01 (-0.01; 0.02)

Abbreviations: SHBG = sex hormone-binding globulin; DHEAS = dihydroepiandrosterone sulfate; IMT = intima-media thickness; PWV = pulse wave velocity.

Model 1: age-adjusted
Model 2: Model 1 plus BMI, smoking, alcohol consumption, antihypertensive medication and lipid-lowering medication (Model 2*: additionally adjusted for mean arterial pressure and heart rate)
Model 3: Model 2 plus systolic blood pressure, HDL-cholesterol, triglycerides and history of diabetes

We further explored whether associations were modified by age (Supplementary Table 1). We observed a significant interaction between age and SHBG. An inverse association between SHBG and PWV was found in men aged > 70 years only (beta = 0.64 (95% CI: 0.09; 1.20) per sd decrease in SHBG, P interaction = 0.04). Age also modified the association between TT and PWV (P interaction = 0.02), although the inverse association in men > 70 years did not reach statistical significance (beta = 0.48 (95% CI -0.09; 1.06)). For cIMT, no significant interactions between age and sex hormones were found (Supplementary Table 1).

The median (interquartile range) follow-up time was 8.8 (0.7) years. Men who participated at follow-up were younger, consumed more alcohol, were more often never-smokers and had a lower prevalence of diabetes and cardiovascular disease at baseline (Table 1). In addition, men who participated at follow-up had a lower systolic and diastolic blood pressure and had lower PWV and cIMT values. Serum concentrations of FT and DHEAS were higher in these participants (Table 1). The mean (sd) of PWV and cIMT at follow-up were 9.05 (2.14) and 0.90 (0.16) respectively, with annual rates of change of 0.03 m/s per year and 0.01 mm per year. Table 3 shows the results of the analyses between sex hormones and longitudinal changes in PWV and cIMT. No association with cIMT and PWV change was observed for any of the sex hormones (Table 3), and no association with carotid plaque score was found (Table 4). Results were similar after multivariable adjustment and there was no evidence of an interaction with age (P interaction all > 0.05). All associations were not materially different in analyses using inverse probability weighting and after excluding prevalent CVD cases and men using antihypertensive and lipid-lowering medication.

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Table 3. Longitudinal associations between baseline sex hormone concentrations and changes in pulse wave velocity and carotid intima-media thickness.

	Rate of change in PWV (m/s per year), (95% CI) (N = 243)			Rate of change in carotid IMT (mm per year), mean (95% CI) (N = 265)		
	Model 1	Model 2 *	Model 3	Model 1	Model 2	Model 3
Total testosterone						
per sd decrease	0.001 (-0.02; 0.02)	0.004 (-0.02; 0.03)	0.004 (-0.02; 0.03)	-0.001 (-0.002; 0.001)	-0.001 (-0.002; 0.001)	-0.001 (-0.002; 0.001)
SHBG						
per sd decrease	-0.01 (-0.03; 0.02)	-0.01 (-0.03; 0.02)	-0.01 (-0.03; 0.02)	-0.0004 (-0.002; 0.001)	-0.0004 (-0.002; 0.001)	-0.0004 (-0.002; 0.001)
Free testosterone						
per sd decrease	0.004 (-0.02; 0.03)	0.01 (-0.02; 0.03)	0.01 (-0.02; 0.03)	-0.0003 (-0.001; 0.001)	-0.0003 (-0.001; 0.001)	-0.0003 (-0.001; 0.001)
Estradiol						
per sd decrease	-0.01 (-0.03; 0.01)	-0.01 (-0.03; 0.01)	-0.01 (-0.03; 0.01)	-0.0001 (-0.001; 0.001)	0.0003 (-0.001; 0.001)	0.0003 (-0.001; 0.001)
Free estradiol						
per sd decrease	-0.004 (-0.02; 0.02)	-0.01 (-0.03; 0.01)	-0.01 (-0.03; 0.01)	0.0001 (-0.001; 0.001)	0.0001 (-0.001; 0.001)	0.0001 (-0.001; 0.001)
DHEAS						
per sd decrease	-0.02 (-0.04; 0.01)	-0.02 (-0.04; 0.01)	-0.02 (-0.04; 0.01)	-0.0004 (-0.002; 0.001)	-0.002 (-0.002; 0.001)	-0.0004 (-0.002; 0.001)

Abbreviations: SHBG = sex hormone-binding globulin; DHEAS = dihydroepiandrosterone sulfate; IMT = intima-media thickness; PWV = pulse wave velocity.

Model 1: age-adjusted
Model 2: Model 1 plus BMI, smoking, alcohol consumption, antihypertensive medication and lipid-lowering medication (Model 2*: additionally adjusted for mean arterial pressure and heart rate)
Model 3: Model 2 plus systolic blood pressure, HDL-cholesterol, triglycerides and history of diabetes

Table 4. Associations between baseline sex hormone concentrations and carotid plaque scores (maximum and mean) at follow-up.

| | Sex hormone concentrations, mean (95% CI) | | | | | | | | | |
| | Max plaque score | | | | | Mean plaque score | | | | |
	0 (N = 44)	1 (N = 105)	≥ 2 (N = 100)	P F-test	P trend	0 (N = 44)	0-0.5 (N = 129)	> 0.5 (N = 76)	P F-test	P trend
Total testosterone (nmol/L)										
Model 1	19.0 (17.3-20.6)	19.0 (18.0-20.0)	18.3 (17.2-19.4)	0.61	0.42	19.0 (17.3-20.7)	19.0 (18.1-20.0)	18.0 (16.7-19.3)	0.41	0.29
Model 2	20.0 (18.1-21.9)	20.6 (19.1-22.0)	19.8 (18.4-21.3)	0.55	0.69	20.2 (18.2-22.1)	20.7 (19.3-22.2)	19.5 (17.9-21.0)	0.23	0.38
Model 3	20.0 (18.2-21.9)	20.5 (19.1-21.9)	20.0 (18.6-21.5)	0.76	0.86	20.2 (18.3-22.1)	20.7 (19.3-22.1)	19.7 (18.2-21.2)	0.35	0.49
SHBG (nmol/L)										
Model 1	37.8 (33.4-42.2)	40.9 (38.2-43.6)	39.4 (36.5-42.3)	0.46	0.78	37.9 (33.5-42.3)	40.9 (38.5-43.4)	38.8 (35.4-42.1)	0.36	0.96
Model 2	41.9 (36.8-47.1)	45.7 (41.8-49.7)	44.2 (40.3-48.2)	0.27	0.61	42.2 (37.0-47.4)	46.1 (42.2-49.9)	43.5 (39.3-47.7)	0.17	0.86
Model 3	42.0 (37.0-47.1)	45.2 (41.4-49.1)	44.4 (40.5-48.2)	0.39	0.53	42.3 (37.2-47.3)	45.5 (41.7-49.3)	43.9 (39.8-47.9)	0.32	0.70
FT (pmol/L)										
Model 1	379.7 (352.6-406.7)	362.2 (345.6-378.9)	357.1 (339.2-374.9)	0.41	0.22	379.9 (352.8-406.9)	362.1 (347.1-377.1)	355.6 (334.8-376.4)	0.40	0.21
Model 2	381.5 (348.4-414.7)	371.2 (346.1-396.4)	366.9 (341.4-392.3)	0.69	0.41	382.7 (349.3-416.0)	372.7 (348.0-397.4)	364.1 (337.3-390.9)	0.59	0.30
Model 3	381.5 (348.2-414.8)	372.2 (346.8-397.5)	370.4 (344.6-396.2)	0.79	0.56	383.1 (349.6-416.7)	375.1 (350.0-400.2)	366.3 (339.4-393.2)	0.64	0.35
Estradiol (pmol/L)										
Model 1	86.9 (80.0-93.8)	89.4 (85.1-93.6)	93.5 (88.9-98.0)	0.26	0.11	87.5 (80.5-94.4)	91.7 (87.9-95.5)	90.5 (85.2-95.8)	0.56	0.62
Model 2	90.0 (81.4-98.5)	92.1 (85.7-98.6)	96.3 (89.8-102.9)	0.27	0.11	90.9 (82.3-99.5)	94.9 (88.5-101.3)	93.2 (86.2-100.1)	0.56	0.74
Model 3	90.3 (81.8-98.9)	92.2 (85.7-98.7)	97.3 (90.7-103.9)	0.18	0.08	91.3 (82.7-99.9)	95.2 (88.7-101.6)	93.9 (87.0-100.8)	0.60	0.46
Free estradiol (pmol/L)										
Model 1	2.24 (2.05-2.42)	2.25 (2.13-2.36)	2.40 (2.28-2.52)	0.18	0.11	2.25 (2.07-2.44)	2.31 (2.21-2.42)	2.32 (2.18-2.47)	0.83	0.59
Model 2	2.24 (2.02-2.47)	2.23 (2.06-2.40)	2.39 (2.22-2.56)	0.16	0.12	2.26 (2.04-2.49)	2.30 (2.14-2.47)	2.31 (2.13-2.49)	0.92	0.73
Model 3	2.25 (2.03-2.48)	2.24 (2.07-2.41)	2.41 (2.23-2.58)	0.12	0.09	2.27 (2.05-2.50)	2.32 (2.15-2.49)	2.32 (2.14-2.51)	0.90	0.72
DHEAS (pmol/L)										
Model 1	6.9 (6.0-7.9)	7.1 (6.5-7.6)	7.3 (6.7-7.9)	0.76	0.47	7.0 (6.1-7.9)	7.3 (6.8-7.8)	7.0 (6.3-7.7)	0.71	0.87
Model 2	7.4 (6.2-8.5)	7.6 (6.7-8.5)	7.9 (7.0-8.7)	0.67	0.37	7.5 (6.3-8.6)	7.8 (7.1-8.7)	7.5 (6.6-8.4)	0.54	0.91
Model 3	7.3 (6.2-8.5)	7.6 (6.7-8.5)	7.8 (7.0-8.7)	0.67	0.37	7.5 (6.3-7.6)	7.9 (7.0-8.8)	7.5 (6.6-8.4)	0.50	0.89

Abbreviations: SHBG = sex hormone-binding globulin; DHEAS = dihydroepiandrosterone sulfate.

Model 1: age-adjusted
Model 2: Model 1 plus BMI, smoking, alcohol consumption, antihypertensive medication and lipid-lowering medication
Model 3: Model 2 plus systolic blood pressure, HDL-cholesterol, triglycerides and history of diabetes

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Discussion

Overall, we found no clear association between endogenous sex hormones and subclinical atherosclerosis, in either cross-sectional or longitudinal analyses. However, for arterial stiffness a significant interaction with age was found cross-sectionally, such that low TT and SHBG concentrations were associated with higher baseline PWV values in elderly men only.

To appreciate our findings, some issues need to be addressed. Strengths of the present study are the longitudinal design, the long-term follow-up and the inclusion of three markers of subclinical CVD representing different stages of the atherosclerotic process. In addition, whereas previous studies focused on elderly men, we also included middle-aged men, which allowed us to assess associations over a broad age range. Furthermore, testosterone and estradiol concentrations were measured by radioimmunoassay after extraction, which is more accurate and sensitive than direct immunoassays. Our study also had several limitations that are inherent to the longitudinal design. First of all, selective loss to follow-up may have occurred in our study. Men who participated at follow-up were younger, had less prevalent disease and had lower cIMT and PWV values at baseline. There were no differences in serum concentrations of TT, SHBG and E2, but participants who attended the re-examination had higher baseline concentrations of FT and DHEAS. However, the participation rate was reasonably good (68%) and controlling for selection bias by inverse probability weighting did not alter the results, making selective loss to follow-up a less likely explanation for our null findings. Another limitation of studies on cIMT and PWV change is the accumulation of repeated measurement errors. We used highly standardised protocols including definitions of anatomic landmarks, and the use of multiple measurements for both PWV and cIMT will have tended to reduce measurement errors. The mean rate of cIMT change in our study (0.01 mm per year) was comparable with that observed in other population-based studies [31, 32], suggesting that the change in cIMT was sufficient to detect a modest association. However, the mean rate of PWV change (0.03 m/s per year) was substantially lower than previously reported rates (0.1-0.2 m/s per year) [33, 34], which may be indicative of repeated measurement errors or less PWV progression in this relatively healthy population. This in turn may have resulted in lower statistical power to detect a longitudinal association. Our inability to detect a longitudinal association could also be explained by changes in medication use during follow-up. In our study, associations remained unchanged after excluding men using antihypertensive and lipid-lowering

medication at baseline and follow-up, suggesting that the lack of an association cannot be attributed to increasing medication use.

Several studies have investigated the association between testosterone and subclinical atherosclerosis. The lack of an association with cIMT is in agreement with previous findings from cross-sectional [35] and longitudinal [36] studies showing no association in apparently healthy men of the same age range. However, in specific subpopulations of elderly men [31, 37], hypogonadal men [38] and men with increased cardiovascular risk [39-41], low testosterone concentrations have been associated with higher cIMT values. As for cIMT, we found no association between testosterone and PWV across the whole age range, but in cross-sectional analysis an inverse relation with TT and SHBG was observed after excluding men aged 70 years and younger. Data on PWV are scarce, but in a small hospital-based study among 55 elderly men an inverse association between FT and PWV has been reported [42]. Existing data on DHEAS are largely based on cross-sectional studies in high-risk populations, showing an inverse association of DHEAS with cIMT [43, 44] and PWV [42, 45]. Again, population-based studies in middle-aged and older men show no association between DHEAS and subclinical atherosclerosis [37, 46]. Results for estradiol are also conflicting and no studies so far have investigated its association with PWV. High estradiol concentrations have been linked to an increase in cIMT progression in middle-aged men [47] and in elderly men a tendency towards a positive relation has been reported [31]. However, an association between estradiol and cIMT has not been confirmed by others [37, 38].

The absence of an overall association between endogenous sex hormones and subclinical atherosclerosis can be interpreted in different ways. The presence of an association in high-risk populations suggests that sex hormones are only linked to more advanced stages of the atherosclerotic process. Our findings of an inverse association with PWV but not with cIMT at baseline do support this notion, as both markers reflect different entities of subclinical CVD. PWV is believed to represent a later stage of the atherosclerotic process than cIMT, as it is more strongly related to carotid plaques [48] and is not detectable in earlier stages of atherosclerosis [49]. This may also explain why previous cross-sectional studies have reported more consistent relationships between sex hormones and markers of more advanced subclinical atherosclerosis including carotid plaques [35, 36] and abdominal aortic calcification [50] in middle-aged and older men. However, we and others [36] found no prospective association between sex hormones and carotid plaques. Altogether these data may suggest that changes in sex hormones are merely a consequence rather than a cause of severe atherosclerosis. Alternatively, the lack of an association in

non-elderly men may be indicative of a threshold-effect. In our study population, circulating testosterone concentrations were relatively high compared to those reported in other population-based settings. Although this can partially be explained by the higher accuracy of the assay methods used, it is well-known that relatively healthy populations like ours are at the higher end of the testosterone distribution [51]. Together with the presence of an association in high-risk populations, these data suggest that testosterone may only influence CVD risk below a certain threshold. This view is supported by preliminary findings from testosterone supplementation studies. Testosterone therapy has been associated with improvements in vascular reactivity in men with coronary artery disease [52] and in hypogonadal men a decrease in PWV has been reported following testosterone replacement [53]. Likewise, androgen deprivation therapy in prostate cancer patients has been associated with an increase in arterial stiffness [54]. Further studies focussing on threshold effects are needed to gain more insight into role of sex hormones in CVD etiology.

In conclusion, our results do not support a major role of endogenous sex hormones in subclinical atherosclerosis in apparently healthy middle-aged and older men. However, the presence of an age-specific association with arterial stiffness is suggestive of a threshold-relationship and merits further investigation.

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Supplementary Table 1. Cross-sectional associations between sex hormone concentrations and pulse wave velocity and carotid intima-media thickness, stratified by age.

	PWV (m/s), mean (95% CI)			cIMT (mm), mean (95% CI)		
	Model 1	Model 2 *	Model 3	Model 1	Model 2	Model 3
Total testosterone (per sd decrease)						
Age ≤ 70 years	0.10 (-0.09; 0.29)	-0.09 (-0.27; 0.10)	-0.08 (-0.26; 0.10)	0.001 (-0.01; 0.01)	-0.005 (-0.02; 0.01)	-0.002 (-0.02; 0.01)
Age > 70 years	0.71 (0.10-1.32)	0.48 (-0.09; 1.06)	0.51 (-0.09; 1.11)	0.01 (-0.02; 0.05)	0.01 (-0.02; 0.05)	0.01 (-0.02; 0.04)
P interaction	0.03	0.02	0.02	0.83	0.61	0.64
SHBG (per sd decrease)						
Age ≤ 70 years	0.07 (-0.13; 0.28)	-0.17 (-0.36; 0.02)	-0.21 (-0.39; -0.02)	-0.002 (-0.02; 0.01)	-0.005 (-0.02; 0.01)	-0.003 (-0.02; 0.01)
Age > 70 years	0.80 (0.22-1.39)	0.64 (0.09; 1.20)	0.65 (0.03; 1.27)	0.02 (-0.01; 0.05)	0.02 (-0.02; 0.05)	0.02 (-0.01; 0.05)
P interaction	0.05	0.04	0.03	0.22	0.23	0.12
Free testosterone (per sd decrease)						
Age ≤ 70 years	0.08 (-0.12; 0.28)	0.02 (-0.16; 0.20)	0.05 (-0.12; 0.22)	0.003 (-0.01; 0.02)	-0.001 (-0.02; 0.01)	0.001 (-0.01; 0.01)
Age > 70 years	0.25 (-0.46; 0.96)	0.09 (-0.54; 0.72)	0.16 (-0.49; 0.81)	0.01 (-0.03; 0.04)	0.01 (-0.03; 0.04)	0.01 (-0.03; 0.04)
P interaction	0.21	0.16	0.30	0.45	0.63	0.42
Estradiol (per sd decrease)						
Age ≤ 70 years	-0.07 (-0.26; 0.12)	-0.07 (-0.24; 0.10)	-0.04 (-0.20; 0.12)	-0.01 (-0.02; 0.003)	-0.01 (-0.02; 0.01)	-0.01 (-0.05; 0.01)
Age > 70 years	0.09 (-0.49; 0.67)	0.04 (-0.51; 0.58)	0.14 (-0.43; 0.70)	-0.01 (-0.04; 0.02)	-0.01 (-0.04; 0.02)	0.003 (-0.03; 0.03)
P interaction	0.54	0.89	0.86	0.60	0.75	0.80
Free estradiol (per sd decrease)						
Age ≤ 70 years	-0.10 (-0.28; 0.09)	-0.01 (-0.18; 0.16)	0.03 (-0.13; 0.20)	-0.01 (-0.02; 0.004)	-0.01 (-0.02; 0.01)	-0.01 (-0.02; 0.01)
Age > 70 years	0.28 (-0.90; 0.34)	-0.30 (-0.92; 0.31)	-0.16 (-0.80; 0.49)	-0.02 (-0.05; 0.01)	-0.02 (-0.05; 0.02)	-0.01 (-0.04; 0.03)
P interaction	0.65	0.44	0.51	0.21	0.36	0.34
DHEAS (per sd decrease)						
Age ≤ 70 years	0.003 (-0.22; 0.22)	0.08 (-0.12; 0.28)	0.09 (-0.10; 0.28)	0.01 (-0.004; 0.02)	0.01 (-0.01; 0.03)	0.01 (-0.03; 0.04)
Age > 70 years	-0.71 (-1.43; 0.01)	-0.83 (-1.47; -0.18)	-0.73 (-0.41; -0.05)	0.003 (-0.03; 0.04)	-0.002 (-0.04; 0.04)	0.01 (-0.03; 0.04)
P interaction	0.62	0.45	0.41	0.94	0.92	0.91

Abbreviations: SHBG = sex hormone-binding globulin; DHEAS = dihydroepiandrosterone sulfate; IMT = intima-media thickness; PWV = pulse wave velocity.

Model 1: age-adjusted
Model 2: Model 1 plus BMI, smoking, alcohol consumption, antihypertensive medication and lipid-lowering medication (Model 2*: additionally adjusted for mean arterial pressure and heart rate)
Model 3: Model 2 plus systolic blood pressure, HDL-cholesterol, triglycerides and history of diabetes

Chapter 11

General discussion

Cardiovascular disease (CVD) is the leading cause of death in most parts of the world and a major determinant of chronic disability. The excess risk of CVD in men and the apparent loss of the female advantage in postmenopausal women and women with hyperandrogenism [1, 2] have led to the hypothesis that endogenous sex hormones may play an important role in CVD etiology. In this thesis we tested this hypothesis by investigating the impact of age at menopause (as a marker of premenopausal endogenous estrogen exposure) and circulating testosterone and SHBG concentrations (as a marker of androgen status) on cardiometabolic risk. In women, we found that an early menopause is associated with a higher risk of type 2 diabetes. We also investigated the reverse association, i.e. the effect of diabetes on menopausal onset. Women with early-onset diabetes (before the age of 20 years) were found to enter menopause at a younger age compared to non-diabetic women. This finding illustrates the complex relationship between menopausal age and diabetes, but does not rule out a potential protective effect of premenopausal estrogen. For androgens, we found evidence for a sex-specific effect, with low testosterone concentrations in men and high testosterone concentrations in women being associated with a higher metabolic risk. Another interesting observation in both men and women was the strong inverse link between SHBG and metabolic disease risk. However, associations between androgens and atherosclerotic markers and cardiovascular endpoints were found to be less consistent. In this chapter, the findings of this thesis are discussed in a broader context and recommendations for further research are made.

Estrogens
Endogenous estrogen concentrations are scarcely studied in premenopausal women, because of the large fluctuations in estrogen concentrations across the menstrual cycle. There have been some efforts relating repeated estrogen measurements to intermediate risk markers, such as the BioCycle Study [3] and the Study of Women's Health Across the Nation (SWAN) [4], but this approach is generally not feasible for large-scale prospective studies on cardiovascular endpoints. In the BioCycle and SWAN study, high concentrations of follicular-phase estrogen have been linked to more favourable levels of inflammatory [5] and lipid markers [6, 7], but data regarding insulin resistance have been inconsistent [8, 9]. Studies relating premenopausal concentrations to markers of subclinical cardiovascular disease are scarce, but in a small Japanese study of 10 premenopausal women with variant angina, endogenous estrogen concentrations were found to correlate positively with flow-mediated vasodilatation, while having an inverse association with ischemic periods [10].

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Because of the practical difficulties with measuring premenopausal estrogen, data on endogenous estrogens primarily come from studies in postmenopausal women. In postmenopausal women, high rather than low circulating estrogen has been linked to an increased risk of type 2 diabetes [11, 12] and the metabolic syndrome [13]. Studies investigating associations with cardiovascular events have produced mixed results with either a positive association [14, 15] or no association [16, 17]. When looking at the discrepant findings in premenopausal and postmenopausal women, it is important to emphasize that postmenopausal estrogen concentrations are only 20% of those before menopause. In the Estrogen in the Prevention of Atherosclerosis Trial (EPAT), high endogenous estrogen concentrations were associated with reduced inflammation and atherosclerosis in postmenopausal women on estrogen replacement only [18, 19]. Thus, endogenous estrogen may only exert a protective effect above a certain threshold, at concentrations present in premenopausal women or in postmenopausal women with supra-physiological estrogen concentrations.

In men, the reported associations with endogenous estrogens are comparable to those seen in postmenopausal women. Estrogen concentrations are elevated in men with diabetes [20] and the metabolic syndrome [21]. Again, associations with cardiovascular endpoints are less clear. We found no evidence of a link between estrogens and subclinical CVD (Chapter 10) and others have reported mixed results for atherosclerotisc markers [22-25] and cardiovascular endpoints [26-28]. This discrepancy for study outcome in both men and postmenopausal women may partially be explained by the multicausal nature of cardiovascular disease, in which associations with metabolic risk factors do not necessarily translate into changes in cardiovascular risk. Another explanation for the more consistent relationship with metabolic risk factors is the presence of reverse causation. Insulin stimulates the ovarian production of estrogens and androgens [29], and the higher estrogen concentrations found in postmenopausal women and men may reflect a higher conversion rate in adipose tissue. Therefore, hard conclusions about the directionality of the associations between endogenous estrogens and cardiometabolic risk cannot be drawn from these observational data.

The conflicting findings for CVD outcomes may also be indicative for the existence of subgroup effects. Recently, it has been suggested that associations with CVD outcomes may differ according to age with associations being stronger in elderly women [15]. However, this hypothesis is speculative and requires further investigation.

Surrogate markers of estrogen exposure

For women, menopause marks the end of estrogen production and its timing may therefore provide more information on the effects of endogenous estrogen than a single measurement of circulating estrogen in either pre- or postmenopausal women. In this thesis, we found that women who enter menopause early have a higher risk of type 2 diabetes (Chapter 2). This observation is in line with previous studies showing an increase in cardiovascular morbidity and mortality with early menopause [30]. Although these findings are suggestive of a beneficial effect of premenopausal estrogen on cardiometabolic risk, there are also data that argue against a protective role of endogenous estrogens. The CVD rate, for instance, does not show a sharp rise around menopause [31, 32], as opposed to the midlife change in the incidence rate of breast cancer, an estrogen-related disease [32, 33]. Moreover, if endogenous estrogens were protective, we would have expected to see a stronger association with reproductive lifespan, which is a more accurate measure of premenopausal endogenous estrogen exposure. Given the uncertainty about the causal role of premenopausal estrogen exposure, it is important to investigate alternative explanations for the observed association with menopausal age. As described in Chapter 2, the menopause transition is not only characterized by estrogen depletion, but also by a shift toward androgen dominance [34, 35]. In the SWAN study menopausal changes in bioavailable testosterone and SHBG have been associated with a higher risk of the metabolic syndrome [36]. Another explanation is that menopausal age is merely a marker of premature ageing, being indirectly associated with cardiometabolic risk. Finally, reverse causation cannot be excluded. Unfavourable levels of premenopausal cardiovascular risk factors have previously been linked to an earlier onset of menopause [37]. We found that diabetes at young age has an accelerating effect on menopausal onset (Chapter 3). However, no clear linear relationship with diabetes duration was observed, which may indicate that this effect is specific to type 1 diabetes and does not apply to metabolic disease in general. Further exploration of these alternative explanations is needed to identify the likelihood of a causal role of premenopausal estrogen in cardiometabolic risk.

Androgens

In men, low endogenous testosterone concentrations have consistently been associated with a wide range of metabolic risk factors [38]. In agreement with this, we observed an inverse relation between testosterone and glycated haemoglobin (Chapter 8) as well as white blood cell count (Chapter 9). Despite considerable heterogeneity, a relationship between low testosterone and the metabolic syndrome

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is evident (Chapter 6 and Chapter 7). Nevertheless, the relation between testosterone and CVD endpoints is less certain [39, 40]. We failed to demonstrate an overall association between testosterone and subclinical atherosclerosis (Chapter 10), and other studies relating testosterone to cardiovascular morbidity and mortality have produced mixed results [39, 40].

Again, the multicausal nature of cardiovascular disease may partly explain the lack of a clear association with CVD outcomes. Reverse causation may also account for some of the discrepant findings. Complex bidirectional relationships between testosterone and metabolic risk factors have been described (Chapter 6), and may explain why the observed association with metabolic risk is more consistent. The conflicting results for CVD outcomes have also produced interest in the investigation of subgroup effects [39, 40]. As described in Chapter 10, low testosterone concentrations show a more consistent relationship with atherosclerotic markers and cardiovascular endpoints in specific populations of hypogonadal men and men with pre-existing disease. Testosterone replacement also seems to be more beneficial in these subgroups, as improvements in cardiometabolic markers are primarily observed in hypogonadal and diabetic men [41, 42] and patients with heart failure [43]. By contrast, evidence for a protective effect of exogenous testosterone in healthy men is less convincing [44-46]. Thus, low testosterone concentrations may only have a detrimental effect on cardiovascular risk when testosterone concentrations are sufficiently low. This explanation is in line with recent observational data on cardiovascular morbidity and mortality [39, 40], which show an inverse association in elderly men only, i.e. men who have low testosterone concentrations.

In postmenopausal women, the role of androgens in cardiometabolic risk is more controversial. As reviewed in Chapter 4, increased androgenicity seems to be associated with a less favourable cardiovascular risk profile. However, these associations appear to be primarily mediated through SHBG and results for cardiovascular events are conflicting showing positive, inverse or null associations. Sources of these heterogeneous findings in women are still unclear and remain to be determined.

Sex hormone-binding globulin
In both men and women, low SHBG concentrations have consistently been associated with an adverse metabolic risk profile, as reviewed in Chapter 5 and Chapter 6. Again, the relation with cardiovascular outcomes is less certain. In men, several studies have reported an association between low SHBG and cardiovascular endpoints [47-49], but

this has not been confirmed by others [50-52]. The few studies that have reported on associations with CVD endpoints in women have yielded conflicting results (Chapter 5). As for estrogens and androgens, the possibility of reverse causation needs to be addressed when interpreting the discrepant findings for metabolic and cardiovascular outcomes. SHBG production and secretion is influenced by adiposity and insulin resistance and this may explain the stronger associations for metabolic risk.

As described in Chapter 5 and Chapter 6, SHBG is often more strongly related to metabolic risk factors than testosterone. The weaker association with testosterone has led to the speculation that SHBG may play a more dominant role in cardiometabolic risk. In observational research, the independency of sex hormone effects is often evaluated by mutual adjustment. Studies with mutual adjustment have produced conflicting results, and therefore the independent role of endogenous testosterone and SHBG is still uncertain [44, 51, 53-55]. Due to the high correlation between testosterone and SHBG and the potential of collinearity, it is not clear whether mutual adjustment is the best approach to assess the independent effects of testosterone and SHBG in cardiometabolic risk. Results from a recent genome-wide association study (GWAS) of testosterone concentrations in men also illustrate the difficulty of disentangling the effects of testosterone and SHBG [56]. In this study, genetic variants at SHBG loci were found to be associated with a substantial variation in testosterone concentrations. An alternative approach, no yet applied in observational studies, is to examine the combined effects of testosterone and SHBG using statistical tests of interaction. If SHBG facilitates the uptake and action of testosterone in target tissues, then cardiometabolic risk in men would be expected to be highest in those with both low testosterone and SHBG concentrations.

Measurement of sex hormone concentrations

The lack of a clear association between sex hormones and cardiometabolic risk may partly be explained by the sensitivity and specificity of the assay methods being used. Mass spectrometry (MS) is the gold standard for the measurement of sex hormones, but most laboratories use direct immunoassays without purification step, as they are more rapid and less time-consuming. Direct assays, however, have a poor sensitivity and specificity, especially at low sex hormone concentrations (i.e. estrogen concentrations in posmenopausal women and men, and testosterone concentrations in women) [57-59]. Extra extraction steps and MS methods have been recommended in these situations, but are still not widely used in large-scale epidemiological studies. Therefore, more attention should be paid to the development of accurate assay

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techniques that are less expensive and labor-intensive than MS methods. In men, most direct testosterone assays have an adequate sensitivity, but are less accurate than MS [57, 60]. Although this may not be problematic when looking at the relative values or ranking of testosterone concentrations, null associations due to random measurement errors can be prevented by using more accurate assays. The use of extraction or MS methods may be particularly relevant when studying cardiovascular outcomes for which associations are expected be less pronounced than for metabolic risk factors.

The difficulty of measuring premenopausal estrogen is reflected by the few studies conducted in premenopausal women. Except for synchronization of sample collections, there are no standardized methods for measuring premenopausal estrogen concentrations. Single measurements are often used in large-scale epidemiological studies but do not seem to be sufficient for capturing long-term estrogen concentrations in premenopausal women [61, 62]. Furthermore, it not clear whether the estrogen peak at ovulation is a good target or whether measurements at different menstrual phases are more informative when investigating the effect of premenopausal estrogen. Given the practical difficulties of multiple blood sampling, urine measurements of estradiol metabolites may offer a solution for large-scale studies on cardiometabolic endpoints. This approach is now increasingly being used in epidemiological studies on breast cancer [63, 64], but its reproducibility and correlation with endogenous estrogen concentrations remains to be determined.

Recommendations for further research

There is increasing evidence for an association between sex hormones and cardiometabolic risk in both men and women, but firm conclusions cannot be drawn. Relationships between sex hormones and cardiometabolic risk are complex, as reflected by the high amount of between-study heterogeneity in the reported associations. Several efforts have been made to identify sources of heterogeneity in men, but more of these are needed to increase our understanding of the inconsistent findings in women. In this context, the possibility of threshold relationships for androgens and estrogens requires further exploration.

Finally, its remains uncertain whether endogenous sex hormones are a causal factor in the etiology of cardiometabolic disease or whether they merely represent a risk marker, being a consequence or by-product of underlying disease processes. Traditional observational studies cannot address this issue because of their inherent limitations (confounding and reverse causation) [65]. Randomized clinical trials (RCTs)

of sex hormone replacement may provide a solution in this context, but do not provide definite answers regarding the role of endogenous sex hormones. Exogenous testosterone, for instance, suppresses the endogenous testosterone production, which makes results from testosterone trials difficult to interpret [66, 67].

An alternative approach that is now commonly used for causal inference of non-traditional CVD risk factors is Mendelian randomization [68]. In Mendelian randomization studies genetic variants are used as instrumental variables to minimize confounding and reverse causation. To date, no such studies have been performed for endogenous sex hormones, but results from the first Mendelian randomization analysis of SHBG are promising as they support a causal role for SHBG in type 2 diabetes [69, 70].

Before this approach can be extended to androgens and estrogens, it is important to emphasize that Mendelian randomization is only a valid method for causal inference when all criteria of an instrumental variable are fulfilled [71]. First of all, a genetic variant should be strongly associated with endogenous sex hormone concentrations. Results from a recent meta-analysis of GWAS data show that genetic variants explain a substantial part of the variation in male testosterone concentrations [56]. The effect size of these genetic variants seems to be comparable to that of well-known determinants of sex hormone concentrations, such as age, smoking and BMI. Twin studies have also reported strong heritability estimates for testosterone concentrations, but the genetic contribution of male estrogen concentrations seems to be minor [72, 73]. In postmenopausal women, the first GWAS analysis revealed no significant association with endogenous sex hormone concentrations [74]. In a candidate gene study, genetic variants in two different loci were found to be associated with postmenopausal estrogen concentrations, but none of these were associated with breast cancer risk [75]. Furthermore, low to moderate heritability estimates have been reported for postmenopausal sex hormone concentrations [76, 77]. Although these data suggest that the genetic influence on postmenopausal sex hormone concentrations might be too small, future GWAS analyses using highly sensitive assay methods are needed for checking the first criterion in postmenopausal women. For premenopausal women, no GWAS data on sex hormone concentrations are currently available, but recently a genetic variant of premenopausal estrogen concentrations was found to be associated with breast cancer risk in a candidate approach [64]. In women, genetic variants predicting menopausal age may further provide more information regarding the cumulative effects of estrogen exposure. Several genetic variants of menopausal age have been identified in a GWAS analysis [78] and are now being investigated for their association with type 2 diabetes risk in the InterAct consortium. The second

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criterion of an instrumental variable implies that a genetic variant should be independent of possible confounders of the association. Main confounders in the sex hormone cardiometabolic risk associations are lifestyle factors (i.e. cigarette smoking, alcohol consumption and physical activity) and their association with genetic variants should thus be considered. Finally, the third criterion states that the genetic variant should only be associated with the outcome via its association with endogenous sex hormone concentrations. This criterion is of special importance here, as there are indications for pleiotrophic effects of genetic variants. As mentioned previously, genetic variants at SHBG loci have been associated with testosterone concentrations [56]. In women, there are also indications for pleiotrophic effects. Recently, a strong shared genetic component was identified among pairs of testosterone and SHBG concentrations which is suggestive of pleiotrophic effects [77]. Thus, for investigating the independent effects of endogenous sex hormones in a Mendelian randomization approach, it is important to rule out any association with other sex hormones.

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Chapter 12

Cardiovascular disease (CVD) is the leading cause of death in most parts of the world. CVD affects men and women differently, with men have a higher incidence and earlier onset of disease. This gender gap has generated considerable interest in the role of sex hormones in CVD etiology. Initially, research was primarily focused on the effects of exogenous sex hormones. Early findings from basic research and case reports suggested a protective role of estrogens and a potential harmful effect of high androgen doses. However, clinical trials of estrogen replacement therapy showed no CVD benefit in postmenopausal women. With this unexpected finding, research has gradually turned more focus towards the role of endogenous sex hormones. In this thesis we examined the association between endogenous sex hormones and cardiometabolic risk in men and women.

Menopausal age and reproductive lifespan (menopausal age minus menarcheal age) are surrogate markers of premenopausal endogenous estrogen exposure. In Chapter 2 we studied the relation between these surrogate markers and risk of type 2 diabetes in 7864 postmenopausal women from the InterAct study. InterAct is a case-cohort study nested in the European Prospective Investigation into Cancer and Nutrition (EPIC) and includes a randomly sampled subcohort and all incident diabetes cases from 8 European countries. We found that an early menopause and a shorter reproductive lifespan were associated with a higher risk of type 2 diabetes. Associations were independent of other diabetes risk factors and were not materially different after excluding women with a hysterectomy and/or oophorectomy and women using hormone therapy.

Thus, menopausal age appears to be associated with a higher diabetes risk, but the reverse, i.e. an effect of diabetes on menopausal timing might also be true. In Chapter 3, we therefore investigated the impact of diabetes and age at diagnosis on menopausal timing in a retrospective study of 255.898 women from EPIC. Overall, no association with age at natural menopause (ANM) was found, but analyses for age at diagnosis showed that women with early-onset diabetes (age at diagnosis < 20 years) entered menopause earlier than non-diabetic women, whereas women with diabetes at age 50 years and older had a later menopause. These associations were independent of other determinants of menopausal age such as smoking, parity and oral contraceptive use. The absence of a linear relationship with age at diagnosis might suggest that the effect on ANM is specific for type 1 diabetes.

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Smoking is an important determinant of menopausal age as well as cardiometabolic risk. In Chapter 4, we examined the association between cigarette smoking habits and endogenous sex hormone concentrations in postmenopausal women. This study was conducted in 2030 women from the EPIC-Norfolk cohort, one of the UK centers participating in EPIC. All women were at least 1 year postmenopausal and not using hormone replacement therapy. We found that cigarette smoking was associated with higher circulating concentrations of androgens, estrogens, 17-hydroxyprogesterone and SHBG. The lack of an association with pack-years and the almost immediate decline of sex hormone concentrations after smoking cessation in formers smokes reflect the acuteness of the smoking effects.

For many years, when studying endogenous sex hormones, the focus has been on estrogens. Only recently, research has concentrated on the role of androgens in cardiometabolic risk. In Chapter 5, we reviewed the literature on testosterone, SHBG and cardiovascular health in postmenopausal women. Despite the lack of large-scale studies on cardiovascular endpoints, increased androgenicity seems to be associated with a higher cardiovascular risk.

It has been suggested that associations between sex hormones and metabolic risk may differ according to sex. Chapter 6 presents the results of a literature-based meta-analysis of endogenous testosterone, SHBG and the metabolic syndrome (MetS). The specific aim of this meta-analysis was to investigate possible sex differences in the association of testosterone and SHBG with MetS. We found evidence of a sex-specific association between testosterone and the metabolic syndrome, with testosterone concentrations being lower in men with MetS, while being higher in women with MetS. However, no sex-specific association was observed for SHBG: SHBG concentrations were lower in both men and women with MetS. Another interesting observation was the high amount of between-study heterogeneity in the reported associations, which could only partly be explained by differences in age, BMI, study design and diagnostic criteria.

In Chapter 7, we further examined possible sources of this heterogeneity in an individual participant data (IPD) meta-analysis. In this meta-analysis combining the original data of 12.811 men from 20 observational studies, we confirmed previous findings of an inverse association of testosterone and SHBG with MetS. BMI appeared to be an important effect modifier with associations being strongest in non-

overweight men. This IPD meta-analysis also enabled us to study associations in more detail, i.e. we found evidence for a dose-relationship with testosterone and SHBG, and abdominal obesity and dyslipidemia turned out to be the core components of the observed associations.

Testosterone concentrations are lowered in men with type 2 diabetes. However, little is known about the relation between testosterone and glycemia in non-diabetic men. Chapter 8 describes the results of a cross-sectional study on testosterone, SHBG and glycated hemoglobin (HbA$_{1c}$) in 1292 men from the EPIC-Norfolk cohort. We found that low circulating concentrations of testosterone and SHBG were associated with higher HbA$_{1c}$ values. Both associations were robust to adjustment for other diabetes risk factors and remained significant after excluding men with type 2 diabetes.

Low tesosterone concentrations have been linked to a wide range of cardiovascular risk factors, but associations with non-traditional risk factors are relatively unexplored. Chapter 9 reports on the relationship between endogenous sex hormones and white blood cell count, an inflammatory and non-traditional cardiovascular risk factor. This study was conducted in 2418 men from the EPIC-Norfolk cohort. TT and SHBG concentrations were found to be inversely associated with total WBC count, an association that was primarily accounted for by the granulocyte count.

Sex hormones have consistently been associated with various cardiovascular risk factors in men, but their association with clinical endpoints is still controversial. In Chapter 10, we therefore examined the cross-sectional and longitudinal associations between endogenous sex hormones and two markers of subclinical atherosclerosis: pulse wave velocity (PWV) and carotid intima media thickness (cIMT). Overall, we found no clear association with subclinical disease, but there was some evidence of a threshold relationship with low testosterone and SHBG concentrations being associated with higher PWV values in elderly men only.

In Chapter 11, the main findings of this thesis are discussed in a broader context and recommendations for further research are made. There is increasing evidence for an association between sex hormones and cardiometabolic risk in both men and women. However, firm conclusions about the directionality of the observed associations cannot be drawn. More intervention and Mendialian randomization studies are needed to determine the causal role of sex hormones in cardiometabolic risk and the clinical significance of the observed associations.

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Chapter 12

Summary
Samenvatting
Dankwoord
Curriculum Vitae
List of publications

Hart- en vaatziekten zijn veruit de belangrijkste doodsoorzaak in de meeste delen van de wereld. Het vóórkomen van hart-en vaatziekten verschilt per geslacht. Zo is bij mannen de incidentie hoger en presenteren hart-en vaatziekten zich op jongere leeftijd. Dit verschil in risico tussen mannen en vrouwen is al jarenlang aanleiding voor onderzoek naar de rol van geslachtshormonen in de etiologie van hart-en vaatziekten. De eerste studies naar geslachtshormonen concentreerden zich voornamelijk op de effecten van exogeen toegediende hormonen. In case-reports en fundamenteel onderzoek werden aanwijzingen gevonden voor een beschermende rol van oestrogenen en een mogelijk schadelijk effect van hoge androgeen doses. De resultaten van klinische studies lieten echter geen beschermend effect van oestrogeentherapie bij postmenopauzale vrouwen zien. Deze onverwachte resultaten hebben geleid tot een toenemende belangstelling voor de rol van endogene geslachtshormoonspiegels. In dit proefschrift wordt onderzoek gedaan naar mogelijke relaties tussen endogene geslachtshormonen en cardiometabole ziekten (o.a. hart-en vaatziekten, diabetes en metabool syndroom) bij mannen en vrouwen.

Menopauzeleeftijd en reproductieve spanne (menopauzeleeftijd minus menarcheleeftijd) zijn surrogaatmarkers voor premenopauzale endogene oestrogeenblootstelling. In **Hoofdstuk 2**, hebben we de relatie tussen deze surrogaatmarkers en diabetesrisico bestudeerd in 7864 postmenopauzale vrouwen uit de InterAct studie. InterAct is een case-cohort studie genest in de European Prospective Investigation into Cancer and Nutrition (EPIC) en omvat incidente diabetesgevallen en een willekeurige groep deelnemers uit 8 Europese landen. Een vroege menopauze en een korte reproductieve spanne waren geassocieerd met een hoger risico op type 2 diabetes. Deze verbanden waren onafhankelijk van de aanwezigheid van andere diabetes risicofactoren en bleven ongewijzigd na exclusie van vrouwen met een voorgeschiedenis van hysterectomie/ovariëctomie en vrouwen die ooit hormoontherapie gebruikten.

Een vroege menopauze is dus geassocieerd met een verhoogd diabetesrisico, maar metabole risicofactoren kunnen mogelijk ook de timing van de menopauze beïnvloeden. In **Hoofdstuk 3**, onderzochten we daarom de relatie tussen diabetes en leeftijd bij natuurlijke menopauze in een retrospectieve studie onder 255.898 vrouwen uit EPIC. Een verband tussen diabetes en menopauze timing was niet direct aantoonbaar, maar in analyses uitgesplitst naar diagnoseleeftijd vonden we dat de menopauze vervroegd was bij vrouwen met diabetes op jonge leeftijd (< 20

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jaar), terwijl de menopauze vertraagd was bij vrouwen met diabetes op late leeftijd (> 50 jaar). Deze verbanden waren onafhankelijk van andere determinanten van menopauzeleeftijd zoals roken, pariteit en pilgebruik. De afwezigheid van een lineair verband met diagnoseleeftijd doet vermoeden dat het effect van diabetes op jonge leeftijd specifiek is voor type 1 diabetes.

Roken is een risicofactor voor zowel cardiometabole ziekten als voor een vroege menopauze. **Hoofdstuk 4** beschrijft de relatie tussen rookgedrag en endogene geslachtshormoonspiegels bij postmenopauzale vrouwen. Deze studie werd uitgevoerd in een populatie van 2030 vrouwen uit het EPIC-Norfolk cohort, een Engelse bijdrage aan de EPIC studie. Alle vrouwen waren tenminste 1 jaar postmenopauzaal en hadden geen voorgeschiedenis van hormoontherapie. Vrouwen die roken hadden hogere spiegels van androgenen en oestrogenen in hun bloed dan niet-rokers. Het effect van roken bleek vrij acuut te zijn. Zo was de cumulatieve blootstelling aan sigarettenrook niet geassocieerd met hormoonspiegels, en waren de geslachtshormoonspiegels van vrouwen die meer dan een jaar geleden gestopt waren gelijk aan die van niet-rokers.

Jarenlang waren oestrogenen de focus van het onderzoek naar geslachtshormonen bij vrouwen. Recent is er meer aandacht voor de rol van androgenen. In **Hoofdstuk 5** wordt een literatuuroverzicht gegeven van de relatie tussen testosteron, sex hormone-binding globulin (SHBG) en cardiovasculair risico bij postmenopauzale vrouwen. Ondanks een beperkt aantal studies naar klinische eindpunten, lijkt een hoge mate van androgeniciteit (hoge testosteron en lage SHBG spiegels) gepaard te gaan met een verhoogd risico op hart- en vaatziekten.

In de literatuur bestaan aanwijzingen voor een geslachtsspecifiek verband tussen geslachtshormonen en cardiometabool risico. **Hoofdstuk 6** beschrijft de resultaten van een meta-analyse van geaggregeerde data naar de relatie tussen testosteron, SHBG en het metabool syndroom. Het doel van deze meta-analyse was het in kaart brengen van mogelijke geslachtsverschillen in dit verband. We vonden aanwijzingen voor een geslachtsspecifiek verband tussen testosteron en het metabool syndroom. Mannen met het metabool syndroom hadden lagere testosteronspiegels, terwijl vrouwen met het syndroom juist hogere testosteronspiegels hadden. De relatie tussen SHBG en het metabool syndroom was niet geslachtsspecifiek. In zowel mannen als vrouwen ging het metabool syndroom gepaard met lagere SHBG spiegels. Een

interessante bevinding was de hoge mate van heterogeniteit in de sterkte van de gevonden verbanden. Deze heterogeniteit kon slechts deels worden verklaard door verschillen in leeftijd, BMI, studiedesign en diagnostische criteria.

In **Hoofdstuk 7** wordt deze heterogeniteit verder onderzocht in een meta-analyse van individuele data. In deze meta-analyse waarin de gegevens van 12.811 mannen uit 20 observationele studies zijn samengevoegd, bevestigden we niet alleen de eerder gevonden verbanden tussen testosteron, SHBG en het metabool syndroom maar toonden we ook aan dat BMI een belangrijke effectmodificator is. De verbanden met testosteron en SHBG waren namelijk sterker bij mannen die geen overgewicht hadden. Een bijkomend voordeel van een meta-analyse van individuele data is dat verbanden in meer detail kunnen worden onderzocht. Zo vonden we aanwijzingen voor een dosis-respons relatie waarbij het risico op het metabool syndroom geleidelijk afneemt met de hoogte van testosteron en SHBG spiegels. Ook kwam uit deze meta-analyse naar voren dat verbanden met het metabool syndroom voornamelijk gemedieerd worden door abdominale obesitas en hypertriglyceridemie.

Testosteronspiegels zijn verlaagd bij mannen met type 2 diabetes. Er is echter vrij weinig bekend over de relatie tussen testosteron en glucosemarkers bij niet-diabeten. **Hoofdstuk 8** beschrijft de resultaten van een cross-sectionele studie naar de relatie tussen testosteron, SHBG en geglycosyleerd hemoglobine (HbA1c) onder 1292 mannen van het EPIC-Norfolk cohort. Mannen met lage testosteron en SHBG spiegels hadden hogere HbA1c waarden. Dit verband was onafhankelijk van andere diabetes risicofactoren en bleef significant na exclusie van mannen met type 2 diabetes.

Lage testosteronspiegels zijn in verband gebracht met een groot scala aan cardiovasculair risicofactoren, maar relatief weinig is bekend over een mogelijke relatie met niet-traditionele risicofactoren. **Hoofdstuk 9** beschrijft de resultaten van een cross-sectionele studie naar de relatie tussen testosteron, SHBG en leukocyten, een ontstekingsmarker en niet-traditionele risicofactor voor hart-en vaatziekten. Deze studie werd uitgevoerd onder 2418 mannen van het EPIC-Norfolk cohort. Lage testosteron en SHBG concentraties waren geassocieerd met een hoger gehalte aan leukocyten. Deze verbanden waren voornamelijk toe te schrijven aan een verhoogde concentratie granulocyten.

Hoewel de relatie tussen geslachtshormonen en cardiometabole risicofactoren in mannen vrij consistent is, zijn de resultaten van epidemiologische studies naar cardiovasculaire eindpunten niet eenduidig. In **Hoofdstuk 10**, onderzochten we daarom de cross-sectionele en longitudinale verbanden tussen geslachtshormoonspiegels en twee markers van subklinisch vaatlijden: de carotis-femoralis polsgolfsnelheid (PWV) en de intima-mediadikte van de halsslagader (cIMT). Uit deze studie bleek dat endogene geslachtshormoonspiegels niet gerelateerd zijn aan makers van subklinisch vaatlijden. Wel vonden we aanwijzingen voor een mogelijk drempeleffect: lage testosteron en SHBG spiegels waren alleen geassocieerd met hogere PWV waarden in oude mannen (> 70 jaar).

In **Hoofdstuk 11** worden de belangrijkste bevindingen van dit proefschrift bediscussieerd en worden suggesties voor vervolgonderzoek gedaan. Er is toenemend bewijs dat geslachtshormoonspiegels een belangrijke determinant zijn van cardiometabool risico in zowel mannen als vrouwen, maar er is nog veel onduidelijk over de directionaliteit van de gevonden verbanden. Meer suppletietrials en genetisch epidemiologische studies zijn nodig om meer inzicht te krijgen in de causaliteit en de klinische toepasbaarheid van de gevonden verbanden.

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Promoveren is een avontuur! In de afgelopen tweeënhalf jaar heb ik mogen proeven aan de wondere wereld van het wetenschappelijk onderzoek. Van dataverzameling tot ingewikkelde statistiche analyses, telkens een nieuwe uitdaging. Al deze ervaringen had ik nooit kunnen opdoen zonder de hulp van velen. Ik wil dan ook graag van de gelegenheid gebruik maken om iedereen te bedanken die heeft bijgedragen aan de totstandkoming van dit proefschrift.

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Judith Simone Maria Brand was born on March 24th 1984 in Roermond. She started her studies in Molecular Life Sciences at Maastricht University. After obtaining her Bachelor of Science degree in 2005 (with distinction) she began her graduate studies in Experimental and Clinical Neuroscience at Utrecht University. As part of this program, she conducted a research project at the Netherlands Institute for Neuroscience (NIN) in Amsterdam where she studied the regulation of estrogen receptor alpha expression in Multiple Sclerosis. In 2008, after receiving her Master of Science degree, she pursued a second master in Clinical Epidemiology at Utrecht University. As part of this study, she conducted a research project on testosterone and glycated haemoglobin at the Department of Public Health and Primary Care at Cambridge University, UK, under supervision of Prof. Kay-Tee Khaw. After completion of her Master of Science degree in Clinical Epidemiology in 2010 (with distinction), she started her PhD studies at the Julius Center for Health Sciences and Primary Care under supervision of Prof. Yvonne van der Schouw. The results of this work are described in this thesis.

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Publications based on studies in this thesis

Chapter 4

1. Brand JS, Chan MF, Dowsett M, Folkerd E, Wareham NJ, Luben RN, van der Schouw YT, Khaw KT. Cigarette smoking and endogenous sex hormones in postmenopausal women. *J Clin Endocrinol Metab.* 2011; 96: 3184-3192.

Chapter 5

2. Brand JS, van der Schouw YT. Testosterone, SHBG and cardiovascular health in postmenopausal women. *Int J Impot Res.* 2010; 22: 91-104.

Chapter 6

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Chapter 8

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Chapter 9

5. Brand JS, van der Schouw YT, Dowsett M, Folkerd E, Luben RN, Wareham NJ, Khaw KT. Testosterone, SHBG and differential white blood cell count in middle-aged and older men. *Maturitas.* 2012; 71: 274-278.