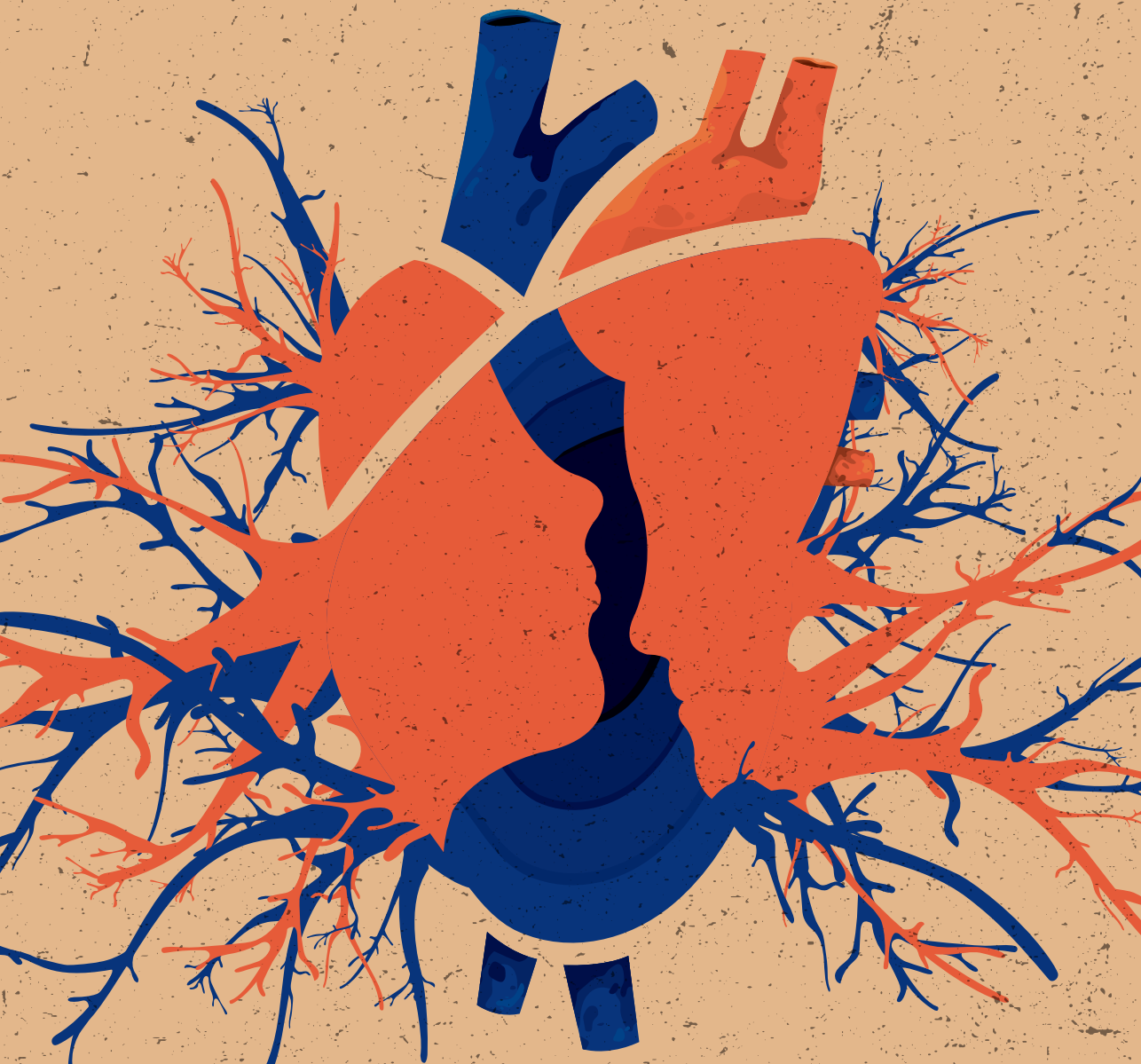
The role of sex hormones in sex differences across the cardiometabolic spectrum:

from menopause to mortality



Hamidreza Raeisidehkordi

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The role of sex hormones in sex differences across the cardiometabolic spectrum: from menopause to mortality

De rol van geslachtshormonen in sekseverschillen binnen het cardiometabole spectrum: van menopauze tot mortaliteit
(met een samenvatting in het Nederlands)

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Chapter 1

General introduction

Epidemiology of CVD

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality worldwide (1). In 2022, CVD accounted for approximately 19.8 million deaths, corresponding to 32% of all global deaths, with 85% attributable to myocardial infarction and stroke (1). In addition, the number of people living with CVD more than doubled worldwide, rising from 311 million in 1990 to 626 million in 2023 (2). CVD remains the leading cause of disability-adjusted life years (DALYs) according to the Global Burden of Disease (GBD) study as well. In 2023, there were an estimated 437 million CVD DALYs globally, representing a 1.4-fold increase from 320 million in 1990 (2).

Sex differences in cardiovascular diseases, risk factors and mortality

Despite major advances in prevention and treatment, substantial differences persist between men and women in the incidence, clinical presentation, and outcomes of CVD. In general, men experience a higher incidence of CVD compared with women across most regions and age groups (3). It has been shown that men have approximately twice the risk of developing coronary heart disease (CHD) compared with women of similar age (4). Men also tend to experience the onset of CVD roughly 7–10 years earlier than women (5).

In contrast, women exhibit an increase in CVD incidence after menopause, and once disease manifests, women frequently face worse short- and long-term prognosis (6).

Similarly, type 2 diabetes (T2D), a major risk factor for CVD, also exhibits important sex differences (7). Globally, the prevalence and incidence of T2D are slightly higher in men than in women (8), and men tend to develop diabetes at a lower body mass index and at a younger age than women. On the other hand, women with diabetes experience a disproportionately greater excess risk of cardiovascular complications (9). Meta-analyses report a 44% greater excess risk of CHD (10), 27% greater excess risk of stroke (11), and 13% higher risk of all-cause mortality (12), compared with men with diabetes.

In addition to the shared CVD risk factors, women have female-specific risk factors, including age of menarche and menopause, polycystic ovary syndrome, adverse pregnancy outcomes which may affect women's cardiovascular risk across their lifespan (13, 14).

Beyond disease-specific outcomes, consistent sex differences in all-cause mortality have been observed across populations and over time. Throughout adulthood, age-standardized mortality rates are generally higher in men than in women, reflecting both higher incidence of fatal diseases such as CVD and poorer survival following disease onset (7, 15).

These observations highlight the need to understand the biological mechanisms that underlie sex differences in cardiometabolic health.

Hormone cascade

Hormones are signaling molecules produced by endocrine glands, including the hypothalamus, pituitary gland, adrenal glands, gonads (e.g., testes and ovaries), thyroid gland, parathyroid glands, and pancreas. Endocrine refers to the process by which these glands, in response to specific stimuli, release their products directly into the bloodstream.

Once a hormone reaches its target cell, its binding to specific receptors triggers a cascade of biochemical reactions that ultimately modify the cell's function or activity (16).

Several major classes of hormones exist, including steroids, amino acid derivatives, polypeptides, and proteins. These classes differ in their molecular structure and, consequently, in their mechanisms of action (16).

Steroid hormones, produced primarily by the gonads and by parts of the adrenal gland (such as the adrenal cortex), have molecular structures similar to cholesterol. Because of their lipid-soluble nature, they can enter target cells and interact with receptors in the cytoplasm or nucleus. The gonads have two major functions: (1) producing germ cells and (2) synthesizing steroid sex hormones. Sex steroid hormones are essential for the development and function of both female and male reproductive organs, as well as secondary sex characteristics (e.g., adult patterns of body hair, such as facial hair in men). They are also critical for pregnancy, childbirth, and lactation. There are three main types of sex steroid hormones: estrogens (e.g., estradiol), progestogens (e.g., progesterone) and androgens (e.g., testosterone) (16, 17).

Sex hormone–binding globulin (SHBG) is a glycoprotein synthesized in the liver and released into the bloodstream, where it binds both androgens and estrogens, with higher affinity for androgens. By binding these hormones, SHBG regulates their circulating availability and modulates their access to target tissues

and cells. Evidence suggests that testosterone in men and estradiol in women play a prominent role in regulating SHBG levels, reflecting their higher relative concentrations in each sex (18).

Sex hormones beyond reproduction: Implications for cardiometabolic health

One of the most plausible explanations for sex differences in cardiometabolic health involves the influence of endogenous sex steroid hormones and SHBG. However, these disparities are multifactorial in origin and may also be shaped by differences in lifestyle factors (such as smoking, physical activity, obesity patterns, and diet), socioeconomic determinants (including education, healthcare access, and cultural roles), and other biological influences (genetic and hormonal factors) (6). Within this multifaceted framework, endogenous sex hormones and SHBG are of particular interest: testosterone (predominant in men), estradiol (predominant in women), dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S), together with SHBG, regulate a wide range of processes relevant to cardiovascular disease and diabetes, including adipose tissue distribution, glucose homeostasis and insulin sensitivity, lipid metabolism, vascular tone, and systemic inflammation (19).

Gaps in current evidence

Despite well-known sex differences in CVD, women remain underdiagnosed, undertreated, and underrepresented in cardiovascular research. They receive fewer diagnostic tests, interventions, and evidence-based therapies than men (20, 21). These disparities contribute to women accounting for more than one-third of global CVD deaths annually.

While there is considerable interest in understanding the role of sex hormones and SHBG in cardiometabolic health, important gaps remain. Observational studies of sex hormones and SHBG often suffer from heterogeneity in design, hormone measurement methods, and analytical approaches, resulting in inconsistent findings. In recent years, Mendelian Randomization (MR) studies have provided new insights by leveraging genetic variants as instruments for hormone levels, thereby strengthening causal inference. For example, MR analyses suggest that higher genetically predicted SHBG and sex hormones are involved in the etiology of T2D (22) or CVD (23). However, MR studies to date have primarily estimated causal effects at the population level and have not incorporated formal mediation analyses; therefore, they cannot fully address whether sex hormones and SHBG mediate observed sex differences in disease incidence and outcomes. In addition, there is limited evidence on sex-specific MR studies on the causal role of sex hormones or SHBG on CVD.

To address this gap, this thesis applies mediation analysis to investigate whether sex hormones or SHBG lie on the causal pathway between sex and cardiometabolic outcomes. Mediation approaches, increasingly used in epidemiology (24, 25), allow quantification of the extent to which the association between sex and outcomes such as T2D or CVD may be explained by differences in hormone profiles. This provides a complementary perspective to MR findings, offering insights into biological pathways that may underlie sex disparities in cardiometabolic health.

In addition, although a number of observational studies have examined associations of endogenous sex hormones or SHBG with risk of T2D or CVD, the majority of this research has been sex-specific**,** focusing on men and women separately (26, 27). Evidence directly comparing men and women within the same analytical framework is scarce. In this thesis, by applying mediation analysis, we sought to go beyond sex-specific analyses and explicitly evaluate whether differences in sex hormone and SHBG profiles mediate the observed sex differences in risk of T2D and CVD. This approach allows for a more integrated understanding of the complex interplay between sex, sex hormones, SHBG, and cardiometabolic outcomes.

This persistent imbalance underscores an urgent need to better understand the sex-specific biological mechanisms underlying cardiovascular risk, including the potential role of endogenous sex hormones and SHBG, which may contribute to the differential susceptibility and outcomes observed between men and women.

Study designs

Systematic reviews and meta-analyses

As part of this thesis, three systematic reviews and meta-analyses were conducted to investigate key aspects of hormonal and cardiovascular health. Specifically, these studies focused on: (1) existing risk prediction models for the onset of natural menopause, (2) the associations between endogenous sex steroid hormones, SHBG, and the risk of all-cause and cause-specific mortality in the general population, and (3) the relationship between circulating sex hormones, SHBG and mortality risk among patients with established CVD.

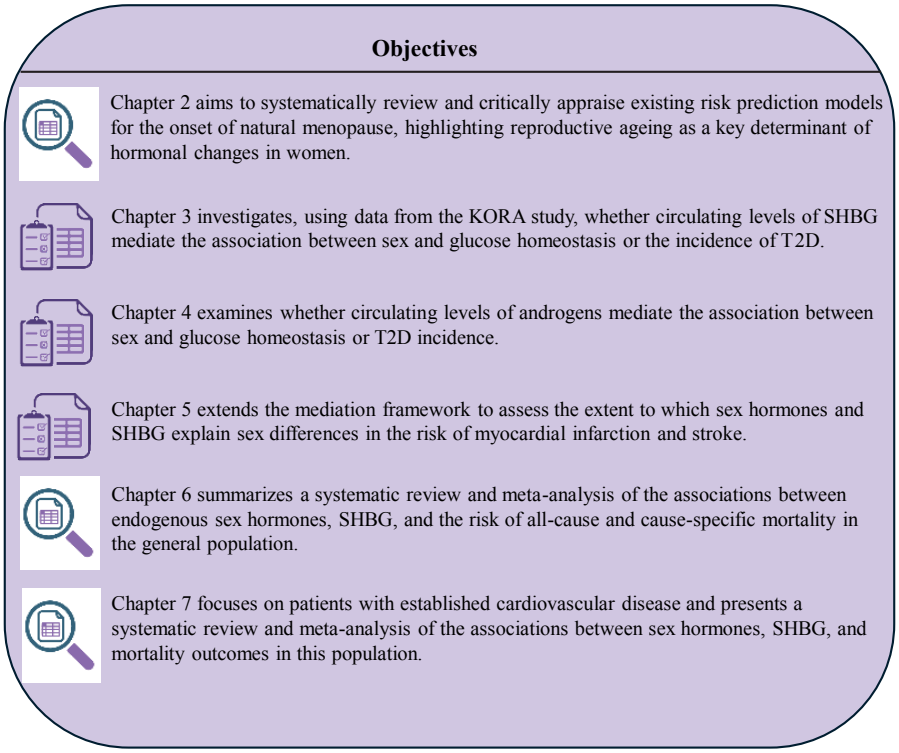
These systematic reviews and meta-analyses were conducted in accordance with established methodological guidelines (28-30). Further details on the methods can be found in the specific chapters.

KORA study

The mediation analyses presented in Chapters 3-5 of this thesis were conducted within the framework of the Cooperative Health Research in the Region of Augsburg (KORA) study. Data were derived from the KORA F4 study (2006–2008) as the baseline and the KORA FF4 study (2013–2014) as the follow-up, both of which are follow-up examinations of the original KORA S4 study (1999–2001) (31). Baseline data from the KORA F4 study and follow-up data were used in this thesis. More details on the methodology and study designs are provided in the specific chapters.

Outline of this thesis

This thesis comprises eight chapters. Chapter 1 provides a general introduction, and Chapter 8 presents the general discussion. Chapters 2–7 each address a specific role of sex hormones or SHBG in relation to cardiometabolic diseases and mortality in men and women. The objectives of these chapters are summarized in Graphical Abstract 1.



Graphical Abstract 1.

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1



Chapter 2

Risk prediction models of natural menopause onset: a systematic review

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Abstract

Context: Predicting the onset of menopause is important for family planning and to ensure prompt intervention in women at risk of developing menopause-related diseases.

Objective: To summarize risk prediction models of natural menopause onset and their performance.

Data Sources and Study Selection: Five bibliographic databases were searched up to March 2022. We included prospective studies on perimenopausal women or women in menopausal transition, that reported either the univariable or multivariable model for risk prediction of natural menopause onset.

Data Extraction: Two authors independently extracted data according to the CHARMS (critical appraisal and data extraction for systematic reviews of prediction modelling studies) checklist. Risk of bias was assessed using PROBAST (prediction model risk of bias assessment tool).

Data Synthesis: Of 8'132 references identified, we included 14 articles based on 8 unique studies comprising 9'588 women (mainly Caucasian) and 3'289 natural menopause events. All the included studies used onset of natural menopause (ONM) as outcome, while four studies predicted early ONM as well. Overall, there were 180 risk prediction models investigated, with age, anti-Müllerian hormone (AMH) and follicle-stimulating hormone (FSH) being the most investigated predictors. Estimated C-statistic for the prediction models ranged from 0.62 to 0.95. Although all studies were rated at high risk of bias mainly due to the methodological concerns related to the statistical analysis, their applicability was satisfactory.

Conclusion: Predictive performance and generalizability of current prediction models on ONM is limited given that these models were generated from studies at high risk of bias and from specific populations/ethnicities. Although in certain settings such models may be useful, efforts to improve their performance are needed as use becomes more widespread.

Keywords: Risk prediction model, Onset of menopause, Perimenopause, Premenopausal women

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PROSPERO registration code CRD42021248771.

Introduction

Menopause as the complete cessation of the menstrual periods, marks the end of a woman's natural reproductive lifespan. This event occurs between the age range of 40-60 year, with a median age of approximately 49 to 51 years (1). The relatively wide age range for the onset of menopause would seem to indicate that females have a highly variable number of oocytes and/or lose them at a highly variable rate (2). With the aging of the population, especially in the last decades, it is estimated that 1.2 billion women will be menopausal (experiencing menopause) or postmenopausal by 2030 (3). Age at natural menopause is a crucial period for women of reproductive age since both an early or late menopause may have some health implications (4). Early menopause is associated with lower bone mass density, osteoporosis, depression and increased risk of type 2 diabetes, cardiovascular diseases, and all-cause mortality (5-8). On the other hand, late menopause has been associated with increased risk of sex-related cancers including breast, ovarian and endometrial cancers (9). Thus, predicting onset of natural menopause (ONM) may not only help clinicians regarding contraception (10), assisted reproduction (11) and management of menorrhagia (12), but also may alert women in reproductive ages of health-related complications due to either early or late menopause. With ongoing lifestyle changes and trends, many women postpone childbearing to the end of their reproductive lifespan. Therefore, an accurate estimation of time of menopause could improve the preventive management of unexpected early infertility (13).

While menopause is inevitable, research has indicated that ONM may vary depending on a wide range of factors including genetic factors, lifestyle and environmental exposures, sociodemographic factors and hormonal mechanisms (14, 15). Thus, considering these known risk factors in univariable or multivariable models, may provide insight into the prediction of natural menopause precisely. The most studied predictor biomarker is the circulating anti-Mullerian hormone (AMH), which as a single biomarker or combined with age and other variables shows a C-statistics ranging from 0.70 to 0.90 (16, 17). Estradiol and Follicle-Stimulating Hormone (FSH) have also been explored as predictor biomarkers of ONM, showing a C-statistic similar to AMH (18, 19). However, most articles have not performed validation (17, 18, 20), neither calibration (17, 18, 21, 22). Calibration is an important step in prediction models because even a good-performing model based on its C-statistics, can over- or underestimate the outcomes risk. In addition, the majority of the current prediction models have not provided clear time prediction period to predict ONM. Uncertainty on generalizability of prediction models to different

populations and ethnicities exists as well. Thus, there is a need to summarize the risk prediction models for ONM and understanding best predictors of natural menopause as well as current gaps in the literature. In the present study, we aimed to perform a comprehensive systematic review to identify prediction models to forecast the timing of ONM and age at menopause of premenopausal women.

Methods

This systematic review was conducted according to a guideline on systematic reviews and meta-analysis (23), and reported according to the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA)-guidelines (24). The study protocol was registered in the International Prospective Register of Systematic Reviews (PROSPERO) database on May 15, 2021 (CRD42021248771).

Search strategy

A systematic literature search was conducted by a library information scientist (BM) in Embase (Ovid), Web of Science, Medline (Ovid), Cochrane CENTRAL and Google Scholar up to February 8, 2021 and was updated on March 25, 2022 using the following Medical Subject Headings (MeSH) and non-MeSH keywords: (i) risk prediction, predictive, prediction model, diagnostic, diagnostic model, predictive ability; (ii) menopause, premature menopause, perimenopause, post menopause, age of menopause, early menopause, late menopause, menopause onset; (iii) accuracy, sensitivity, specificity, positive predictive value PPV, negative predictive value NPV, likelihood ratio, area under the ROC curve (AUC), Youden's index, diagnostic odds ratio (DOR), C-statistics. Furthermore, we made a forward citation tracking and also hand-searched the reference list of eligible studies. The selection of studies was not restricted based on date and language. The detailed search strategy is presented in **Supplementary Material 1** (25)**.**

Selection criteria

Published original studies fulfilling the following criteria were included: (i) prospective cohort studies, case-cohort, and nested case-control studies (i.e., studies with prospective data collection); (ii) conducted on premenopausal or perimenopausal women or women in menopausal transition; (iii) had followed women over time for the occurrence of menopause and reported ONM; (iv) reported either the univariable model or multivariable model for risk prediction of ONM; (v) had presented at least one of the following outcomes: accuracy, sensitivity, specificity, positive predictive value (PPV), negative predictive value

(NPV), likelihood ratio, area under the curve (AUC), Youden's index, diagnostic odds ratio (DOR), and C-statistics.

Studies were excluded if they (i) were performed in animals; (ii) were cross-sectional or case-control studies without prospective data collection; (iii) were focused on menopause due to surgery, radiotherapy, or chemotherapy; (iv) provided data about model performance beyond our inclusion criteria like hazard ratios. Titles and abstracts of publications were manually screened by independent reviewers (HR-D, SK, PFR, GD, PET). Subsequently, the full-text screening was performed. Disagreements in judgment were resolved by discussion with a third reviewer (MG or TM).

Data extraction

For each included article, we extracted the following data: study and population characteristics (year of publication, country, study design, funding source, number of participants, follow up time, exclusion criteria, age, menopause status), variables considered for the prediction (hormones, imaging-like transvaginal ultrasound, menstrual cycle irregularities), number of natural menopause events, definition of menopause and early natural menopause, prediction time and performance of the model (C-statistics, sensitivity, specificity, AUC) using a standardized data extraction form based on the Critical Appraisal and Data Extraction for Systematic Reviews of Prediction Modelling Studies (CHARMS)-checklist (26). In the case of multiple publications, the most up-to-date or comprehensive paper was included unless the article investigated different predictors and study outcomes in different time periods within the same study (e.g., 2 years or 1 year before the onset of the last menstruation (22, 27)) . Two reviewers (HR-D and SK) independently extracted all of the required data. Possible discrepancies were resolved by consulting a third reviewer (MG or TM).

Quality assessment

Risk of bias (ROB) and applicability to the intended population and setting was examined by using "A tool to assess risk of bias and applicability of prediction model studies PROBAST"(28). PROBAST contains main domains including (participants, predictors, outcome, and analysis) and assesses the ROB for the four domains and applicability, except for the analysis domain. Each domain is characterized by specific questions (20 questions overall). Questions may be answered as yes, probably yes, probably no, or no information, depending on the provided information of each included study. A domain is at high ROB when at least one of its items is judged as not appropriate (no or probably no). The overall risk of bias is considered as high ROB when one or more domains are considered to be at high risk.

Results

Study selection

A flow chart of the study selection process is shown in **Figure 1**. A total of 8'132 citations were obtained from the initial database search. After removal of duplicates, 4'415 unique references remained for screening of the titles and abstracts, of which 95 articles underwent full-text reviewing. Fourteen articles based on eight unique studies (16-22, 27, 29-34) describing 180 prediction models of ONM satisfied the inclusion criteria and were selected for data extraction and synthesis **(Supplementary Table 2)** (25).

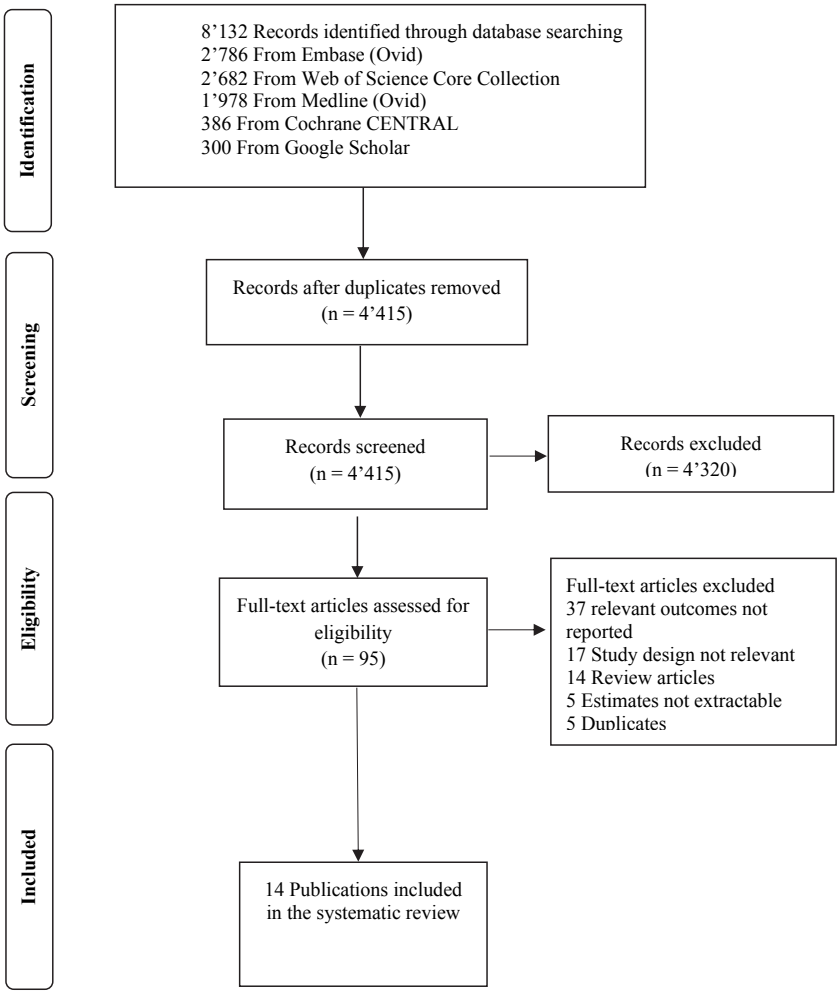


Figure 1. Study selection procedure.

Study characteristics

Population: Of the 14 articles included, six (16-21) used data from the Netherlands, four from USA (21, 23, 29, 32), three (22, 30, 34) from Iran and one (32) article combined data from the Netherlands and Iran. Some cohorts were used multiple times for model development. The Doetinchem Cohort Study was used in De Kat et al. (16) and Dolleman et al. 2015 (21). The SWAN cohort (The Study of Women's Health Across the Nation) was used in Finkelstein et al. (22) and Greendale et al. (27). The article by Depmann et al. 2016 is a follow-up study of Broer et al. (the Scheffer, van Rooij, de Vet (SRV) cohort study). The article of Dolleman et al. 2014 (20) consists of two cohorts, one of which is the SRV cohort. The TLGS cohort (Teheran Lipid and Glucose Study) was used in Gohari et al. (29), Tehrani et al. 2020 (31) and Tehrani et al. 2021(34). The study by Tehrani et al. 2014 (32) was performed using data from two cohorts, TLGS and the SRV cohort. Thus, 14 articles (16-22, 27, 29-34) based on 8 unique study populations (16, 17, 19, 20, 22, 29, 30, 33) were included for final analysis.

All articles included premenopausal women with ages ranging from 18 to 63 years at baseline. Ten articles included Caucasians (16-21, 29, 31, 32, 34), while two articles included a black or white population (30, 33). Two articles included cohorts of The Study of Women's Health Across the Nation (SWAN) (22, 27), which included different ethnicities (Caucasian, African American, Hispanic, Chinese & Japanese). The proportion of hormone therapy users (either hormone replacement therapy or hormonal contraception) varied from 16 to 33% (16, 21, 30). The ONM was self-reported in all the included studies.

Women who underwent gynaecological surgery (oophorectomy, hysterectomy) or regularly took hormones (oral contraception, hormonal replacement therapy) were excluded from most studies. Other exclusion criteria of individual studies were pregnancy, infertility, unproved fertility, other endocrine disorders, or irregular/unpredictable menstrual cycles at the time of first assessment. The exclusion criteria for each study are summarized in **Supplementary Table 1** (25).

Prediction models: Risk prediction models of included articles used biomarkers (AMH, FSH, Estradiol, Inhibin B), imaging (transvaginal ultrasonography to obtain antral follicle count), menstrual cycle irregularities/climacteric symptoms, or lifestyle factors (body mass index (BMI), smoking). All biomarkers were assessed at a single time point, which is usually at baseline. More than half of the articles (n = 8) developed and validated both the univariable and multivariable model (16-20, 29, 30). For each of these individual studies, at first, the accuracy of prediction was checked for the

individual predictors. Next, the predictors with the best accuracy were combined to explore whether it improved the prediction of ONM. Five articles elaborated only multivariable models (22, 27, 29, 32, 34), and one article (33) provided univariable model. With the exception of one article (27), all articles had the prediction of ONM as an outcome. Menopause in the included articles was defined as 12 consecutive months without menstruation (3), and was based on self-reporting. Greendale et al. (27) validated risk prediction models that predicted the probability of a woman having crossed 2 years before, 1 year before, and the final menstrual period (FMP) itself. Four studies also provided the risk prediction of onset of early menopause (16, 21, 31, 34). Nine articles reported C-Statistic (16-18, 20, 21, 30-32, 34), one article reported AUC (19) and another one only sensitivity/specificity (33), and three articles reported AUC/sensitivity/specificity (22, 27, 29). A 95% confidence interval was reported in eight articles (16, 19, 20, 22, 31-34).

Prediction horizon: The majority (8 of 14) of articles (16-18, 20, 21, 31, 32, 34) did not report the prediction time of menopause onset. Finkelstein et al. (22) and Greendale et al. (27) used prediction times of 1, 2 and 3 years till women became menopausal, whereas Gohari et al. (29) provided forecast information in 3, 4 or 5 years. Kim et al. (30) and van Rooij et al. (19) predicted the time to menopause to be 5 and 4 years, respectively. Taylor et al. (33) used a prediction time that ranged within 2 or 4 years.

Study design: All articles were of prospective cohort design.

Sample size: The number of participants ranged from 81 to 2434. The mean follow-up period was 10.2 years. During follow-up, a mean of 34.3% of women reached menopause. The number of events (occurrence of menopause) were clearly reported in 10 articles (16-18, 20, 21, 29-32, 34), and ranged from 46 to 1'298. Four articles (19, 22, 27, 33) did not report the number of women experiencing menopause during the follow-up.

Outcome assessment: One model (33) was developed using only self-reported data (menstrual cycle irregularities). However, most of the models (13 of 14) were developed using a combination of self-reported (interview or questionnaire) and biological measurements (e.g., hormones and antral follicle count).

Predictors: There were overall 180 models tested. In total, 20 different predictors were included. Most models included a set of similar predictors, consisting of age, (n = 121), Anti-Müllerian hormone (AMH) (n = 87), and follicle-stimulating hormone

(FSH) (n = 44). Other commonly identified predictors are shown in **Supplementary Figure 1** (25).

Predictors of ONM in risk prediction models

Most common predictors

The three most common predictors were age, AMH and FSH, and findings on these predictors are summarized in **Table 1.**

Age: Age was the most common investigated predictor, used in 121 models (from a total of 180 models). Age as the only predictor was used in 7 studies (17-21, 30, 33); in 6 studies age as a sole predictor showed AUC or C-statistics ranging from 0.82 – 0.88 (17-21, 30). Another study performed by Taylor et al. (33) showed poor sensitivity and good specificity of age in forecasting menopause within 2 or 4 years (sensitivity 42.4% (95% CI 26.8-60.0) and 40% (95% CI 28.3-52.3), specificity 87.4% (95% CI 82.9-91.1) and 94.8% (95% CI 90.2-97.4), respectively (33).

AMH: AMH was used in 87 predictive models, making it the second most common investigated predictor. AMH as a sole predictor of menopause onset provided a C-statistics ranging from 0.70 to 0.87 (16-19). Adding age into the models using AMH improved predictive ability across all articles (C-statistics/AUC 0.86 – 0.90) (17-19) **Supplementary Materials 2** (25).

FSH: FSH was used in 44 prediction models (of which 3 in univariable models) (17-19). The univariable models of Broer et al. (17) and Depmann et al. 2016 (18) using FSH as a predictor (18) showed C-statistics of 0.70 and 0.66, respectively. The AUC of van Rooij et al. (19) was 0.72 (95% CI 0.56-0.88). After adjusting for age, the model performance of all three mentioned articles improved (Depmann 0.85, Broer 0.88, and van Rooij 84) **Supplementary Materials 2** (25).

Table 1. Most common predictors of menopause onset.

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
Broer, 2011	185	48 (25.9%)	11	1996-2010	n.r.	Age	-	-	-	87
						AMH	-	-	-	86
						FSH	-	-	-	70
De Kat, 2019	2434 a	1298 (53%)	20	1987-2019	n.r.	AMH	-	-	-	70 (68-72)
Depmann, 2016	155	81 (52.2%)	14	1992-2013	n.r.	Age	-	-	-	85
						AMH	-	-	-	78
						FSH	-	-	-	66
Dolleman, 2014 (study group 2)	150	46 (30.6%)	12	n.r.	n.r	Age	-	-	-	84 (78-90)
						AMH	-	-	-	86 (81-91)
Dolleman, 2015	1163 b	527 (45.3%)	10	1993-2007	n.r	Age	-	-	-	88
Kim, 2016	426 c	n = 55 (13%)	10	2001-2011	5	Age	-	-	-	83
	359 d	n.r.				Age	-	-	-	82
	327 e					Age	-	-	-	81
	306 f					Age	-	-	-	86
Taylor, 2004	326	n.r.	4.8	1993-1997	Within 2 years	Age ≥ 50 years	42.4 (26.8-60.0)	87.4 (82.9-91.1)	-	-
					Within 4 years	Age ≥ 50 years	40 (28.3-52.3)	94.8 (90.2-97.4)	-	-

Table 1. Continued

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
Van Rooij, 2004	81	N = 14 had cycle fluctuations	5	1996-?	4	Age	-	-	82 (71-93)	-
						AMH	-	-	87 (79-96)	-
						FSH	-	-	72 (56-88)	-

Abbreviations: CI, Confidence interval; AUC, Area under the curve; FSH, Follicle stimulation hormone; AMH, Anti-Mullerian Hormone; AFC, Antral follicle count; ANM, age at natural menopause; OC, oral contraceptive; ANM, age at natural menopause; n.r., not reported.

[a] Women who were using oral contraceptives were also included in the final analysis. [b] Pregnant women, women who were taking hormonal replacement therapy and OC users were also included in the final analysis. [c] All women. [d]Women not using estrogen. [e] Women with regular menses only

[f] No women with dominant follicle.

Final menopause prediction models

In the following section, we present the final models reported by the authors and /or the models with the best performance generated from the included studies; the findings and whether these final models were validated and/ or calibrated are summarized in **Table 2**. Overall C-statistics/AUC ranged from 0.710 – 0.987, while one article (33) showed a sensitivity and specificity of 93.9% (95% CI 80.6-98.9) and 90.8% (95% CI 86.7-94.0), respectively. Age and AMH were present in most included studies. Of the included studies, only six (16, 19, 21, 22, 30, 31) and one (32) studies validated their models internally and externally, respectively, and 5 studies performed calibration (16, 27, 29, 31, 32). Of which, while all of the above-mentioned studies performed validation for their final models (16, 19, 21, 22, 30-32), only the study by Gohari et al. (29) performed calibration for their final model.

Best model performance of Broer et al. (17) and Tehrani et al. 2014 (32), consisting of age and AMH, showed a C-statistics of 0.90 and 0.88 (95% CI 0.83 – 0.94), respectively. De Kat et al. (16) and Gohari et al. (29) models both included AMH at a certain age (35 and 43 years, respectively). The C-statistics and AUC were 0.71 (95% CI 0.69 – 0.72) and 0.987, respectively. Addition of AMH decline rate, oral contraception use or smoking to De Kat's model did not have any impact on the model performance. Best model performance presented by Depmann et al. 2016 (18) included age, AMH and smoking (C-statistic 0.87). There was no change in performance between the different time-points of age or smoking (baseline versus follow up). Dollemann's (2014) (20) final model including daughter's age and AMH and mother's age of natural menopause (ANM) presented a C-statistic of 0.92 (95% CI 0.88 – 0.96). The study showed that AMH was a more accurate menopause predictor than mother's age at menopause. Dolleman et al., 2015 (21) presented a multivariable model with good performance consisting of age, AMH, BMI, packyears of smoking and menstrual cycle status (C-statistic 0.91). A similar model by Finkelstein et al. study with age, AMH and BMI presented an AUC of 0.896 (95% CI 0.889 – 0.903) (22).

The final model by Greendale et al. (27) included FSH, Estradiol, age, menopause transition stage, race/ethnicity and time of venipuncture (in early follicular phase) either 2 years before final menstrual period (FMP), 1 year before FMP or at FMP. The models with different prediction times showed AUC of 0.902 – 0.945.

Kim et al. combined age with different hormones (AMH and FSH) and AFC and presented a C-statistic of 0.95.

Taylor (33) used six definitions of menstrual variability for predicting time to natural menopause within 2 or 4 years (i. more than 90 days since the most recent menstrual period; ii. 60 or more days of amenorrhea during the previous years; iii. cycle lengths that varied by 19 or more days; iv. cycle lengths too variable to report a usual length; v. cycles less regular than they had been at age 40; vi. change in the duration or heaviness of menstrual flow compared with age 40). The second definition of menstrual variability showed the highest sensitivity (93.9%, 95% CI 80.6 - 98.9%) and specificity (90.8%, 95% CI 86.7 – 94%) within 2 years.

Tehrani et al. 2020 (31) showed that prediction of age at menopause could be improved by multiple AMH measurements. Their final prediction model included age, AMH, annual AMH decline rate and sample storage time, and showed a C-statistic of 0.81 (95% CI 0.79 – 0.83). Van Rooij's (19) prediction model, which included age, AMH and Inhibin B, resulted in AUC of 0.92 (95% CI 0.86 – 0.99).

In a recent study done by Tehrani et al. 2021 (34), AMH measurement at different time points, baseline age, storage time of AMH and annual AMH decline rate were included in the final model, resulting in a C-statistic of 0.85 (95% CI 0.83-0.87).

Detailed explanation of the risk prediction models of the included studies are presented in **Supplementary Table/ Materials 2** (25).

Table 2. Final menopause prediction models.

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
Broer, 2011	185	48 (25.9%)	11	1996-2010	n.r.	AMH + age*	-	-	-	90
De Kat, 2019[d]	2434[a]	1298 (53%)	20	1987-2019	n.r.	AMH at age 35*	-	-	-	71 (69-72)
Depmann, 2016	155	81 (52.2%)	14	1992-2013	n.r.	AMH + age + smoking*	-	-	-	87
Dolleman, 2014 (**study group 2**)	150	46 (30.6%)	12	n.r.	n.r	Daughter's age + mother's ANM + daughter's AMH*	-	-	-	92 88-96
Dolleman, 2015[d]	1163[b]	527 (45.3%)	10	1993-2007	n.r	AMH + age + BMI + packyears of smoking + menstrual cycle status	-	-	-	91
Finkelstein, 2020[d]	1537	n.r	2	1996-1998	3	AMH + age + BMI*	-	-	89.6 (88.9-90.3)	-
Gohari, 2016[f]	266	63 (23.7 %)	6.5	1999-2006	3	AMH + Age at making prediction was 43*	-	-	98.7	-

Table 2. Continued

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
Greendale, 2013[d]	552	n.r.	10	1996-2007	2 years before FMP [c]	FSH + Estradiol + age + menopause transition stage + race/ethnicity + time of venipuncture (in early follicular phase = cycle days 2-5 = in-window)	-	-	90.2	-
					1 year before FMP [d]	FSH + Estradiol + age + menopause transition stage + race/ethnicity + time of venipuncture (in early follicular phase = cycle days 2-5 = in-window)	-	-	92.6	-
					FMP [e]	FSH + Estradiol + age + menopause transition stage + race/ethnicity + time of venipuncture (in early follicular phase = cycle days 2-5 = in-window)	-	-	94.5	-
Kim, 2016[d]	306 [c]	n.r.	10	2001-2011	5	Age + AMH + FSH + AFC*	-	-	-	95

Table 2. Continued

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
Taylor, 2004	326	n.r.	4.8	1993-1997	Within 2 years	60 or more days of amenorrhea during the previous year*	93.9 (80.6-98.9)	90.8 (86.7-94.0)	-	-
Tehrani, 2014[e]	266	n = 63, 23.7%	6	n.r.	n.r.	Age + AMH (TLGS model on TLGS data)*	-	-	-	88 (83-94)
Tehrani, 2020[d]	959	n = 529, 55.2%	14	1998-?	n.r.	Age + AMH + annual AMH decline rate + sample storage time	-	-	-	81 (79-83)
Tehrani, 2021	901	522	18	1998-2016	n.r.	AMH value + Log (annual AMH decline rate) (adjusted for age at baseline and storage time of AMH)	-	-	-	0.85 (0.83–0.87)
Van Rooij, 2004[d]	81	n = 14 had cycle fluctuations	5	1996-?	4	Age + AMH + Inhibin B	-	-	92 (86-99)	-

Abbreviations: CI, Confidence interval; AUC, Area under the curve; FSH, Follicle stimulation hormone; AMH, Anti-Mullerian Hormone; AFC, Antral follicle count; ANM, age at natural menopause; OC, oral contraceptive; ANM, age at natural menopause; n.r., not reported

[a] Women who were using oral contraceptives were also included in the final analysis.

[b] Pregnant women, women who were taking hormonal replacement therapy and OC users were also included in the final analysis.

[c] No women with dominant follicle

[d] Studies that performed internal validation for their final models.

[e] Studies that performed external validation.

[f] Studies that performed calibration for their final models.

* These models were selected for their best performance. Models without labelling were called final models by the authors.

ROB assessment

The detailed summary of ROB assessment based on each domain and its related sub-domains for the included studies is presented in **Supplementary Table 3** (25). All included studies were classified as high ROB (**Figure 2 and Figure 3**) (16-22, 27, 29-34). ROB assessment based on each domain showed that all of the included articles had a low ROB for "participants" domain, demonstrating that participants enrolled in the articles were representative for the models' targeted populations (**Figure 2**).

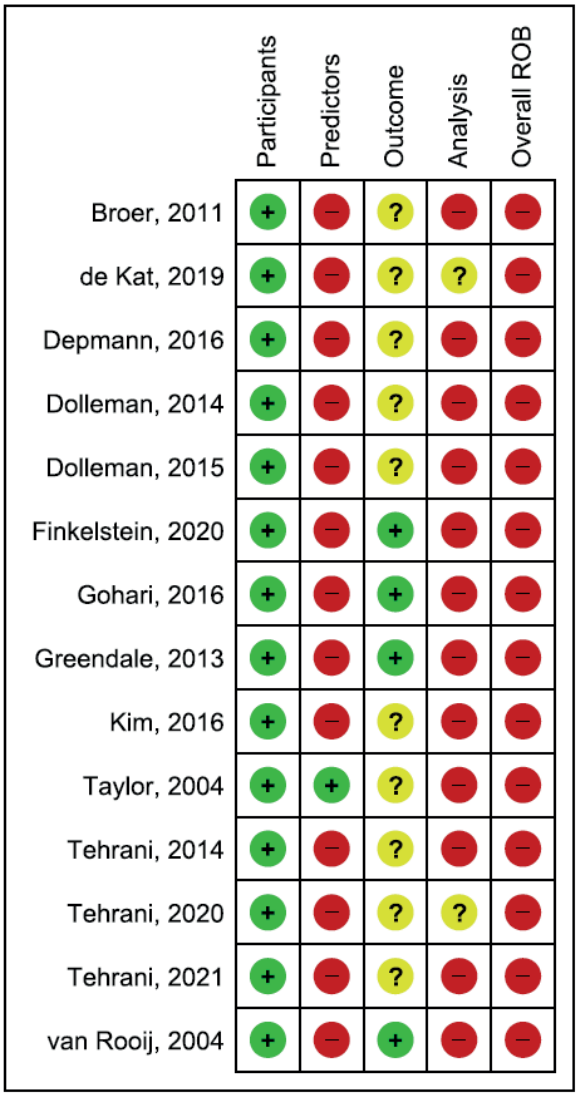


Figure 2. Risk of bias assessment (ROB) summary of the included studies.

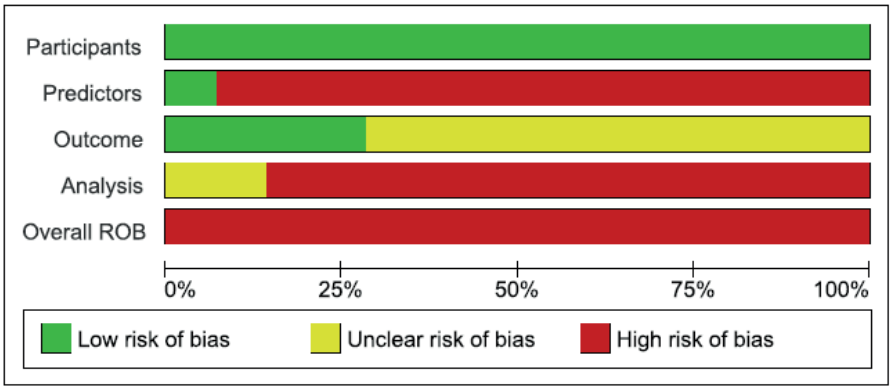


Figure 3. Risk of bias graph of the included studies.

Thirteen articles (16-22, 27, 29-32, 34) had a high ROB for "predictors" domain as predictors were not defined in a similar way for all participants or different assessment methods were used or since all of the predictors would not be available at the time the model intended to be used for prediction. The study performed by Taylor et al. (33) was the only study that all of its predictors would be available at the time the model is intended to be used for prediction (**Figure 2**). The ROB was unclear for 10 articles for "outcome" domain (**Figure 2**) (16-18, 20, 21, 30-34); Eight out of 10 articles did not provide any information on the time interval between predictor assessment and outcome determination (16-19, 22, 29, 31, 34). The article done by Kim et al. did not provide any information on whether a prespecified or standard outcome definition (onset of menopause) was used or whether predictors were excluded from outcome definition (30). In addition, another article had an unclear ROB for this domain due to the lack of information on whether predictors were excluded from the outcome definition or whether outcome was determined without knowledge of predictors information (33). Twelve of the 14 articles were at high ROB for the "analysis" domain, and the two remained articles were scored as unclear ROB for this domain (**Figure 2**). Number of participants with the outcome (those who reached menopause) was not provided in 4 articles and subsequently the ROB was considered as unclear for this sub-domain (19, 22, 27, 33), while the article performed by Kim et al. (30) was at high ROB due to the limited number of participants with the outcome relative to the number of candidate predictor parameters. Three articles did not provide information on whether all enrolled participants were included in the analysis and were scored as unclear for this sub-domain (19, 27, 29), nevertheless, the article done by Finkelstein et al. (22) was at high ROB for this sub-domain due to the inappropriate exclusion of 1108 enrolled women from the final analysis. Only three articles presented sufficient

information on handling the participants with missing data appropriately and were scored as low ROB (16, 21, 34). Only one study was at high ROB due to the selection of predictors based on univariable analysis (33), while the other 13 articles were at low ROB for this sub-domain (16-22, 27, 29-32, 34). Although four articles did not present information on complexities in the data analysis (21, 29, 31, 33) and four articles used inappropriate analytical methods (19, 22, 27, 30) and were scored as unclear or high ROB, respectively, six articles accounted for complexities in the data analysis appropriately (16-18, 20, 32, 34). Regarding the complexities in the data analysis, three out of six articles (17, 18, 32) have used a Weibull survival model analysis which accounts for the length of follow-up and delayed entry of participants rather than logistic models in which predictions are not specific to a particular time period and the length of follow-up is being ignored. In addition, three other articles (16, 20, 34) have used a time-to-event analysis, such as Cox regression, which accounts for censored participants up to the end of their follow-up rather than logistic regression models which simply exclude participants with incomplete follow-up. Nine articles did not assess calibration and were at high ROB (17-22, 30, 33, 34), indicating that the performance or the ability of their model to provide accurate individuals probabilities is unsatisfactory. Seven articles have used some form of validation and were scored as low ROB (16, 19, 21, 22, 30-32). Of which, 6 studies have performed internal validation methods such as bootstrapping (16, 19, 21, 22, 30, 31) and only one study used cross-validation method (32). Only three articles reported that the predictors and regression coefficients in the final model correspond to reported results from multivariable analysis and subsequently were scored as low ROB (20, 21, 31).

Concerns regarding applicability of the presented models are presented in **Supplementary Figures 2 and 3** (25). Assessment of applicability to "participants", "predictors", and "outcome" domains revealed that four, two, and eight articles were rated with high, unclear, and low overall applicability concern, respectively.

Discussion

Summary of main findings

This study systematically reviewed the literature and identified 14 articles describing 180 either univariable or multivariable risk prediction models of menopause onset. These models varied significantly regarding participant inclusion, sample size, predictors, endpoints, and model performance. The different predictors included demographics, lifestyle, biomarkers, imaging, and clinical features of menopause

transition. In total, 13 of the 14 articles used more than one predictor in their models. The most commonly used predictor was age, followed by AMH and FSH.

While there are some narrative reviews on this topic, they have major limitations. First, they have not performed a systematic appraisal of evidence, which could lead to bias. Our study provides the first comprehensive systematic review on the topic, using well-defined inclusion and exclusion criteria. Second, previous studies have focused on a limited number of predictors like AMH (35) or restricted their results to Western European countries (36). Of the 14 included articles, most were conducted on populations from the Netherlands (n = 7), the USA (n = 4) or Iran (n = 5). Risk prediction models may, however, act differently in populations of different racial or ethnic backgrounds. Systematic efforts to validate models in other populations are essential. Women who were using hormone therapy for medical reasons or as contraceptives or hormonal replacement therapy were in general excluded from the articles. However, De Kat et al., Dolleman et al. 2015 and Kim et al. included women who were taking estrogen (oral contraception, hormonal replacement therapy) (16, 21, 30). Dolleman et al. 2015 also included pregnant women (21). Pregnancy or exogenously supplied hormones affect the level of hormones, which can affect model`s performance.

In addition, more than half of the included studies did not report the time interval between the assessment of predictors and outcome. The interpretation of the results is strongly dependent on the prediction horizon, and among the articles reporting time of prediction, the focus was on short-term prediction of menopause onset. While long-term prediction models can be useful in terms of public health interventions to identify women at risk of developing early menopause, such models are lacking. In addition, predicting the onset of menopause becomes more difficult the longer this time lies in the future and is therefore more prone to error, which needs to be explored and addressed by future studies.

While overall, the presented prediction models were at high ROB, all the included articles were at low concern regarding inclusion and exclusion of participants and none of them included participants who already had the outcome (menopausal women). Inclusion of participants who already have the outcome may result in biased and overestimated predictive performance. Most of the articles suffer from serious analytical limitations. For instance, 10 articles excluded participants with missing data from the final analysis, which may cause biased predictor-outcome associations and result in misleading model performance. In addition, three articles only presented classification measures, including sensitivity and specificity, to

show model predictive performance without providing C-statistics (22, 27, 33). It is worth mentioning that the estimation of classification requires the consideration of one or more thresholds in the range of the model-predicted probabilities, which typically leads to misinformation since the whole range of predicted probabilities of the model is not fully considered, and also, the selection of thresholds might be data-driven rather than prespecified clinical cut-offs. The introduction of thresholds helps reporting the performance of predictive models at probability thresholds that may be clinically relevant, which does not include the entire range of the model-predicted probabilities. This approach may result in critical bias in the estimated classification measures, especially when thresholds are selected to show the maximum performance (37, 38). Calibration was not assessed in nine studies, and it is worth mentioning that only the study by Gohari et al. (29) performed calibration for all models, while the remaining four studies reported calibration for a limited number of models (16, 27, 31, 32), which indicates that the performance of their prediction models to provide accurate probabilities are doubtful. Poorly calibrated or not calibrated risk prediction models can be misleading and potentially harmful for clinical decision-making guidelines. Moreover, while over half of the included articles presented some form of internal validation that accounts for the overfitting of the developed model and optimism in its predictive performance, the evidence from the external validation of the presented models is still unsatisfactory. Only one article performed by Tehrani et al. 2014 (32) replicated their models into another cohort in a cross-validation approach. In addition, most of the presented models did not provide 95% CIs of the estimates. The majority of articles did not test whether the differences between models in predicted estimates (e.g., AUC) were significantly different, thus precluding the interpretation of results.

Strengths and limitations

To our best knowledge, the current study is the first study summarizing the risk prediction models of menopause onset. Due to a large number of prediction models (n = 180) and the limited number of included articles (n = 14), we were not able to perform a meta-analysis. In addition, only eight articles reported a 95% confidence interval of sensitivity, specificity, AUC and C-statistics. While we applied a comprehensive search strategy and screened references of the final included articles to capture all relevant studies, we cannot exclude the possibility there has been underreporting of negative results. Although all of the included studies were at high ROB, their applicability according to PROBAST was satisfactory. Applicability concerns whether the review question and the included studies are a good match regarding its domains (participants, predictors, outcome). Eight out of 14 studies were rated as low concern (16, 19, 21, 22, 27, 29, 31, 34) and two (30, 33) and

four studies (17, 18, 20, 32) were rated as unclear and high concern with regard to applicability, respectively. Reviewing the risk prediction models with high applicability indicates that most of them have included age and AMH in their models. Thus, such predictors can be used by clinicians to get insights on prediction of ONM to make better clinical judgment for health-related consequences of menopause onset. In case of limited resources to measure biomarkers, the models presented by Taylor et al. (32) may be used by clinicians to predict ONM considering that the models were based on simple questions related to menstrual variability which can be obtained during the physical examination. However, it is important that clinicians and patients understand the limitation of such prediction models when interpreting the results.

Future prospective

Due to increased longevity, there is a trend of postponing childbearing; therefore, prediction of menopause onset could improve early informed decision-making regarding family planning. Predicted early menopause could emphasize the need for timely prevention of bone demineralization and cardiovascular and neurological disease, whereas the prediction of late menopause would open options for preventive management of breast, endometrial and ovarian cancer. Future studies should be performed in different countries and include premenopausal women of different ethnicities with wider age ranges (that accounts for both early and late menopause) to improve the model generalizability. Further, increased knowledge of the variation of menopausal onset and performance of prediction models across different ethnicities and medical history will allow for better personalized prediction, that is, providing an accurate prediction for each single patient. Efforts are required to avoid poor calibration when developing prediction models and evaluate calibration when validating them, and improvement of methods that deal with analysis. Currently, there is limited evidence in the prediction of early onset of menopause, and/or long-term prediction of menopause onset.

Conclusion

This systematic review summarizes the available literature trying to predict the risk of ONM in different settings and highlights numerous methodological deficiencies of the current prediction models. While the predictive performance of the presented models is limited, their applicability is satisfactory. Age and AMH were the most used predictors in the presented models and in general showed to have high predictability for menopause onset. The current study can help guide future studies to develop methodologically sound predictors of ONM.

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Supplementary materials: Risk prediction models of natural menopause onset: a systematic review

Supplementary Material 1. Detailed search strategy of the current study in each database.

Medline (Ovid)

1 ((predict* or risk) adj3 (early or onset or age or ages or time or timing or stage or stages or premature or late or natural) adj3 (menopaus* or perimenopaus* or peri-menopaus* or postmenopaus* or post-menopaus* or climact*)).ab,ti.
2 (predict* adj3 (menopaus* or climact*)).ab,ti.
3 ((age adj3 menopaus*) or ((time or timing) adj3 menopaus*)).ab,ti.
4 (onset adj3 menopaus*).ab,ti.
5 cessation of menstruation*.ab,ti.
6 final mens*.ab,ti.
7 (climact* adj3 (chang* or stage*)).ti,ab.
8 (((ovarian or reproductive*) adj (failur* or declin* or reserve* or aging* or ageing*)) and (menopaus* or climact*)).ti,ab.
9 late reproductive age*.ab,ti.
10 (loss adj1 fertil*).ab,ti.
11 (menopaus* adj3 transition*).ab,ti.
12 (approach* adj3 menopaus*).ab,ti.
13 "sensitivity and specificity"/ or "predictive value of tests"/ or roc curve/ or odds ratio/ or proportional hazards models/
14 (accuracy or ((risk or predict* or cox or hazard) adj5 (model* or abilit* or analys*)) or sensitivity or specifity or (predic* adj1 value*) or likelihood ratio* or c-statistic* or multivariab* or AUC or ((area-under or ROC) adj3 curve) or ((odd* or risk) adj3 ratio*)).ab,ti.
15 (diagnos* adj2 (performance* or accurac* or utilit* or value* or efficien* or effectiveness)).ab,ti.
16 cohort studies/ or follow-up studies/ or exp longitudinal studies/ or prospective studies/
17 (cohort* or case-cohort* or nested case-control* or prospectiv* or (observ* adj (study or studies)) or longitudinal or follow-up or followup or followed-up or multivariate*).ab,ti.
18 exp animals/ not humans/
19 (letter or news or comment or editorial or congress or abstracts).pt.
20 or/3-12
21 or/13-15

22 or/16-17
23 20 and 21 and 22
24 1 or 2 or 23
25 24 not (18 or 19)

Embase (Ovid)

1 *menopause/ and (diagnostic accuracy/ or predictive value/)
2 "age at natural menopause"/ or "time to menopause"/ or (onset age/ and exp "menopause and climacterium"/)
3 ((predict* or risk) adj5 (early or onset or age or ages or time or timing or stage or stages or premature or late or natural) adj5 (menopaus* or perimenopaus* or peri-menopaus* or postmenopaus* or post-menopaus* or climact*)).ab,ti.
4 (predict* adj5 (menopaus* or climact*)).ab,ti.
5 ((age adj3 menopaus*) or ((time or timing) adj3 menopaus*)).ab,ti.
6 (onset adj3 menopaus*).ab,ti.
7 cessation of menstruation*.ab,ti.
8 final mens*.ab,ti.
9 (climact* adj3 (chang* or stage*)).ti,ab.
10 (((ovarian or reproductive*) adj (failur* or declin* or reserve* or aging* or ageing*)) and (menopaus* or climact*)).ti,ab.
11 late reproductive age*.ab,ti.
12 (loss adj1 fertil*).ab,ti.
13 (menopaus* adj3 transition*).ab,ti.
14 (approach* adj3 menopaus*).ab,ti.
15 "sensitivity and specificity"/ or "predictive value"/ or predictor variable/ or prediction/ or accuracy/ or diagnostic accuracy/ or diagnostic test accuracy study/ or receiver operating characteristic/ or odds ratio/ or proportional hazards model/
16 (accuracy or ((risk or predict* or cox or hazard) adj5 (model* or abilit* or analys*)) or sensitivity or specifity or (predic* adj1 value*) or likelihood ratio* or c-statistic* or multivariab* or AUC or ((area-under or ROC) adj3 curve) or ((odd* or risk) adj3 ratio*)).ab,ti.
17 (diagnos* adj2 (performance* or accurac* or utilit* or value* or efficien* or effectiveness)).ab,ti.
18 "cohort analysis"/ or follow-up/ or exp longitudinal study/ or prospective study/ or exp case control study/ or exp case study/
19 (cohort* or case-cohort* or nested case-control* or prospectiv* or (observ* adj (study or studies)) or longitudinal or follow-up or followup or followed-up or multivariate*).ab,ti.

20 (exp animal/ or nonhuman/) not exp human/
21 (Editorial or Letter or Note or Erratum or Conference Paper or Conference Abstract or Conference Review).pt.
22 2 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14
23 15 or 16 or 17
24 18 or 19
25 22 and 23 and 24
26 1 or 3 or 4 or 25
27 25 or 26
28 27 not (20 or 21)

Cochrane Central Register of Controlled Trials

ID Search Hits
#1 ((predict* or risk) NEAR/5 (early or onset or age or ages or time or timing or stage or stages or premature or late or natural) NEAR/5 (menopaus* or perimenopaus* or peri-menopaus* or postmenopaus* or post-menopaus* or climact*))
#2 (predict* NEAR/5 (menopaus* or climact*))
#3 ((age NEAR/3 menopaus*) or ((time or timing) NEAR/3 menopaus*))
#4 (onset NEAR/3 menopaus*)
#5 "cessation of menstruation"
#6 final NEXT mens*
#7 (climact* NEAR/3 (chang* or stage*))
#8 (((ovarian or reproductive*) NEXT (failur* or declin* or reserve* or aging* or ageing*)) AND (menopaus* or climact*))
#9 "late reproductive age"
#10 (loss NEAR/1 fertil*)
#11 (menopaus* NEAR/3 transition*)
#12 (approach* NEAR/3 menopaus*)
#13 #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12
#14 (accuracy or ((risk or predict* or cox or hazard) NEAR/5 (model* or abilit* or analys*)) or sensitivity or specifity or (predic* NEAR/1 value*) or likelihood ratio* or c-statistic* or multivariab* or AUC or ((area-under or ROC) NEAR/3 curve) or ((odd* or risk) NEAR/3 ratio*))
#15 (diagnos* NEAR/2 (performance* or accurac* or utilit* or value* or efficien* or effectiveness))
#16 #14 OR #15
#17 (cohort* or case-cohort* or nested case-control* or prospectiv* or (observ* NEXT (study or studies)) or longitudinal or follow-up or followup or followed-up or multivariate*)

#18 #13 AND #16 AND #17
#19 #1 OR #2 OR #18

Web of Science Core Collection

#23 #21 AND #22
#22 DT=(article)
#21 #19 NOT #20
#20 TS=((animal* OR plant* OR rats OR mice OR murine OR rabbits OR pigs OR primate*) NOT (human* OR patient*))
#19 #1 OR #2 OR #18
#18 #13 AND #16 AND #17
#17 TS=((cohort* or case-cohort* or nested case-control* or prospectiv* or (observ* NEAR/0 (study or studies)) or longitudinal or follow-up or followup or followed-up or multivariate*))
#16 #14 OR #15
#15 TS=(diagnos* NEAR/2 (performance* or accurac* or utilit* or value* or efficien* or effectiveness))
#14 TS=((accuracy or ((risk or predict* or cox or hazard) NEAR/5 (model* or abilit* or analys*)) or sensitivity or specifity or (predic* NEAR/1 value*) or likelihood ratio* or c-statistic* or multivariab* or AUC or ((area-under or ROC) NEAR/3 curve) or ((odd* or risk) NEAR/3 ratio*)))
#13 #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12
#12 TS=(approach* NEAR/3 menopaus*)
#11 TS=(menopaus* NEAR/3 transition*)
#10 TS=((loss NEAR/1 fertil*))
#9 TS=("late reproductive age")
#8 TS=(((ovarian or reproductive*) NEAR/0 (failur* or declin* or reserve* or aging* or ageing*)) AND (menopaus* or climact*))
#7 TS=(climact* NEAR/3 (chang* or stage*))
#6 TS=(final NEAR/0 mens*)
#5 TS=("cessation of menstruation")
#4 TS=((onset NEAR/3 menopaus*))
#3 TS=(((age NEAR/3 menopaus*) or ((time or timing) NEAR/3 menopaus*)))
#2 TS=((predict* NEAR/5 (menopaus* or climact*)))
#1 TS=(((predict* or risk) NEAR/5 (early or onset or age or ages or time or timing or stage or stages or premature or late or natural) NEAR/5 (menopaus* or perimenopaus* or peri-menopaus* or postmenopaus* or post-menopaus* or climact*)))

Google scholar 300 most relevant results (out of 17'000 results in total)

Prediction|predict|predicting|predictor|predictors|determinate|determinant|determine|timing|diagnosis "age at menopause"|"age at natural menopause"|"menopause onset"|"onset of menopause"|"menopause stage" study|trial -rat -rats -mouse –mice

Supplementary material 2. Detailed explanation of all prediction models in the included studies.

Age

We identified seven articles (1-7) examining age as sole predictor of MP onset. The C-statistics of age in predicting MP onset in five articles ranged from 0.83 to 0.87 (2, 3, 8-10), while other articles (5, 6) showed an AUC of 0.82 (95% confidence interval CI 0.71-0.93), and poor sensitivity and good specificity of age in forecasting MP within 2 or 4 years (sensitivity 42.4% (95% CI 26.8-60.0) and 40% (95% CI 28.3-52.3), specificity 87.4% (95% CI 82.9-91.1) and 94.8% (95% CI 90.2-97.4), respectively.

Minor improvements were observed in prediction models by adding other predictors to age. The model performance of Dolleman *et al.* 2014 (3) improved minimally by adding mothers' ANM from C-statistics of 0.84 (95% CI 0.78-0.90) to 0.85 (95% CI 0.79-0.91). They also studied ANM of the mother as a sole predictor (C-statistic 0.63, 95% CI 0.54-0.72). Dolleman *et al.* 2015 (9) showed a minor improvement by including body mass index (BMI), packyears of smoking and menstrual cycle status (C-statistic from 0.88 to 0.89).

Anti-Müllerian Hormone (AMH) AMH as predictor of ONM was used in 12 articles (1-5, 7-12). AMH as a sole predictor of MP onset provided a C-statistics ranging from 0.70 to 0.87. (1-5, 7-12). Adding age into the models using AMH improved predictive ability in all articles (C-statistics 0.70 – 0.91) (1, 2, 4, 7, 8, 10, 12).

Some articles developed risk prediction models by determining AMH at a certain age or age group. De Kat *et al.* (1) measured AMH at age 20, 25, 30 and 35 with moderate C-statistics (0.62 –0.71). Finkelstein *et al.* (11) tested prediction models by using different AMH thresholds (< 10 pg/ml vs. < 100 pg/ml) for prediction times of 1, 2 and 3 years. Sensitivity would depend on age, with AMH threshold < 100 pg/ml showing better sensitivity (65% – 76%) in the age group < 48 years, and AMH threshold < 10 pg/ml showing better sensitivity (70%– 82%) at age group > 51 years. The performance of the model developed by Gohari *et al.* (4) was higher at age 43 with time prediction of 3 years (AUC 0.987). The AUC deteriorated with increasing age

(45, 47, and 49 years of age) at which the blood samples were taken, but still showed a good model performance.

Several articles (1, 2, 9, 11) combined AMH with lifestyle factors like oral contraception use, smoking or BMI. The multivariable model (AMH, age + smoking either at baseline or follow-up) of Depmann *et al.* (2) showed a C-statistic of 0.87, which corresponded to an improvement of performance relative to the univariable model (AMH, C-statistic 0.78). One article tested the predictive ability of AMH, age and BMI, reporting an AUC varying between 0.881 and 0.896; the prediction was similar across different times of follow-up (1, 2 or 3 years) (11). Dolleman's model (9) included age, BMI and packyears of smoking as well as menstrual cycle status (i.e., regular/irregular, pregnant or taking oral contraceptives) and presented high C-statistics (0.91). The article of de Kat *et al.* (1), which did not include age in their risk prediction model, showed the worst model performance (C-statistic 0.70).

Only two articles (7, 10) included age and other biomarkers besides AMH and demonstrated a very good model performance by predicting MP onset in 5 and 4 years, respectively. The C-statistics of the final model (including AMH, age, FSH and AFC) of Kim *et al.* (10) was 0.91, whereas the AUC of the model of van Rooij *et al.* (7) including AMH, age and Inhibin B was 0.92 (95% CI 0.86-0.99).

Tehrani *et al.* 2014 (5) published a cross-validation article of two existing models that predict menopausal age with AMH and age. The C-statistics ranged from 0.72 to 0.88. It is one of the articles that performed the calibration. After calibration of the SRV (Scheffer, van Rooij and de Vet) model on the TLGS (Tehran Lipid and Glucose Study) data, the slope was 0.99, the intercept -0.26 and the shape parameter 1.13. The TLGS model on the SRV data had a slope of 0.30, an intercept of 12.66 and a shape parameter of 0.62.

AMH decline rate Tehrani *et al.* 2020 (12), Tehrani *et al.* 2021 (13) and de Kat *et al*. (1) integrated AMH decline rate into their models. The multivariable models developed by Tehrani *et al.* 2020 (12) showed a C-statistics ranging from 0.72 to 0.81. The latter value resulted due to the addition of sample storage time to the model. De Kat *et al.* (1) presented a moderate model performance when only AMH decline rate was integrated in the model (C-statistic 0.65, 95% CI 0.63-0.67). By adding either age or lifestyle factors, C-statistics improved slightly (C-statistics 0.69 to 0.70). Tehrani, *et al.* 2021(13) presented their prediction models using two common statistical models, including time-dependent Cox regression and Cox proportional-hazards regression models. Using Cox proportional-hazards models, the predictive performance of their

model including age at baseline and baseline AMH values showed a C-statistics of 0.71 (95% CI 0.69-0.73). Adding the AMH annual decline rate to the previous model improved the C-statistics by 3% (C-statistics (0.74, 95% CI 0.71-0.76). Findings based on time-dependent Cox regression analysis including AMH measurement at each time point and baseline age as predictors found a C-statistic of 0.64 (95% CI 0.61-0.67). Adding annual AMH decline rate to the previous model improved the C-statistic by 21% (C-statistics (0.85, 95% CI 0.83-0.87).

Follicle-stimulating hormone (FSH) The univariable models of Broer *et al*. (8) and Depmann *et al.* (2) using FSH as a predictor (2) showed C-statistics of 0.70 and 0.66, respectively. The AUC of van Rooij *et al.* (7) was 0.72 (95% CI 0.56-0.88). After adjusting for age, the model performance of all three mentioned articles improved (Depmann 0.85, Broer 0.88, and van Rooij 84). The multivariable model of Kim *et al.* (10) including FSH and age showed a C-statistics of 0.86.

Two articles (2, 11) added as predictors a lifestyle factor (either smoking or BMI) to their model with FSH and age. Inclusion of smoking at baseline additionally to FSH and age did not improve C-statistics (0.85) in the article by Depmann *et al.* (2), while the addition of smoking at follow up slightly improved C-statistics (0.86). The multivariable model (FSH, age and BMI) of Finkelstein *et al.* (11) showed good performance (AUC: 0.871-0.885) in predicting 1, 2 or 3 years of MP onset. The article did not provide information on individual markers.

First, Greendale *et al.* (14) determined candidate marker (Estradiol, FSH or N-telopeptide) or a combination that best predicted different landmarks (2 years before final menstrual period (FMP), one year before FMP and FMP itself), then a multivariable model was developed. The article only provided AUC of N-telopeptide in their univariable model (see below), but not of FSH or Estradiol. Their multivariable model, consisting of FSH and Estradiol, showed AUC ranging from 0.775 – 0.855 (depending on the landmarks they set, see above). By adding N-telopeptide to FSH and Estradiol, AUC slightly improved (0.778-0.863). Finally, the best prediction performance showed the multivariable model consisting of FSH, Estradiol, age, menopausal transition stage, race/ethnicity and time of venipuncture (AUC 0.902-0.945).

N-telopeptide Only one of the included articles developed a risk prediction model using N-telopeptide (14), in which the area under the curve at 2 years before the FMP, 1 year before the FMP and at the time FMP was 0.594, 0.617 and 0.669, respectively.

Inhibin B Of the included articles, only van Rooij *et al*. (7) tested Inhibin B as a predictor of MP onset with a prediction time of 4 years. The AUC of the biomarker alone was 0.76 (0.63-0.89), while adding age in the model improved the AUC to 0.88 (95% CI 0.78-0.98) after taking the age of the women into account.

Estradiol van Rooij *et al.* (6) showed in their article that Estradiol alone had poor predictive accuracy (AUC 0.55, 95% CI 0.35-0.75). After adjusting for age, the AUC was greatly improved to 0.88 (95% CI 0.78-0.98).

Antral follicle count (**AFC**) Broer *et al.* (8) and Depmann *et al.* (2) presented a C-statistic for AFC of 0.84 and 0.79, respectively. The van Rooij article (7) found an AUC of 0.80 (95% CI 0.69-0.91). After adding age to the prediction models, the accuracy improved in all above-mentioned studies (Broer: 0.88, Depmann: 0.85 and van Rooij: 0.84, 95% CI 0.73-0.94). Depmann et al. (2) pointed out no difference between adding age and smoking either at baseline or at follow up to the AFC (both with C-statistic of 0.85). The risk prediction model developed by Kim *et al*. (10) slightly improved by adding FSH to AFC and age (C statistic of 0.86 compared to 0.84); Additionally, a combination of age, AMH and AFC resulted in a highest predictive performance (C-statistic 0.91).

Menstrual cycle irregularities and vasomotor symptoms Taylor *et al.* (6) tested whether 6 menstrual cycle irregularities (listed alphabetically below) and climacteric symptoms would forecast MP within 2 or 4 years.

If the most recent menstrual period was more than 90 days ago (a), the sensitivity and specificity for predicting natural MP within 2 or 4 years were 40.6% (95% CI 24.7-58) and 99.2% (95% CI 97.2-99.9), 21.9% (95% CI 13.1-33.5) and 100% (95% CI 97.8-100), respectively.

If amenorrhoea of 60 days or more occurred during the previous year (b), the sensitivity was 93.9% (MP within 2 years, 95% CI 86.6-98.9) and 64.6% (95% CI 52.3-75.5) and specificity 90.8% (MP within 4 years, 95% CI 86.7-94.0) and 97.1% (95% CI 93.4-98.9).

If women reported cycle length that varied by ≥ 19 days (c), the sensitivity and specificity for developing ONM within 2 years were 100% (95% CI 89.5-100) and 76.1% (95% CI 70.3-81.2), and for MP within 4 years 76.2% (95% CI 64.4-85.3) and 87.7% (95% CI 81.7-92.1), respectively.

Risk prediction models of women with a variable cycle length (d) showed very poor sensitivity (48.5%, 95% CI 31.6-65.3 and 29.3%, 95% CI 19.0-41.5) with good specificity (97.3%, 95% CI 94.5-98.7 and 98.8%, 95% CI 95.7-99.8) within 2 and 4 years, respectively.

Women with a cycle less regular than it had been at age 40 (e) showed sensitivity/specificity for prediction of MP within 2 and 4 years of 87.1% (95% CI 71.2-95.5)/74.2% (95% CI 68.4-79.3) and 72.6% (CI 95% 59.7-82.4)/84.8% (95% CI 78.4-89.6), respectively.

If a change in the duration or heaviness of menstrual flow compared with age 40 (f) has been detected, the sensitivity was good (MP within 2 years: 90.9% (95% CI 76.1-97.5), MP within 4 years: 87.7% (95% CI 77.1-94.2), accompanied by poor specificity (MP within 2 years: 23% (95% CI 18.2-28.5), MP within 4 years: 26% (95% CI 19.9-33.2)).

The sensitivity was poor when using climacteric symptoms like hot flashes or night sweats during the previous week (30.3% (95% CI 16.1-48.5) and 23.1% (95% CI 14.3-35.3)), while the specificity was good (86.2% (95% CI 81.4-89.9) and 87.1% (95% CI 81.3-91.6)) within 2 years and 4 years, respectively.

Supplementary Table 1. Details of the prediction models of 14 included studies to predict menopause onset.

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
Broer, 2011	185	48 (25.9%)	11	1996-2010	n.r.	Univariable:	-	-	-	+
						Age	-	-	-	0.87
						AMH	-	-	-	0.86
						AFC	-	-	-	0.84
						FSH	-	-	-	0.70
						Multivariate (adjusted for age):	-	-	-	+
						AMH	-	-	-	0.90
						AFC	-	-	-	0.88
						FSH	-	-	-	0.88
de Kat, 2019	2434[a]	1298 (53%)	20	1987-2019	n.r.	AMH	-	-	-	0.70 (0.68-0.72)
						AMH + OC use + and smoking	-	-	-	0.70 (0.68-0.72)
						AMH decline rate	-	-	-	0.65 (0.63-0.67)
						AMH + OC use + smoking + decline rate	-	-	-	0.70 (0.68-0.72)
						AMH + OC use + smoking + decline rate + decline rate × age interaction	-	-	-	0.70 (0.68-0.71)
						AMH at age 20	-	-	-	0.62 (0.60-0.64)
						AMH at age 25	-	-	-	0.64 (0.62-0.66)
						Decline rate age between age 20-25	-	-	-	0.70 (0.68-0.71)

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
						AMH and decline rate between age 20-25	-	-	-	0.69 (0.67-0.70)
						AMH at age 30	-	-	-	0.70 (0.69-0.72)
						Decline rate between age 25-30	-	-	-	0.70 (0.68-0.71)
						AMH and decline rate between age 25-30	-	-	-	0.70 (0.68-0.71)
						AMH at age 35	-	-	-	0.71 (0.69-0.72)
						Decline rate between age 30-35	-	-	-	0.70 (0.68-0.72)
						AMH and decline rate between age 30-35	-	-	-	0.70 (0.69-0.72)
Depmann, 2016	155	81 (52.2%)	14	1992-2013	n.r.	Univariable:	-	-	-	+
						Age at baseline	-	-	-	0.85
						FSH	-	-	-	0.66
						AMH	-	-	-	0.78
						AFC	-	-	-	0.79
						Multivariate (adjusted for age at baseline):	-	-	-	+
						FSH	-	-	-	0.85
						AMH	-	-	-	0.86
						AFC	-	-	-	0.85
						Multivariate (adjusted for age and smoking at baseline):	-	-	-	+
						FSH	-	-	-	0.85
						AMH	-	-	-	0.87

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
						AFC	-	-	-	0.85
						Multivariate (adjusted for age and smoking at follow-up):	-	-	-	+
						FSH	-	-	-	0.86
						AMH	-	-	-	0.87
						AFC	-	-	-	0.85
Dolleman, 2014 (study group 2)	150	46 (30.6%)	12	n.r.	n.r	Univariable:	-	-	-	+
						Daughter's age	-	-	-	0.84 (0.78-0.90)
						Mother's ANM	-	-	-	0.63 (0.54-0.72)
						Daughter's AMH	-	-	-	0.86 (0.81-0.91)
						Multivariate:	-	-	-	+
						Daughter's age + mother's ANM	-	-	-	0.85 (0.79-0.91)
						Daughter's age + daughter's AMH	-	-	-	0.91 (0.88-0.94)
						Daughter's age + mother's ANM + daughter's AMH	-	-	-	0.92 (0.88-0.96)
Dolleman, 2015	1163 [b]	527 (45.3%)	10	1993-2007	n.r	Age	-	-	-	0.88
						Age + BMI + packyears of smoking + menstrual cycle status	-	-	-	0.89
						AMH + age + BMI + packyears of smoking + menstrual cycle status	-	-	-	0.91

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
Finkelstein, 2020	1537	n.r	2	1996-1998	1	AMH + age + BMI	-	-	0.881 (0.873-0.889)	-
						FSH + age + BMI	-	-	0.885 (0.876-0.893)	-
					2	AMH + age + BMI	-	-	0.891 (0.884-0.900)	-
						FSH + age + BMI	-	-	0.877 (0.869-0.885)	-
					3	AMH + age + BMI	-	-	0.896 (0.889-0.903)	-
						FSH + age + BMI	-	-	0.871 (0.864-0.880)	-
					1	AMH <10 pg/mL for women age <48 years	71 (64-78)	-	-	-
					2	AMH <10 pg/mL for women age <48 years	60 (53-66)	-	-	-
					3	AMH <10 pg/mL for women age <48 years	47 (42-53)	-	-	-
					1	AMH <10 pg/mL for women age 48-51 years	73 (68-77)	-	-	-
					2	AMH <10 pg/mL for women age 48-51 years	62 (58-66)	-	-	-
					3	AMH <10 pg/mL for women age 48-51 years	54 (50-57)	-	-	-

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
					1	AMH <10 pg/mL for women age ≥51 years	82 (78-85)	-	-	-
					2	AMH <10 pg/mL for women age ≥51 years	75 (72-79)	-	-	-
					3	AMH <10 pg/mL for women age ≥51 years	70 (66-73)	-	-	-
					1	AMH <100 pg/mL for women age <48 years	-	65 (61-68)	-	-
					2	AMH <100 pg/mL for women age <48 years	-	70 (66-73)	-	-
					3	AMH <100 pg/mL for women age <48 years	-	76 (72-80)	-	-
					1	AMH <100 pg/mL for women age 48-51 years	-	46 (42-50)	-	-
					2	AMH <100 pg/mL for women age 48-51 years	-	57 (52-62)	-	-
					3	AMH <100 pg/mL for women age 48-51 years	-	68 (62-74)	-	-
					1	AMH <100 pg/mL for women age ≥51 years	-	27 (22-32)	-	-
					2	AMH <100 pg/mL for women age ≥51 years	-	39 (31-47)	-	-
					3	AMH <100 pg/mL for women age ≥51 years	-	53 (42-64)	-	-

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
Gohari, 2016	266	63 (23.7 %)	6.5	1999-2006	3	AMH + Age at making prediction was 43	-	-	0.987	-
						AMH + Age at making prediction was 45	-	-	0.932	-
						AMH + Age at making prediction was 47	-	-	0.961	-
						AMH + Age at making prediction was 49	-	-	0.881	-
					4	AMH + Age at making prediction was 43	-	-	0.969	-
						AMH + Age at making prediction was 45	-	-	0.939	-
						AMH + Age at making prediction was 47	-	-	0.939	-
						AMH + Age at making prediction was 49	-	-	0.863	-
					5	AMH + Age at making prediction was 43	-	-	0.967	-
						AMH + Age at making prediction was 45	-	-	0.948	-
						AMH + Age at making prediction was 47	-	-	0.867	-
						AMH + Age at making prediction was 49	-	-	0.807	-
Greendale, 2013	554	n.r	10	1996-2007	2 years before FMP [c]	FSH + Estradiol Treshold > 0.3 Treshold > 0.4 Treshold > 0.5 Treshold > 0.6	85 75 67 59	77 89 96 98	0.775	-

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
					1 year before FMP [d]	FSH + Estradiol Treshold > 0.3 Treshold > 0.4 Treshold > 0.5 Treshold > 0.6	80 75 72 62	92 95 96 97	0.819	-
					FMP [e]	FSH + Estradiol Treshold > 0.3 Treshold > 0.4 Treshold > 0.5 Treshold > 0.6	89 84 70 52	90 92 94 97	0.855	-
	472	n.r.	10	1996-2007	2 years before FMP [c]	N-telopeptide	-	-	0.594	-
					1 year before FMP [d]	N-telopeptide	-	-	0.617	-
					FMP [e]	N-telopeptide	-	-	0.669	-
					2 years before FMP [c]	FSH + Estradiol + N-telopeptide	-	-	0.778	-
					1 year before FMP [d]	FSH + Estradiol + N-telopeptide	-	-	0.822	-
					FMP [e]	FSH + Estradiol + N-telopeptide	-	-	0.863	-
	552	n.r.	10	1996-2007	2 years before FMP [c]	FSH + Estradiol + age + menopause transition stage + race/ethnicity + time of venipuncture (in early follicular phase = cycle days 2-5 = in-window)	-	-	0.902	-
					1 year before FMP [d]	FSH + Estradiol + age + menopause transition stage + race/ethnicity + time of venipuncture (in early follicular phase = cycle days 2-5 = in-window)	-	-	0.926	-

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
					FMP [e]	FSH + Estradiol + age + menopause transition stage + race/ethnicity + time of venipuncture (in early follicular phase = cycle days 2-5 = in-window)	-	-	0.945	-
Kim, 2016	426 [f]	n = 55, 13%	10	2001-2011	5	Age	-	-	-	0.83
						Age + AMH	-	-	-	0.91
						Age + AFC	-	-	-	0.84
						Age + FSH	-	-	-	0.86
						Age + AMH + FSH	-	-	-	0.91
						Age + FSH + AFC	-	-	-	0.86
						Age + AMH + AFC	-	-	-	0.91
						Age + AMH + FSH + AFC	-	-	-	0.91
	359 [g]	n.r.	10	2001-2011	5	Age	-	-	-	0.82
						Age + AMH	-	-	-	0.91
						Age + AFC	-	-	-	0.84
						Age + FSH	-	-	-	0.86
						Age + AMH + FSH	-	-	-	0.92
						Age + FSH + AFC	-	-	-	0.87
						Age + AMH + AFC	-	-	-	0.91
						Age + AMH + FSH + AFC	-	-	-	0.92
	327 [h]	n.r.	10	2001-2011	5	Age	-	-	-	0.81
						Age + AMH	-	-	-	0.91
						Age + AFC	-	-	-	0.82
						Age + FSH	-	-	-	0.84

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
						Age + AMH + FSH	-	-	-	0.91
						Age + FSH + AFC	-	-	-	0.84
						Age + AMH + AFC	-	-	-	0.91
						Age + AMH + FSH + AFC	-	-	-	0.91
	306 [i]	n.r.	10	2001-2011	5	Age	-	-	-	0.86
						Age + AMH	-	-	-	0.94
						Age + AFC	-	-	-	0.86
						Age + FSH	-	-	-	0.89
						Age + AMH + FSH	-	-	-	0.94
						Age + FSH + AFC	-	-	-	0.89
						Age + AMH + AFC	-	-	-	0.95
						Age + AMH + FSH + AFC	-	-	-	0.95
Taylor, 2004	326	n.r.	4.8	1993-1997	Within 2 years	More than 90 days since the most recent menstrual period	40.6 (24.7-58)	99.2 (97.2-99.9)	-	-
					Within 4 years	More than 90 days since the most recent menstrual period	21.9 (13.1-33.5)	100 (97.8-100)	-	-
					Within 2 years	60 or more days of amenorrhea during the previous year	93.9 (80.6-98.9)	90.8 (86.7-94.0)	-	-
					Within 4 years	60 or more days of amenorrhea during the previous year	64.6 (52.3-75.5)	97.1 (93.4-98.9)	-	-
					Within 2 years	Cycle lengths that varied by 19 or more days	100 (89.5-100)	76.1 (70.3-81.2)	-	-
					Within 4 years	Cycle lengths that varied by 19 or more days	76.2 (64.4-85.3)	87.7 (81.7-92.1)	-	-
					Within 2 years	Cycle length too variable to report a usual length	48.5 (31.6-65.3)	97.3 (94.5-98.7)	-	-

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
					Within 4 years	Cycle length too variable to report a usual length	29.3 (19.0-41.5)	98.8 (95.7-99.8)	-	-
					Within 2 years	Cycles less regular than they had been at age 40	87.1 (71.2-95.5)	74.2 (68.4-79.3)	-	-
					Within 4 years	Cycles less regular than they had been at age 40	72.6 (59.7-82.4)	84.8 (78.4-89.6)	-	-
					Within 2 years	Change in the duration or heaviness of menstrual flow compared with age 40	90.9 (76.1-97.5)	23 (18.2-28.5)	-	-
					Within 4 years	Change in the duration or heaviness of menstrual flow compared with age 40	87.7 (77.1-94.2)	26 (19.9-33.2)	-	-
					Within 2 years	Hot flashes or night sweats during the previous week	30.3 (16.1-48.5)	86.2 (81.4-89.9)	-	-
					Within 4 years	Hot flashes or night sweats during the previous week	23.1 (14.3-35.3)	87.1 (81.3-91.6)	-	-
					Within 2 years	Age ≥ 50 years	42.4 (26.8-60.0)	87.4 (82.9-91.1)	-	-
					Within 4 years	Age ≥ 50 years	40 (28.3-52.3)	94.8 (90.2-97.4)	-	-
Tehrani, 2014	185	n = 48, 25.9%	11	n.r.	n.r.	Age + AMH (SRV model on SRV data)	-	-	-	73 (65-82)
	266	n = 63, 23.7%	6	n.r.	n.r.	Age + AMH (SRV model on TLGS data)	-	-	-	82 (75-88)
	266	n = 63, 23.7%	6	n.r.	n.r.	Age + AMH (TLGS model on TLGS data)	-	-	-	88 (83-94)
	185	n = 48, 25.9%	11	n.r.	n.r.	Age + AMH (TLGS model on SRV data)	-	-	-	72 (63-81)

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
Tehrani, 2020	959	n = 529, 55.2%	14	1998-?	n.r.	Age + AMH	-	-	-	70 (67-71)
						Age + AMH + annual AMH decline rate	-	-	-	78 (75-80)
						Age + Age-specific-AMH + annual AMH decline rate	-	-	-	72 (69-74)
						Age + AMH + annual AMH decline rate + sample storage time	-	-	-	81 (79-83)
van Rooij, 2004	81	N = 14 had cycle fluctuations	5	1996-?	4	Age	-	-	82 (71-93)	-
						AMH	-	-	87 (79-96)	-
						Age + AMH	-	-	89 (81-97)	-
						AFC	-	-	80 (69-91)	-
						Age + AFC	-	-	84 (73-94)	-
						FSH	-	-	72 (56-88)	-
						Age + FSH	-	-	84 (74-94)	-
						Inhibin B	-	-	76 (63-89)	-
						Age + Inhibin B	-	-	88 (78-98)	-

Study	Sample size	Number and percentage of women reaching menopause	Follow up period (years)	Time period	Prediction time (years)	Model	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	C-Statistics (95% CI)
						Estradiol	-	-	55 (35-75)	-
						Age + Estradiol	-	-	83 (72-94)	-
						Age + AMH + Inhibin B	-	-	92 (86-99)	-

Abbreviations: CI, Confidence interval; AUC, Area under the curve; FSH, Follicle stimulation hormone; AMH, Anti-Mullerian Hormone; AFC, Antral follicle count; ANM, age at natural menopause; OC, oral contraceptive; ANM, age at natural menopause; n.r., not reported

[a] Women who were using oral contraceptives were also included in the final analysis.

[b] Pregnant women, women who were taking hormonal replacement therapy and OC users were also included in the final analysis.

[c] Equals 3 years before menopause onset

[d] Equals 2 years before menopause onset

[e] Equals 1 year before menopause onset

[f] All women

[g] Women not using estrogen

[h] Women with regular menses only

[i] No women with dominant follicle

Supplementary Table 2. The detailed summary of risk of bias (ROB) assessment for included studies according to the Prediction model Risk Of Bias Assessment Tool (PROBAST).

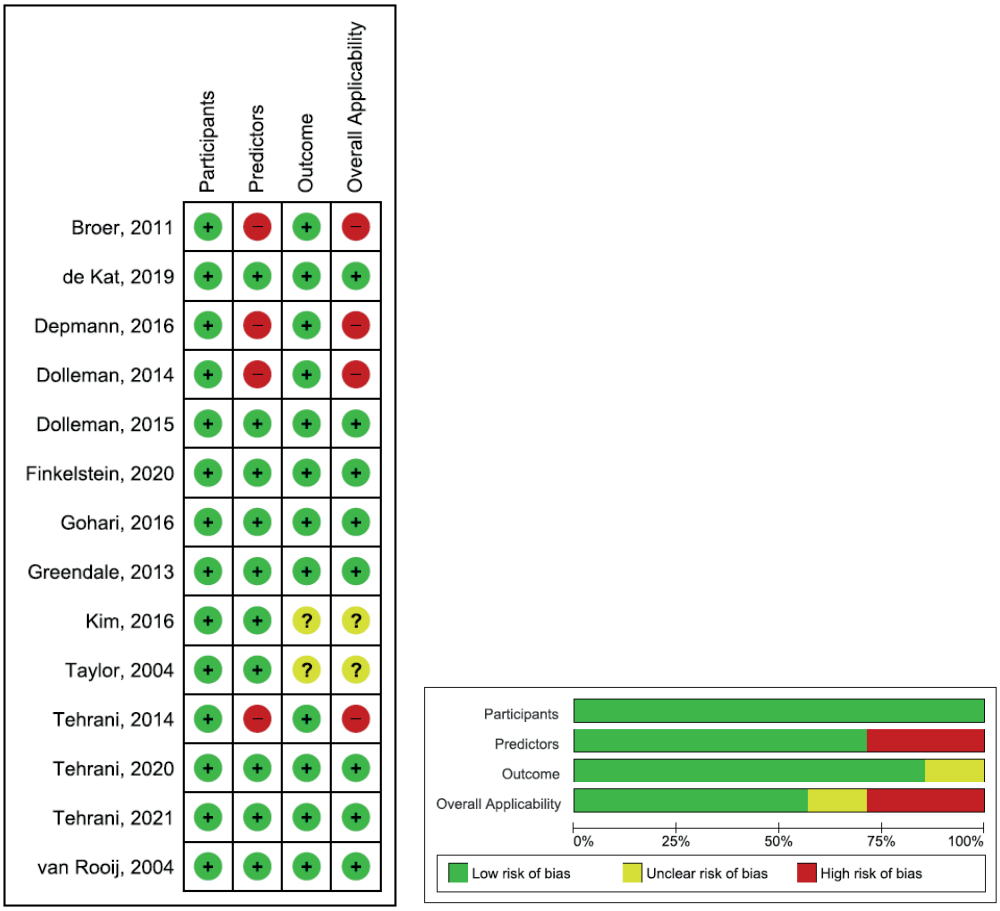
Study	Participants		ROB	Applicability	Predictors			ROB	Applicability	Outcome						ROB	Applicability	Analysis									ROB	Overall Applicability	Overall ROB
Broer, 2011	✓	✓	✓	✓	✗	✓	✗	✗	✗	✓	✓	✓	✓	✓	?	?	✓	✓	✓	✓	✗	✓	✓	✗	✗	?	✗	✗	✗
de Kat, 2019	✓	✓	✓	✓	✓	✓	✗	✗	✓	✓	✓	✓	✓	✓	?	?	✓	✓	✓	✓	✓	✓	✓	✓	✓	?	?	✓	✗
Depmann, 2016	✓	✓	✓	✓	✗	✓	✗	✗	✗	✓	✓	✓	✓	✓	?	?	✓	✓	✓	✓	✗	✓	✓	✗	✗	?	✗	✗	✗
Dolleman, 2014	✓	✓	✓	✓	✗	✓	✗	✗	✗	✓	✓	✓	✓	✓	?	?	✓	✓	✓	✓	✗	✓	✓	✗	✗	✓	✗	✗	✗
Dolleman, 2015	✓	✓	✓	✓	✓	✓	✗	✗	✓	✓	✓	✓	✓	✓	?	?	✓	✓	✓	✓	✓	✓	?	✗	✓	✓	✗	✓	✗
Finkelstein, 2020	✓	✓	✓	✓	✓	✓	✗	✗	✓	✓	✓	✓	✓	✓	✓	✓	✓	?	✓	✗	✗	✓	✗	✗	✓	?	✗	✓	✗
Gohari, 2016	✓	✓	✓	✓	✓	✓	✗	✗	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	?	✗	✗	?	✓	✗	?	✗	✓	✗
Greendale, 2013	✓	✓	✓	✓	✓	✓	✗	✗	✓	✓	✓	✓	✓	✓	✓	✓	✓	?	✓	?	✗	✓	✗	✓	✗	?	✗	✓	✗
Kim, 2016	✓	✓	✓	✓	✓	✓	✗	✗	✓	✓	?	?	✓	✓	✓	?	?	✗	✓	✓	✗	✓	✗	✗	✓	?	✗	?	✗
Taylor, 2004	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	?	✓	?	✓	?	?	?	✓	✓	✗	✗	?	✗	✗	?	✗	?	✗
Tehrani, 2014	✓	✓	✓	✓	✗	✓	✗	✗	✗	✓	✓	✓	✓	✓	?	?	✓	✓	✓	✓	✗	✓	✓	✓	✓	?	✗	✗	✗
Tehrani, 2020	✓	✓	✓	✓	✓	✓	✗	✗	✓	✓	✓	✓	✓	✓	?	?	✓	✓	✓	✓	?	✓	?	✓	✓	✓	?	✓	✗
van Rooij, 2004	✓	✓	✓	✓	✓	✓	✗	✗	✓	✓	✓	✓	✓	✓	✓	✓	✓	?	✓	?	✗	✓	✗	✗	✓	✗	✗	✓	✗
Tehrani, 2021	✓	✓	✓	✓	✓	✓	✗	✗	✓	✓	✓	✓	✓	✓	?	?	✓	✓	✓	✓	✓	✓	✓	✗	✗	?	✗	✓	✗

Abbreviations: ROB, risk of bias

✓ indicates low ROB/low concern regarding applicability

✗ indicates high ROB/high concern regarding applicability

? indicates unclear ROB/unclear concern regarding applicability



Supplementary Figures 1 and 2. Summary of applicability of 14 included studies.

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Chapter 3

Sex hormone-binding globulin may explain sex differences for glucose homeostasis and incidence of type 2 diabetes: the KORA study

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Abstract

Background: Research has indicated that sex hormone-binding globulin (SHBG) is associated with glucose homeostasis and may play a role in the etiology of type 2 diabetes (T2D). While it is unclear whether SHBG may mediate sex differences in glucose control and subsequently, incidence of T2D.

Methods: We used observational data from the German population-based KORA F4 study (n =1937, mean age: 54 years, 41% women) and its follow-up examination KORA FF4 (median follow-up 6.5 years, n = 1387). T2D was initially assessed by self-report and validated by contacting the physicians and/ or reviewing the medical charts. Mediation analyses were performed to assess the role of SHBG in mediating the association between sex (women vs men) and glucose- and insulin-related traits (cross-sectional analysis) and incidence of T2D (longitudinal analysis).

Results: After adjustment for confounders, (model 1: adjusted for age; model 2: model 1 + smoking + alcohol consumption + physical activity), women had lower fasting glucose levels compared to men (β = -4.94 (mg/dl), 95% CI: -5.77, -4.11). SHBG levels were significantly higher in women than in men (β = 0.47 (nmol/l), 95% CI:0.42, 0.51). Serum SHBG may mediate the association between sex and fasting glucose levels with a proportion mediated (PM) of 30% (CI: 22-41%). Also, a potential mediatory role of SHBG was also observed for sex differences in incidence of T2D (PM= 95% and 63% in models 1 and 2, respectively).

Conclusions: Our novel findings suggest that SHBG may partially explain sex-differences in glucose control and T2D incidence.

Keywords: sex differences; sex hormone-binding globulin; glycemic control; type 2 diabetes; mediation analysis.

Introduction

Ample evidence indicates that the alarming rise in obesity will continue to drive a devastating increase in the prevalence and burden of type 2 diabetes (T2D) over the next decades (1). The prevalence and incidence of T2D and the associated risk factors such as obesity, glucose and insulin impairments differ according to the sex (2). In general, prevalence of diabetes is higher in men than women while aging reduces this difference (3). There is a growing body of evidence that sex differences exist in diabetes outcomes and related complications, highlighting the need for further sex-specific research (4).

Certainly, some of these sex differences are determined by genetics, socio-cultural and most largely by biological factors (5). Sex hormones and sex hormone binding globulin (SHBG) have been suggested as potential determinants of these sex differences (6). The association of sex hormones (testosterone, estradiol, etc.) with T2D have been widely addressed (7), however, the independent role of SHBG is still a topic of debate. While SHBG has traditionally been viewed as a passively binding protein that simply regulates the levels of free sex hormones, in recent years its independent biological properties have been highlighted (5). Emerging evidence suggests that SHBG may directly influence various physiological processes and disease states, offering a promising avenue for further investigation and potential therapeutic development (8). Typically, men have lower levels of SHBG compared to women (5). In adult men, SHBG levels are stable for many years but tend to increase with age (9) while SHBG progressively decreases in adult women between the ages of 20 and 60 and then begins to increase (10).

Findings from a large systematic review and meta-analysis of observational studies indicated that lower levels of SHBG are associated with insulin resistance and higher risk of T2D, with stronger associations seen in women compared to men (7). Mendelian randomization studies have supported a causal role of SHBG in T2D, although the causal effects have been shown to be weaker than the estimates observed in observational studies (11, 12). However, there is limited evidence on sex-specific mendelian randomization studies on causal role of SHBG in T2D (13). Therefore, our study lays the groundwork for future research to explore the potentially distinct causal pathways in men and women.

We hypothesized that SHBG levels may explain sex differences for glucose homeostasis and incidence of T2D. To test this hypothesis, we aimed to (i) investigate the association of sex with glucose- and insulin-related traits and incidence of T2D; (ii) to investigate

the associations of sex with SHBG and SHBG with glucose- and insulin-related traits and incidence of T2D; and (iii) to assess the potential mediating role of SHBG and its extent in the association of sex with incidence of T2D in a population-based setting of middle-aged and elderly adults. Particularly, we also focused to identify and quantify SHBG's potential mediating role in broader aspects of glucose homeostasis, such as glucose- and insulin-related traits, an area that has received less attention.

Methods

Setting and study population

This study was conducted among participants of the prospective population-based Cooperative Health Research in the Region of Augsburg (KORA) study, selected from population registries in the city of Augsburg (Germany) and two surrounding counties. A total of 4261 middle-aged and older adults, aged 25-74 years, were included at baseline between 1999 and 2001 (KORA S4). Follow-up examinations were performed after 7 years, between 2006-2008 (KORA F4) and after 14 years, between 2013-2014 (KORA FF4). The KORA F4 study enrolled 3080 participants of whom 2161 were followed up in KORA FF4. All study participants have provided written informed consent. The study was approved by the Ethics Committees of the Bavarian Chamber of Physicians (Ethical Approval Number 06068) adhering to the declaration of Helsinki. Details of the study population and data collection have been reported elsewhere (14).

The present analysis includes data from the KORA F4 at baseline and KORA FF4 at follow-up. We excluded participants who withdrew consent (n=3), non-fasting participants and participants with missing information on fasting status (n=21), participants with T2D (n=217) and type 1 (n=7), participants with unclear diabetes diagnosis (n=75), participants newly diagnosed with diabetes by oral glucose tolerance test (OGTT) (n=115), participants diagnosed with medication induced diabetes (n=1), participants with missing information on diabetic medications (n=2), participants taking external hormone therapy (including estrogen and/or progestin, anti-estrogens) (n=240), participants with surgeries (including hysterectomy, oophorectomy) (n=232), participants with missing information on surgeries (n=1), and participants with missing information on SHBG, sex hormones and glucose- and insulin-related traits (fasting glucose levels, insulin levels, 2-h glucose levels, homeostatic model assessment for insulin resistance (HOMA-IR)) (n=229). Thus, 1937 individuals were included in the cross-sectional analysis, while 1387 participants were included for the longitudinal analysis. (**Figure 1**).

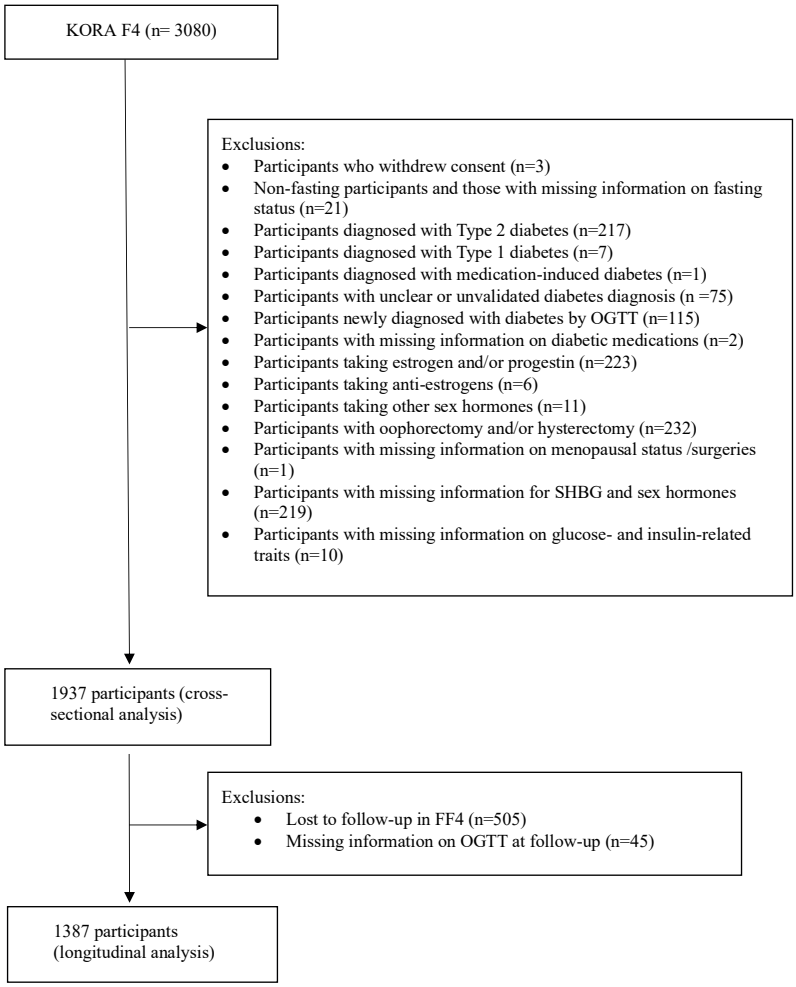


Figure 1. Flowchart for the selection of study participants

Sexual hormone binding globulin, glucose homeostasis and T2D assessments

SHBG was measured in serum that was stored at -80 °C, until being assayed. Measurements of SHBG in serum were performed using the ARCHITECT SHBG assay, a chemiluminescent microparticle immunoassay (CMIA) for absolute quantification of SHBG (Abbott Diagnostics) (measuring range SHBG: 0-250 nmol/L, with intra-assay coefficient of variation: 4.29 % and inter-assay coefficient of variation range: 6.39-10.3%). Known T2D was self-reported, validated by a physician or medical record review, or self-reported current use of glucose-lowering medication. Participants

without known T2D were given a standard 75g oral glucose tolerance test. Among those receiving an OGTT, newly diagnosed diabetes was defined according to the 1999 World Health Organization diagnostic criteria (i.e. fasting glucose > 6.9 mmol/L and/or 2h glucose > 11.0 mmol/L) (15). Incident diabetes at follow-up was a combination of diabetes clinically diagnosed during the follow-up period plus those who had newly diagnosed diabetes based on OGTT data at FF4 among those who did not have diabetes at baseline. Fasting glucose was measured in fresh serum using hexokinase-G6PD (GLUFlex; Dade Behring, USA). Fasting insulin was measured in thawed serum by an elctrochemiluminescence immunoassay (Cobas e602 Immunoassay Analyser; Roche Diagnostics GmbH, Germany).

Assessment of covariates

Information on age, sex, medication use (antihypertensive medications and lipid-lowering medications (yes/ no)), hypertension (yes/no), smoking status (regular smoker, irregular smoker, ex-smoker, never-smoker), alcohol consumption (g/day), physical activity (inactive/ active) was collected by trained medical staff using a standardized interview (16). Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Waist circumference (cm) was measured at the level midway between the lower rib margin and the iliac crest while the participants breathed out gently. High C-reactive protein was quantified in plasma using a high-sensitivity latex-enhanced nephelometric assay (BN II Analyzer, Dade Behring). Thyroid-stimulating hormone was measured using electrochemiluminescent methods (Dimension Vista Systems; Siemens, Germany). Total cholesterol was measured in fresh serum by enzymatic methods (CHOL Flex).

Statistical analyses

Continuous data are presented as mean ± SD or as median (inter quartile range (IQR)) when the variables are non-normally distributed. Categorical data are shown as percentages. We log-transformed non-normally distributed variables prior to further regression and subsequently mediation analysis. Differences in baseline characteristics of men and women were assessed with independent-sample t-test or Mann-Whitney U-test for continuous variables and the chi-squared test for categorical variables.

Multivariable linear regression models were used to investigate the association of sex (women vs men) with SHBG (exposure-mediator) and glucose- and insulin-related traits (fasting glucose levels, 2h glucose levels, fasting insulin levels, and HOMA-IR) (exposure-outcome), as well as SHBG with glucose- and insulin-related traits (mediator-outcome). In addition, the association of sex and SHBG with

incidence of T2D was assessed using multivariable logistic regression analysis. In model 1, all analyses were adjusted for age and additionally for physical activity, alcohol consumption and smoking in model 2. These models were selected based on potential confounders according to the published literature and the second one was considered as the main model in the mediation analysis.

We performed the mediation analysis to determine whether SHBG is a potential mediator in the association of sex (women vs men) with glucose- and insulin-related traits and incidence of T2D and if so to what extent. The hypothesized causal structure of the association between sex (the exposure) and the outcome (glucose- and insulin-related traits / incidence of T2D) with SHBG as a mediator are shown as a Directed Acyclic Graph (DAG) in **Supplementary Figures 1-3**.

We performed several sensitivity analyses to investigate the robustness of our results. 1) We defined a third model with additional adjustments for waist circumference, systolic blood pressure, total cholesterol, high C-reactive protein, thyroid-stimulating hormone, antihypertensive mediations and lipid lowering mediations. We did not consider model 3 as one of our main models since the covariates in this model are more likely to play an intermediate role in the pathways of investigated associations, rather than being confounders. Thus, the findings of the third model should be interpreted with caution as stated by VanderWeele (17, 18) - the confounding assumptions for mediation analysis are extremely important and violations in these assumptions can give rise to misleading results.

2) To investigate the effect of age, we divided the participants into two groups based on the median age of 53 and repeated the analyses. 3) To account for the effect of weight, we repeated the mediation analysis for subset of individuals with normal weight (BMI < 25) and with overweight and obesity (BMI ≥ 25). 4) To investigate the independent role of SHBG from testosterone, we added testosterone to the main models and repeated the mediation analysis. This sensitivity analysis was run only for fasting glucose levels as an outcome, and we were not able to rerun the analyses for incidence of T2D as the incidence of T2D in the subsamples was limited.

The direct effect (DE), indirect effect (IE), total effect (TE) and proportion mediated (PM) were estimated using regression-based approach in a counterfactual framework developed by Valeri and VanderWeele (19). Non-parametric bootstrapping (200 times) was used to estimate 95% CI and P values. The proportion mediated (%) was estimated as $(OR^{Direct} \times (OR^{Indirect} - 1)/ (OR^{Direct} \times OR^{Indirect} - 1) \times 100$ in the case of a binary outcome (odds ratio (OR)) or as $(\beta^{Indirect}/\beta^{Total}) \times 100$ in the case of a continuous

outcome (β coefficient (β)) (18). The DE can be conceived of as the exposure effect on the outcome at a fixed level of the mediator variable, which is different from the TE, the latter representing the overall effect of exposure on the outcome. The IE can be conceived of as the effect on the outcome resulting from the changes of the exposure due to different mediator levels (SHBG). Of note, DE and IE should operate in the same direction in order for the PM to provide meaningful summary (20).

Multiple imputations were done to handle missing values on covariates. Statistical analyses were performed using R statistical software, version 4.2.2 with CMA verse package (21). All results were considered statistically significant at a p-value < 0.05.

Results

Baseline characteristics of the overall population and stratified by sex are shown in **Table 1**. Overall, in the cross-sectional analysis 1937 men (n= 1130) and women (n= 807) women with a mean age of 54.0 ± 12.8 years and median BMI of 26.5 kg/m^2 (IQR: 24.2, 29.6) were included. The overall mean and SD of fasting glucose and 2h-glucose were 94.2 ± 9.4 106.7 ± 30.1 mg/dl, respectively. Fasting glucose levels, 2h-glucose levels, fasting insulin levels, and HOMA-IR were significantly higher in women compared to men ($p < 0.001$). No significant difference was observed in the level of physical activity between men and women. Alcohol consumption and intake of medications were higher in men and a sex difference was observed in smoking status ($p < 0.001$). Over a median follow-up of 6.5 years, 99 incident T2D cases (70 men, 29 women) were recorded.

Sex, SHBG, glucose- and insulin-related traits and T2D

Supplementary Table 1 presents the association of sex (women vs men) with SHBG and glucose- and insulin-related traits. SHBG levels were significantly higher in women than in men. Women had lower fasting glucose, fasting insulin and HOMA-IR levels in both model 1 and 2. No significant sex differences were observed for 2h-glucose levels.

The association of SHBG and glucose- and insulin-related traits are shown in **Supplementary Table 2.** Inverse associations of SHBG with fasting and 2h-glucose levels, fasting insulin, and HOMA-IR were observed in both model 1 and 2.

Sex differences were observed for the incidence of T2D based on our model 2 (OR: 0.56, CI: 0.34, 0.92) (**Supplementary Table 3**). An inverse association was seen

between SHBG and T2D in model 1 (OR: 0.38, CI: 0.23, 0.62) and model 2 (OR: 0.37, CI: 0.22, 0.60) (**Supplementary Table 3**).

Table 1. Baseline characteristics of study participants of KORA F4 in the overall population and stratified by sex.

Characteristic	Total sample (n= 1937)	Men (n= 1130)	Women (n= 807)	p-value
Age	54.0 ± 12.8	54.8 ± 12.8	52.99 ± 12.7	0.001
Body weight (kg)	79.3 ± 15.4	85.4 ± 13.8	70.87 ± 13.5	< 0.001
BMI (kg/m^2)	26.5 (24.2, 29.6)	27.0 (24.86, 29.64)	25.5 (23, 29.55)	< 0.001
WC (cm)	93.2 (84.4, 102.1)	97.2 (90.8, 105.2)	84.5 (76.6, 94.5)	< 0.001
Fasting glucose levels (mg/dl)	94.2 ± 9.4	96.6 ± 8.9	90.9 ± 9.0	< 0.001
2h-glucose levels (mg/dl)	106.7 ± 30.1	108.2 ± 30.3	104.4 ± 29.7	0.006
Fasting insulin levels (µU/ml)	8.5 (6.1, 12)	8.8 (6.5, 12)	7.8 (5.6, 11)	< 0.001
HOMA-IR	1.9 (1.3, 2.8)	2.11 (1.5, 3.0)	1.7 (1.2, 2.5)	< 0.001
SHBG (nmol/l)	58.1 (40.2, 81)	47.9 (34.9, 64.8)	76.4 (55.9, 101.5)	< 0.001
Alcohol consumption (g/day)	8.5 (0.0, 22.8)	16.0 (2.8, 31.4)	2.8 (0.0, 12.3)	< 0.001
Physically active n, (%)	1097 (56%)	639 (56%)	458 (57%)	1.00
Smoking				
Regular smoker	331 (17%)	207 (18%)	124 (15%)	< 0.001
Irregular smoker	51 (2%)	29 (2%)	22 (2%)	
Ex-smoker	783 (40%)	534 (47%)	249 (30%)	
Never-smoker	769 (39%)	358 (31%)	411 (51%)	
SBP (mmHg)	121.3 ± 18.0	126.4 ± 16.9	114.1 ± 17.0	< 0.001
Total cholesterol (mg/dl)	215.5 ± 38.5	215.1 ± 37.8	216.10 ± 39.5	0.58
C-reactive protein (mg/l)	1.0 (0.4, 2.0)	1.0 (0.5, 2.1)	0.9 (0.4, 2.0)	0.29
TSH (mIU/l)	1.2 (0.8, 1.8)	1.2 (0.8, 1.8)	1.2 (0.8, 1.9)	0.61
Use of antihypertensive medications	468 (24%)	300 (26%)	168 (20%)	0.004
Use of lipid lowering medications	194 (10%)	131 (11%)	63 (7%)	0.007

Data are presented as mean ± SD for normally distributed continuous variables, median (IQR) for non-normally distributed variables or numbers (percentage) for categorical variables. P-values were generated by independent-sample t-test or Mann-Whitney U-test for continuous variables and chi-square test for categorical variables. P-values < 0.05 are shown in bold.

Abbreviations: BMI, body mass index; WC, waist circumference; HOMA-IR, homeostatic model assessment for insulin resistance; SHBG, sex hormone-binding globulin; SBP, systolic blood pressure; TSH, thyroid stimulating hormone.

Mediation analysis

Mediation analysis was performed to assess whether, and to what extent, the sex differences (women vs men) in glucose- and insulin-related traits and incidence of T2D were mediated by serum SHBG levels.

The results of the mediation analysis on glucose- and insulin-related traits are shown in **Table 2**. The findings of the mediation analysis showed a significant mediatory effect of SHBG on the association between sex and fasting glucose levels in both DE and IE. DE were (β= -3.68, 95% CI: -4.53, -2.86) in model 1 and (β= -3.42, 95% CI: -4.42, -2.61) in model 2. IE were (β= -1.56, 95% CI: -1.99, -1.18) and (β= -1.51, 95% CI: -1.93, -1.13) in model 1 and 2, respectively. In both models, serum SHBG was estimated to mediate up to PM 30% (CI: 22-41%) of the association.

The DE and IE were in opposite directions for mediation analysis of SHBG on the association between sex and 2h-glucose levels, fasting insulin levels and HOMA-IR (known as inconsistent mediation). In the second model, IE of SHBG for 2h-glucose levels, fasting insulin levels and HOMA-IR were (β= -5.17, 95% CI: -6.84, -3.89), (β= -0.16, 95% CI: -0.19, -0.14) and (β= -5.17, 95% CI: -6.84, -3.89), respectively.

Table 2. Mediation analysis of SHBG on the association between sex (women vs men [Reference]) and glucose- and insulin-related traits among participants of KORA F4.

Effects	ß (95% CI) model 1	p-value	ß (95% CI) model 2	p-value
Fasting glucose levels				
DE	-3.68 (-4.53, -2.86)	<0.001	-3.42 (-4.42, -2.61)	<0.001
IE	-1.56 (-1.99, -1.18)	<0.001	-1.51 (1.93, -1.13)	<0.001
TE	-5.24 (-6.02, -4.52)	<0.001	-4.93 (-5.71, -4.24)	<0.001
PM	0.30 (0.22-0.39)	<0.001	0.30 (0.22-0.41)	<0.001
Fasting insulin levels				
DE	0.06 (0.01, 0.12)	0.01	0.03 (-0.01, 0.08)	0.15
IE	-0.17 (-0.20, -0.14)	<0.001	-0.16 (-0.19, -0.14)	<0.001
TE	-0.10 (-0.15, -0.06)	<0.001	-0.13 (-0.17, -0.08)	<0.001
PM	1.6 (1.12, 2.98)	<0.001	1.28 (0.91, 1.89)	<0.001
2h-glucose levels				
DE	3.33 (0.50, 6.34)	0.05	2.67 (-0.51, 5.28)	0.11
IE	-5.47 (-6.71, -4.18)	<0.001	-5.17 (-6.84, -3.89)	<0.001
TE	-2.13 (-4.36, 0.59)	0.08	-2.49 (-5.03, -0.13)	0.04
PM	2.56 (-4.66, 29.69)	0.08	2.07 (0.76, 12.86)	0.04
HOMA-IR				

Table 2. Continued

Effects	ß (95% CI) model 1	p-value	ß (95% CI) model 2	p-value
DE	0.02 (-0.02, 0.07)	0.35	0.00 (-0.04, 0.05)	0.95
IE	-0.18 (-0.22, -0.16)	<0.001	-0.18 (-0.21, -0.15)	<0.001
TE	-0.16 (-0.21, -0.11)	<0.001	-0.18 (-0.24, -0.13)	<0.001
PM	1.16 (0.86-1.59)	<0.001	1 (0.78-1.38)	<0.001

Model 1: Age.
Model 2: Model 1+ smoking+ alcohol consumption+ physical activity.
SHBG, fasting insulin levels and HOMA-IR are log-transformed.
Abbreviations:DE, direct effect; IE, indirect effect; TE, total effect; PM, proportion mediated; HOMA-IR, homeostatic model assessment for insulin resistance.

The mediation analysis of SHBG on the association between sex and incidence of T2D is presented in **Table 3**. The DE of this association was not statistically significant in neither model 1 (OR: 0.98, 95% CI: 0.61, 1.60) nor model 2 (OR: 0.82, 95% CI: 0.49, 1.28), while the IE was statistically significant in both models 1 (OR: 0.63, 95% CI: 0.50, 0.80) and model 2 (OR: 0.63, 95% CI: 0.50, 0.83), showing the mediatory effect of SHBG on this association. PM was estimated to be 95% in model 1 and 63% in model 2. The results of all sensitivity analyses are provided in **Supplementary information 1**.

Table 3. Mediation analysis of SHBG on the association between sex (women vs men [Reference]) and incidence of T2D between KORA F4 and FF4.

Effects	OR (95% CI) model 1	p-value	OR (95% CI) model 2	p-value
DE	0.98 (0.61, 1.60)	0.96	0.82 (0.49, 1.28)	0.47
IE	0.63 (0.50, 0.80)	<0.001	0.63 (0.5, 0.83)	<0.001
TE	0.62 (0.42, 0.91)	0.04	0.52 (0.32, 0.81)	<0.001
PM	0.95 (0.23, 4.18)	0.04	0.63 (0.22, 2.44)	<0.001

Model 1: adjusted for age.
Model 2: Model 1+ smoking+ alcohol consumption+ physical activity.
SHBG is log-transformed.
Abbreviations: OR, odds ratio; SHBG, sex hormone-binding globulin; T2D, type 2 diabetes; DE, direct effect; IE, indirect effect; TE, total effect; PM, proportion mediated.

Discussion

To our best knowledge, the current study is the first investigation examining the mediatory role of SHBG, an important biological factor, in explaining sex differences in glucose metabolism and incidence of T2D. Our results indicated a greater level of SHBG in women than in men, which may explain sex differences in glucose levels

and incidence of T2D. We observed a high estimated proportion mediated (PM) by SHBG in the association between sex (women vs men) and fasting glucose levels (30%) and incidence of T2D (63%).

Our findings on sex differences for SHBG, glucose homeostasis and T2D are in line with previous studies, showing in general higher SHBG levels (22), lower glucose levels (23), lower insulin resistance (23) and lower incidence of T2D (24) in women compared to men.

Our results reinforce previous evidence on the association between SHBG, glucose biomarkers and T2D incidence. The results of a large systematic review and meta-analysis of observational studies on endogenous sex hormones and risk of T2D showed an inverse association of SHBG and risk of T2D in both sexes, although the findings were stronger in women than in men (7); the same inverse association of SHBG with T2D was found in another systematic review and meta-analysis performed exclusively on women (25). In support of observational evidence, mendelian randomization studies have also found a causal role of SHBG on T2D (12, 13). Above all, our study underscores the necessity of developing sex-specific mendelian randomization studies.

A novel finding of our study is the potential mediating role of SHBG on glucose homeostasis. To our knowledge, the current study is the first study that identified SHBG as a potential mediator in the association between sex and glucose hemostasis. We found the mediating effect of SHBG, which was independent of confounding factor and some of potential intermediate factors including age and obesity. Findings of a recent study based on a large population-based cohort on aging, cardiovascular risk and SHBG, found a clear sex-specific pattern of SHBG levels with age. These novel findings highlighted the importance of considering the age-related changes in SHBG levels to avoid controversial results (22). Thus, we tried to perform the analysis for different subsets of individuals based on median age. Of note, the potentially mediatory role of SHBG on sex differences in glucose levels was observed in participants < 53 years of age and >= 53 years, with a lower PM in those aged 53 and over. The median (IQR) of SHBG levels for individuals aged < 53 were 40.8 (29.6-55.2) and 81.9 (61.4-105.5) and for those >= 53 years were 54.3 (40.2-71.2) and 69.7 (50-97.3) for men and women, respectively. The SHBG levels in men increased with aging, while they decreased in women, resulting in the narrower variation of SHBG levels between men and women in adults aged 53 years and over. This has resulted in lower PM (19-22%) in older individuals compared to higher PM (29-30%) in younger adults.

We also stratified the mediation analysis by BMI categories, given the robust evidence linking overweight and obesity to altered SHBG levels (26). Interestingly, while the mediation effect of SHBG on sex-differences in glucose levels was strongest in individuals with normal weight, it remained significant across both BMI subsets. The lower PM observed in overweight and obese individuals could be attributed to the well-established reduction in SHBG levels associated with increased adiposity (27, 28).

Various mechanisms have been proposed regarding sex differences in risk of T2D, with steroid hormones being the important ones. Research has indicated that SHBG may interfere with the pathogenesis and development of T2D by regulating the biologic effects of sex hormones (testosterone and estrogen) on peripheral tissues (e.g. muscle, fat and liver).

Increased risk of T2D with low SHBG levels may represent the stronger effects and possible interactions of more bioavailable testosterone and estradiol, and thus, explaining the sex-dependent associations of SHBG. Some studies have proposed a sex-dependent association of SHBG with risk of T2D. For instance, the study done by Haffner et al. indicated that SHBG levels, independent of insulin levels, predict the development of T2D in women but not in men (29). Another observational study found that SHBG was associated with higher risk of T2D in women rather than men (30). The findings of a recent mediation analysis on the Masstricht study, investigating the mediatory role of SHBG on the association between intrahepatic lipid content (IHL) and T2D showed greater mediatory role of SHBG in women compared to men, with a PM of 17.2% and 50.9% of SHBG on the association of IHL and T2D for men and women, respectively (31).

Rather than interaction with other sex hormones, there is also strong evidence supporting independent effect of SHBG on T2D (32). Studies have found several polymorphisms in the SHBG gene to be associated with insulin resistance and T2D, showing that altered SHBG physiology may trigger the pathogenesis of T2D (11-13). Additionally, it has been shown that SHBG may mediate cell-surface signaling, cellular delivery, and biological action of sex hormones via activation of a specific plasma receptor directly (12, 33). To test this hypothesis, we repeated the mediation analysis additionally adjusting for testosterone levels in model 1 and 2 and found that the mediatory role of SHBG was still significant and large for sex differences in glucose levels and T2D. In support of some previous literature, our findings suggest that SHBG may play a more significant role in T2D risk rather than previously recognized mechanisms linked to androgens, which warrants further

investigations. However, it's important to exercise caution when interpreting these results, as our mediation analysis model was adjusted for testosterone, a potential mediator, compromising as such the mediation analysis assumptions (17, 18).

Other novel findings of the current study are the inconsistent mediatory role of SHBG on fasting insulin levels, 2-h glucose levels and HOMA-IR. While we found no significant associations for the DE of sex on these traits, the IE for the above-mentioned outcomes were significant and considerable, which is sometimes called inconsistent mediation (20).

Our findings could provide more insights to implement randomized clinical trials targeting SHBG in women suffering from low levels of SHBG. Although a large number of clinical trials have been investigating the effect of different interventions like dietary interventions (34), medications (35) and hormonal therapy (36) on SHBG levels, to our knowledge, no clinical trial has investigated the effect of SHBG administration on health outcomes.

This current study has several strengths and limitations. This was the first study to report the mediatory role of SHBG on sex differences in glucose metabolism and T2D risk. By using data from the KORA study, we were able to investigate the mediatory role of SHBG on the association of sex and glucose homeostasis in a large cohort of individuals both cross-sectionally and longitudinally with a limited proportion of missing values for confounders. OGTT were performed at both baseline and follow-up which helped us to explore the development of clinically diagnosed T2D and also newly OGTT-diagnosed T2D. Having detailed data on menopausal status (e.g. experiencing oophorectomy or hysterectomy) and on hormonal therapy (e.g. anti-estrogens, estrogens, progestin, hormone replacement therapy, androgens, antiandrogens, enzyme inhibitors, gonadotropin releasing hormones, and anabolic steroids) was helpful to account for these important confounding factors which may not be well-collected in other cohort studies. As a limitation, we were not able to repeat the sensitivity analysis stratified by age and BMI categories for T2D due to the limited number of incident cases. Lack of replication and not considering the non-alcoholic fatty liver disease in our statistical models are other limitations of our study. In addition, we could not perform other preferable modeling such as Cox regression analysis, due to the lack of exact time to event data. Finally, this study was conducted among a German population, which limits the generalizability of our findings to other ethnicities.

In conclusion, in a large-scale population-based study we showed that serum SHBG is a potential mediator in the association between sex and glucose levels as well as incident T2D. Whether SHBG could be a target therapy for reducing sex differences in diabetes needs larger and well-designed clinical studies.

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Supplementary materials: Sex hormone-binding globulin may explain sex differences for glucose homeostasis and incidence of type 2 diabetes: The KORA study

Results-sensitivity analysis- Supplementary information 1

We additionally adjusted the association between sex (women vs men) and SHBG and between sex and glucose- and insulin-related traits for other cardiovascular risk factors as model 3. Similar to previous models, women had lower fasting glucose levels and higher SHBG levels than men (**Supplementary Table 4).** We also found an inverse association between SHBG and fasting glucose levels in this model (**Supplementary Table 5**). No association was observed between sex (women vs men) and SHBG with incidence of T2D in the third model (**Supplementary Table 6**). Also, we observed the mediatory role of SHBG on glucose levels while adjusting for further cardiovascular risk factors as well (PM 30%, CI: 22-39%, **Supplementary Table 7**).

We also performed another sensitivity analysis by adding testosterone to model 1 and 2 (**Supplementary Table 8**). PM of SHBG for fasting glucose levels was estimated up to 52% in model 1 and 53% in model 2. Also, PM of SHBG for T2D was up to 36% in model 1 and 31% in model 2.

Moreover, we observed the potential mediatory role of SHBG on the sex differences in fasting glucose levels in both age categories based on median of age (**Supplementary Table 9**). For individuals younger than 53 years old, the PM was 29% (CI:17-40%) in model 1 and the PM 30% (CI:19-43%) in model 2, whereas for those aged 53 and older, the PM was lower (PM 22%, CI:12-36% in model 1 and PM 19%, CI:10-34% in model 2).

Additionally, findings of mediation analysis of SHBG on the association between sex (women vs men) and fasting glucose levels in different categories of BMI showed a PM of 28% (CI:12-49%) in model 1 and PM 29% (CI:12-55%) in model 2 in normal weight individuals while the PM by SHBG for fasting glucose levels were 17% (CI:09-28%) in model 1 and 18% (CI:10-31%) in model 2 in overweight and obese individuals (**Supplementary Table 10**).

Supplementary Table 1. Association of sex (women vs men [Reference]) with SHBG and glucose- and insulin-related traits among participants of KORA F4.

Variable	ß (95 % CI) Model 1	ß (95 % CI) Model 2
SHBG (nmol/l)	0.47 (0.43, 0.52)	0.47 (0.42, 0.51)
Glucose- and insulin-related traits		
Fasting glucose levels (mg/dl)	-5.24 (-6.01, -4.47)	-4.94 (-5.77, -4.11)
Fasting insulin levels (µU/ml)	-0.10 (-0.15, -0.06)	-0.13 (-0.18, -0.08)
2h-glucose levels (mg/dl)	-2.13 (-4.66, 0.39)	-2.50 (-5.21, 0.19)
HOMA-IR	-0.16 (-0.21, -0.11)	-0.18 (-0.23, -0.13)

Model 1: Age.
Model 2: Model 1 + smoking + alcohol consumption + physical activity.
Fasting insulin levels, SHBG and HOMA-IR are log-transformed.
Abbreviations: SHBG, sex hormone-binding globulin; HOMA-IR, homeostatic model assessment for insulin resistance.

Supplementary Table 2. Association of SHBG with glucose- and insulin-related traits among participants of KORA F4.

Variable	ß (95 % CI) model 1	ß (95 % CI) model 2
Fasting glucose levels (mg/dl)	-3.25 (-4.08, -2.42)	-3.22 (-4.05, -2.38)
Fasting insulin levels (µU/ml)	-0.36 (-0.40, -0.31)	-0.35 (-0.40, -0.31)
2h-glucose levels (mg/dl)	-11.39 (-14.09, -8.69)	-11.04 (-13.75, -8.33)
HOMA-IR	-0.39 (-0.44, -0.34)	-0.39 (-0.44, -0.34)

Model 1: Age.
Model 2: Model 1+ smoking+ alcohol consumption+ physical activity.
Fasting insulin levels, SHBG and HOMA-IR are log-transformed.
Abbreviations: SHBG, sex hormone-binding globulin; HOMA-IR, homeostatic model assessment for insulin resistance.

Supplementary Table 3. Association of sex (women vs men [Reference]) and SHBG with incidence of T2D between KORA F4 and FF4.

Variable	OR (95% CI) model 1	OR (95% CI) model 2
Sex (women vs men [Reference])	0.67 (0.42, 1.05)	0.56 (0.34, 0.92)
SHBG (nmol/l)	0.38 (0.23, 0.62)	0.37 (0.22, 0.60)

Model 1: Age.
Model 2: Model 1+ smoking+ alcohol consumption+ physical activity.
SHBG is log-transformed.
Abbreviations: OR, odds ratio; T2D; type 2 diabetes; SHBG, sex hormone-binding globulin.

Supplementary Table 4. Association of sex (women vs men [Reference]) with SHBG and glucose- and insulin-related traits among participants of KORA F4 (model 3).

Variable	ß (95 % CI) model 3
SHBG (nmol/l)	0.31 (0.27, 0.36)
Glucose- and insulin-related traits	
Fasting glucose levels (mg/dl)	-1.68 (-2.62, -0.73)
Fasting insulin levels (µU/ml)	0.14 (0.10, 0.19)
2h-glucose levels (mg/dl)	7.05 (3.94, 10.15)
HOMA-IR	0.12 (0.07, 0.17)

Model 3: Model 2 + waist circumference + SBP + antihypertensive medications + lipid lowering medications + total cholesterol + CRP + TSH. SHBG, fasting insulin and HOMA-IR are log-transformed.
Abbreviations: SHBG, sex hormone-binding globulin; HOMA-IR, homeostatic model assessment for insulin resistance.

Supplementary Table 5. Association of SHBG with glucose- and insulin-related traits among participants of KORA F4 (model 3).

Variable	ß (95 % CI) model 3
Fasting glucose levels (mg/dl)	-1.46 (-2.34, -0.59)
Fasting insulin levels (µU/ml)	-0.14 (-1.91, -0.10)
2h-glucose levels (mg/dl)	-5.98 (-8.85, -3.12)
HOMA-IR	-0.16 (-0.21, -0.11)

Model 3: Model 2+ waist circumference + SBP + antihypertensive Medications + lipid lowering medications + total cholesterol + CRP + TSH. SHBG, fasting insulin levels, and HOMA-IR are log-transformed.
Abbreviations: SHBG, sex hormone-binding globulin; HOMA-IR, homeostatic model assessment for insulin resistance.

Supplementary Table 6. Association of sex (women vs men [Reference]) and SHBG with incidence of T2D between KORA F4 and FF4. (model 3).

Variable	OR (95% CI) model 3
Sex (women vs men [Reference])	0.92 (0.51, 1.63)
SHBG (nmol/l)	0.61 (0.35, 1.04)

Model 3: Model 2+waist circumference+ SBP+ antihypertensive medications+ lipid lowering medications+ total cholesterol + CRP+ TSH. SHBG is log-transformed.
Abbreviations: OR, odds ratio; T2D; type 2 diabetes; SHBG, sex hormone-binding globulin; SBP, systolic blood pressure; TSH, thyroid stimulating hormone.

Supplementary Table 7. Mediation analysis of SHBG on the association between (women vs men [Reference]) and glucose- and insulin-related traits (model 3).

Effects	ß (95% CI) model 3	p-value
Fasting glucose levels		
DE	-3.68 (-4.53, -2.86)	<0.001
IE	-1.56 (-1.99, -1.18)	<0.001
TE	-5.24 (-6.02, -4.52)	<0.001
PM	0.30 (0.22-0.39)	<0.001
Fasting insulin levels		
DE	0.06 (0.01, 0.12)	0.01
IE	-0.17 (-0.20, -0.14)	<0.001
TE	-0.10 (-0.15, -0.06)	<0.001
PM	1.6 (1.12, 2.98)	<0.001
2h-glucose levels		
DE	3.33 (0.50, 6.34)	0.05
IE	-5.47 (-6.71, -4.18)	<0.001
TE	-2.13 (-4.36, 0.59)	0.08
PM	2.56 (-4.66, 29.69)	0.08
HOMA-IR		
DE	0.02 (-0.02, 0.07)	0.35
IE	-0.18 (-0.22, -0.16)	<0.001
TE	-0.16 (-0.21, -0.11)	<0.001
PM	1.16 (0.86-1.59)	<0.001

Model 3: Model 2+ waist circumference + SBP + antihypertensive
medications + lipid lowering medications + total cholesterol + CRP + TSH.
SHBG, fasting insulin levels and HOMA-IR are log-transformed.
Abbreviations:DE, direct effect; IE, indirect effect; TE, total effect; PM, proportion mediated.

Supplementary Table 8. Mediation analysis of SHBG on the association between (women vs men [Reference]) and fasting glucose levels and T2D with adding testosterone in the main models.

Mediation analysis of SHBG and fasting glucose levels				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-2.85 (-5.42, -0.48)	0.02	-2.60 (-4.88, -0.45)	0.03
IE	-3.1 (-4.26, -2.15)	<0.001	-3.00 (-4.20, -2.12)	<0.001
TE	-5.96 (-8.47, -3.79)	<0.001	-5.6 (-7.9, -3.78)	<0.001
PM	0.52 (0.32, 0.88)	<0.001	0.53 (0.32, 0.89)	<0.001
Mediation analysis of SHBG and T2D				
Effects	**OR (95% CI) model 1**	**p-value**	**OR (95% CI) model 2**	**p-value**
DE	0.49 (0.18, 1.2)	0.15	0.43 (0.16, 0.99)	0.04
IE	0.45 (0.28, 0.65)	<0.001	0.43 (0.27, 0.68)	<0.001
TE	0.22 (0.08, 0.51)	0.01	0.19 (0.08, 0.48)	0.01
PM	0.36 (0.10, 1.17)	0.01	0.31 (0.08, 0.84)	0.01

Model 1: Age + testosterone.
Model 2: Model 1+ smoking + alcohol consumption + physical activity + testosterone.
SHBG is log-transformed.
Abbreviations: OR, odds ratio; SHBG, sex hormone-binding globulin; T2D, type 2 diabetes; DE, direct effect; IE, indirect effect; TE, total effect; PM, proportion mediated.

Supplementary Table 9. Mediation analysis of SHBG on the association between (women vs men [Reference]) and fasting glucose levels stratified by median age (53 years).

Mediation analysis for individuals < 53 years				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-4.44 (-5.46, -3.30)	<0.001	-4.18 (-5.33, -2.99)	<0.001
IE	-1.82 (2.40, -1.13)	<0.001	-1.79 (-2.52, -1.10)	<0.001
TE	-6.27 (-7.06, -5.25)	<0.001	-5.97 (-6.90, -5.01)	<0.001
PM	0.29 (0.17-0.40)	<0.001	0.30 (0.19-0.43)	<0.001
Mediation analysis for individuals >= 53 years				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-3.28 (-4.45, -1.99)	<0.001	-3.33 (-4.71, -2.21)	<0.001
IE	-0.93 (-1.31, -0.51)	<0.001	-0.82 (-1.28, -0.43)	<0.001
TE	-4.22 (-5.40, -3.03)	<0.001	-4.16 (-5.47, -3.07)	<0.001
PM	0.22 (0.12-0.36)	<0.001	0.19 (0.10-0.34)	<0.001

Model 1: Age.
Model 2: Model 1+ smoking + alcohol consumption + physical activity.
SHBG is log-transformed.
Abbreviations: SHBG, sex hormone-binding globulin; DE, direct effect; IE, indirect effect; TE, total effect; PM, proportion mediated.

Supplementary Table 10. Mediation analysis of SHBG on the association between (women vs men [Reference]) and fasting glucose levels stratified by BMI.

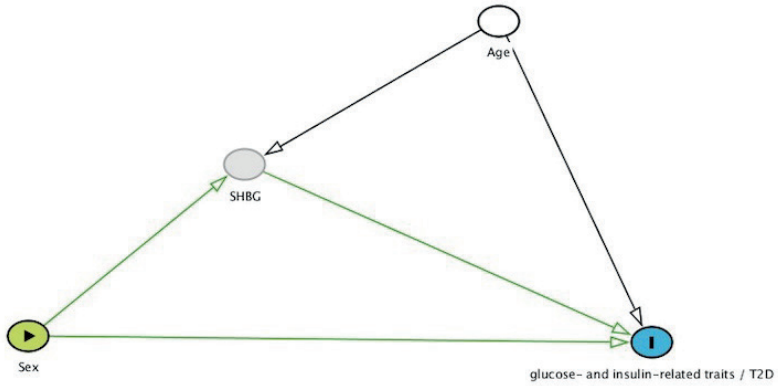
Mediation analysis for individuals with normal body weight (BMI < 25 kg/m²)				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-3.28 (-4.72, -1.94)	<0.001	-3.13 (-4.61, -1.55)	<0.001
IE	-1.33 (-2.06, -0.62)	<0.001	-1.30 (-2.14, -0.50)	<0.001
TE	-4.61 (-5.73, -3.54)	<0.001	-4.43 (-5.75, -3.28)	<0.001
PM	0.28 (0.12, 0.49)	<0.001	0.29 (0.12, 0.55)	<0.001
Mediation analysis for overweight and obese individuals (BMI >= 25 kg/m²)				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-3.85 (-4.65, -2.81)	<0.001	-3.48 (-4.51, -2.40)	<0.001
IE	-0.82 (-1.30, -0.43)	<0.001	-0.81 (-1.17, -0.44)	<0.001
TE	-4.68 (-5.51, -3.60)	<0.001	-4.29 (-5.27, -3.22)	<0.001
PM	0.17 (0.09, 0.28)	<0.001	0.18 (0.10, 0.31)	<0.001

Model 1: adjusted for age.
Model 2: Model 1+ smoking + alcohol consumption + physical activity.
SHBG is log-transformed.
Abbreviations: SHBG, sex hormone-binding globulin; DE, direct effect; IE, indirect effect; TE, total effect; PM, proportion mediated.

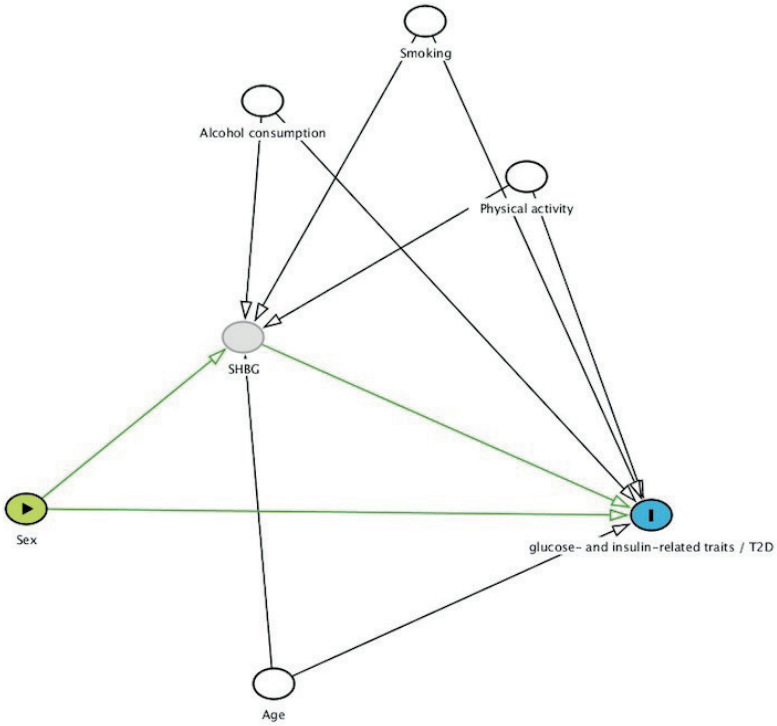
Supplementary Table 11. Mediation analysis of SHBG on the association between (women vs men [Reference]) and incidence of T2D (model 3).

Effects	**OR (95% CI) model 3**	**P value**
DE	1.02 (0.59, 1.60)	0.91
IE	0.87 (0.73, 1.05)	0.15
TE	0.89 (0.51, 1.50)	0.69
PM	1.27 (-8.4, 10.9)	0.73

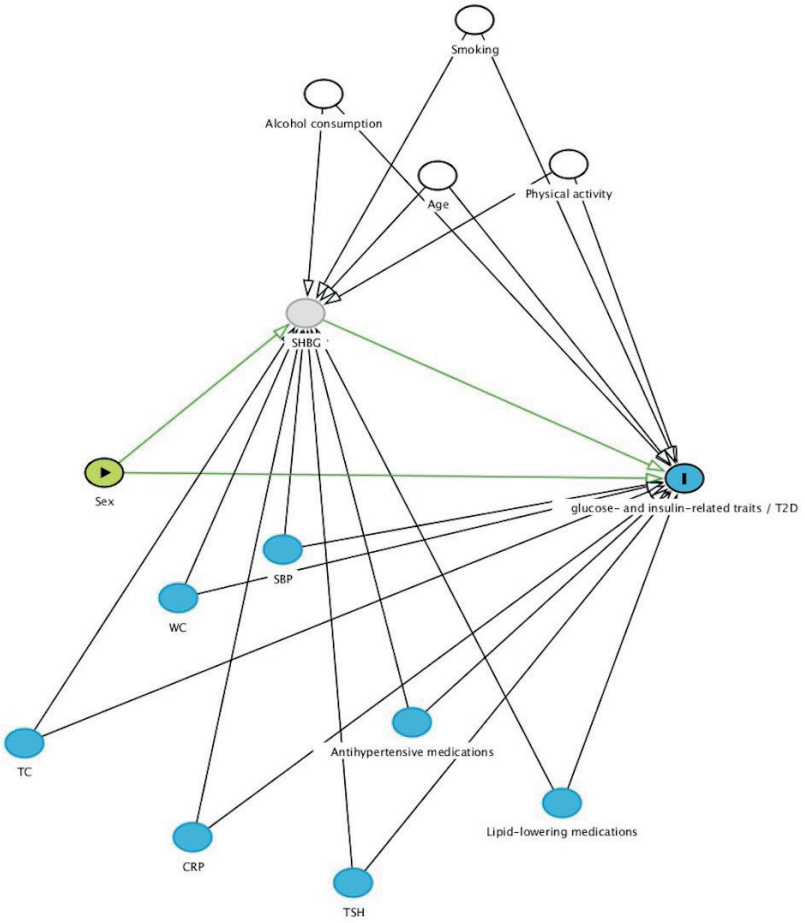
Model 3: Model 2 + waist circumference + SBP + antihypertensive
medications + lipid lowering medications + total cholesterol + CRP + TSH.
SHBG is log-transformed.
Abbreviations: OR, odds ratio; T2D, type 2 diabetes; SHBG, sex hormone-binding globulin;
DE, direct effect; IE, indirect effect; TE, total effect; PM, proportion mediated.



Supplementary Figure 1. Directed acyclic graph (DAG) for the association of sex through, sex hormone binding globulin (SHBG), on glucose- and insulin-related traits / type 2 diabetes (T2D). Study DAG for model 1, exposure = Sex, mediator = SHBG, outcome = glucose- and insulin-related traits / T2D. for **model 1**, adjustment = age.



Supplementary Figure 2. Directed acyclic graph (DAG) for the association of sex, through sex hormone binding globulin (SHBG), on glucose- and insulin-related traits / type 2 diabetes (T2D). Study DAG for **model 2**, exposure = sex, mediator = SHBG, outcome= glucose- and insulin-related traits / T2D, adjustment variables = age + alcohol consumption + smoking + physical activity.



Supplementary Figure 3. Directed acyclic graph (DAG) for the association of sex, through sex hormone binding globulin (SHBG), on glucose- and insulin-related traits / type 2 diabetes (T2D). Study DAG for **model 3**, further adjustments for possible mediators (variables in blue). Exposure = Sex, mediator = SHBG, outcome = glucose- and insulin-related traits / T2D, adjustment variables = age + alcohol consumption + smoking, physical activity + waist circumference + SBP + antihypertensive medications+ lipid lowering medications + total cholesterol + CRP+ TSH.



Chapter 4

The mediatory role of androgens on sex differences in glucose homeostasis and incidence of type 2 diabetes: the KORA study

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Abstract

Background: Sex differences exist in type 2 diabetes (T2D), and androgens have been implicated in the etiology of T2D in a sex-specific manner. We therefore aimed to investigate whether androgens play a role in explaining sex differences in glucose homeostasis and incidence of T2D.

Methods: We used observational data from the German population-based KORA F4 study (n =1975, mean age: 54 years, 41% women) and its follow-up examination KORA FF4 (median follow-up 6.5 years, n = 1412). T2D was determined through self-reporting and confirmed by contacting the physicians and/or reviewing the medical charts. Multivariable linear and logistic regression models were employed to explore associations, while mediation analyses were performed to assess direct effects (DE), indirect effects (IE), and the mediating role of androgens (total testosterone (TT), dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulfate (DHEAs)) in the association between sex (women vs. men) and glucose- and insulin-related traits (cross-sectional analysis) and incidence of T2D (longitudinal analysis).

Results: After adjustment for confounders, (model 1: adjusted for age; model 2: model 1 + smoking + alcohol consumption + physical activity), women had lower levels of TT, DHEAs, fasting glucose levels, fasting insulin levels, 2h-glucose levels and HOMA-IR, compared to men. An inverse association was observed for TT and glucose- and insulin-related traits in men, while a positive association was observed for TT and fasting glucose levels in women. We found a mediatory role of TT on the association of sex with fasting glucose levels (IE: β= 3.08, 95% CI: 2.04, 4.30), fasting insulin levels (IE: β= 0.39, 95% CI:0.30, 0.47), 2h-glucose levels (IE: β= 12.77, 95% CI: 9.01, 16.03) and HOMA-IR (IE: β= 0.41, 95% CI: 0.33, 0.50). Also, the inconsistent mediatory role of TT was seen on the association of sex with incidence of T2D (DE: 0.12, 95% CI: 0.06, 0.20 and IE: OR= 7.60, 95% CI: 3.43, 24.54). The opposing DE and IE estimates suggested that the association between sex and either glucose homeostasis or the incidence of T2D may differ when TT is considered as a potential mediator, with higher TT levels being beneficial for glucose metabolism or incidence of T2D in men, while in women, detrimental. No mediatory role was observed for either DHEA or DHEAs on glucose homeostasis or the incidence of T2D.

Conclusions: The dimorphic mediatory role of TT highlights its complex role in metabolic health, contributing differently to the glucose dysregulation and risk of T2D in men and women.

Keywords: sex differences; sex hormones; androgens; glycemic control; type 2 diabetes; mediation analysis.

Introduction

While the prevalence of type 2 diabetes (T2D) is increasing in both sexes, the diabetes rates are higher in men than women (1). Sex differences have been also found within diagnosis and prognosis of T2D. For instance, men are usually diagnosed at a younger age with lower body mass index; on the other hand, women have a higher risk factor burden including excess weight gain and hypertension (2). Sex differences in T2D complications are more complex. For instance, evidence indicates that women with T2D have a higher relative risk of cardiovascular disease and mortality. However, the absolute risk remains higher in men than in women (3, 4).

Sex hormone-binding globulin and sex hormones have been attributed for sex differences in glucose homeostasis and incidence of T2D (5, 6). In women, hyperandrogenic conditions such as polycystic ovarian syndrome (PCOS) have been associated with insulin resistance and glucose intolerance and increased risk of T2D (7-9), while hypoandrogenism in men has been linked to obesity and increased glucose and insulin levels (7, 10). A large systematic review and meta-analysis of prospective observational studies found a lower risk of T2D in men with higher total testosterone (TT) levels; conversely, in women, elevated TT levels were linked to an increased risk of T2D (11). A recent Mendelian randomization study found a causal role of TT in the pathogenesis of T2D which was more prominent in men than women (12). Results of another Mendelian randomization study on sex hormones and metabolic traits revealed a beneficial causal effect of bioavailable testosterone on glucose metabolism in men but not women (13). A large randomized controlled clinical trial involving 1007 men with impaired glucose tolerance or newly diagnosed T2D found that 2 years of testosterone treatment significantly reduced the proportion of individuals with T2D beyond the effects of a lifestyle program (14). On the other hand, findings from another large controlled clinical trial examining the effects of testosterone replacement therapy in hypogonadal men with prediabetes or diabetes showed no significant differences in the incidence of progression from prediabetes to diabetes or in glycemic control improvements between the treatment and placebo groups (15).

The evidence on the sex-specific association of other androgens including dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulfate (DHEAs) and risk of T2D is limited. An observational cohort study on 1258 community-dwelling individuals aged > 65 years showed lower risk of T2D with higher DHEAs in men, while no association was observed for women (16). Other cross-sectional and prospective studies on women have also found no association between DHEA or DHEAs levels and risk of T2D (17-19). The findings from another prospective cohort study involving 5189 middle-aged and elderly men and women indicated a reduced risk of T2D associated with higher levels of DHEA, but not DHEAs (20).

Thus, we hypothesized that androgens (TT, DHEA and DHEAs) may explain sex differences for glucose homeostasis and incidence of T2D. To test this hypothesis, we aimed to (i) investigate the association of sex with glucose- and insulin-related traits and incidence of T2D; (ii) to investigate the associations of sex with androgens and each sex hormones with glucose- and insulin-related traits and incidence of T2D; and (iii) to assess the potential mediating role of androgens and, if any, to what extent they mediate the association between sex and the incidence of T2D in a population-based setting of middle-aged and elderly adults.

Methods

Data were obtained from the Cooperative Health Research in the Region of Augsburg (KORA) study, with KORA F4 study as baseline (2006-2008) and KORA FF4 study as follow-up (2013-2014), which both are follow-up examinations of the KORA S4 study (1999-2001). All study participants have provided written informed consent. The study was approved by the Ethics Committees of the Bavarian Chamber of Physicians (Ethical Approval Number 06068) adhering to the declaration of Helsinki. The study design has been described previously in detail (21).

The following participants were excluded from the analysis: participants who withdrew consent (n= 3), participants with missing information on fasting status and non-fasting participants (n= 21), already known /newly identified diabetes patients (n= 340), participants with an unclear or missing information on diabetes status (n= 77), participants taking sex hormones (e.g. anti-estrogens, estrogens, progestin, hormone replacement therapy, androgens, antiandrogens, enzyme inhibitors, gonadotropin releasing hormones, and anabolic steroids) (n= 240), participants with surgeries (including hysterectomy, oophorectomy) (n= 233) and finally, participants with missing information on sex hormones (TT, DHEA, and

DHEAs) (n= 181) and glucose- and insulin-related traits (fasting glucose levels, insulin levels, 2h-glucose levels, homeostatic model assessment for insulin resistance (HOMA-IR)) (n= 10). Thus, 1975 individuals were included in the cross-sectional analysis. For the longitudinal analysis, we further excluded participants with missing information on OGTT (n= 46) and who were lost to follow-up in the KORA FF4 (n= 517) which resulted in 1412 participants. The study flowchart of included participants is shown in **Figure 1**.

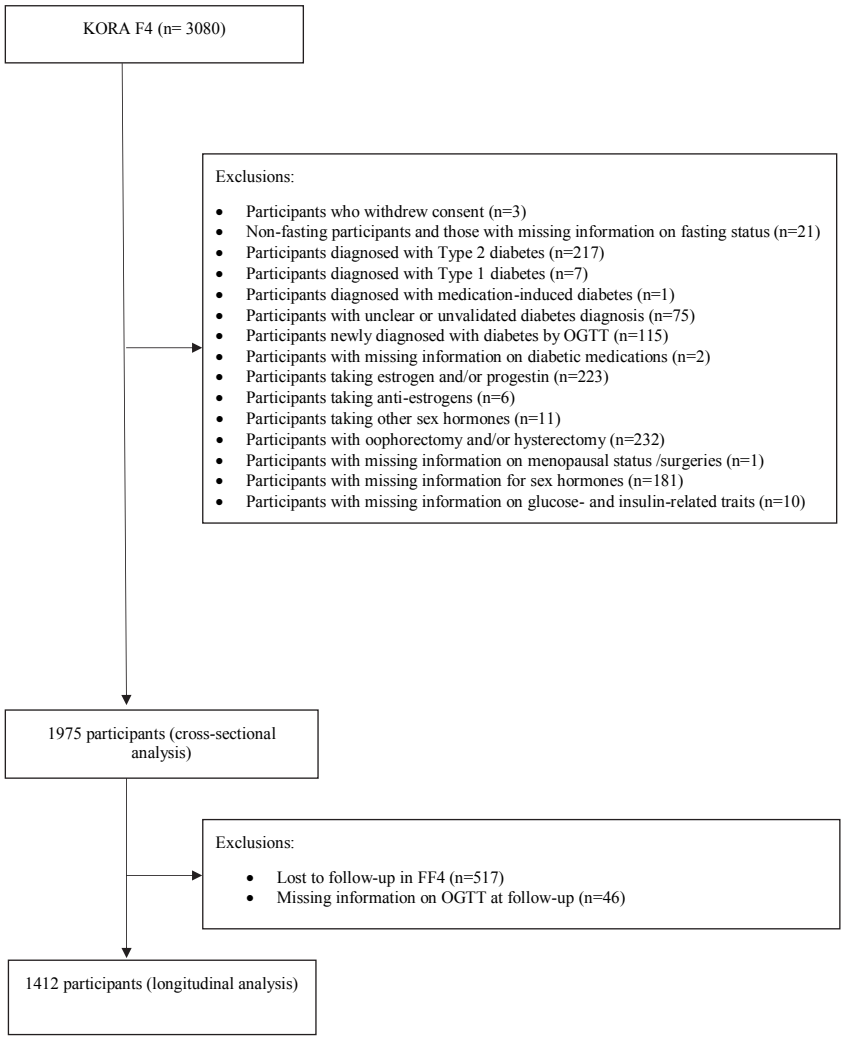


Figure 1. Flowchart for the selection of study participants

Androgens, glucose homeostasis and T2D measurements

Sex hormones (TT, DHEA and DHEAs) were measured in the plasma or serum samples which were stored at -80 °C until being assayed. The detailed assessment procedure was described elsewhere (22). Samples were prepared and sex hormones were quantified by AbsoluteIDQTM Stero17 Kit and electrospray ionization liquid chromatography-mass spectrometry (ESI-LC-MS/MS). The acquisition and quantification methods of AbsoluteIDQTM Stero17 Kit have been proved to follow the European Medicines Agency's Guideline on bioanalytical method validation (July 21st, 2011). Sample preparation and LC-MS/MS measurements were performed as described in the manual UM-STERO17 by the manufacturer (23). Mass spectrometric analyses were done on a QTRAP 5500 triple quadrupole system (Sciex Deutschland GmbH, Darmstadt, Germany) paired with a 1260 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a HTC PAL auto sampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6.2. The MultiQuant 3.0.1 software (Sciex) and the MetIDQTM software package were used to perform data evaluation for the quantification of sex hormone concentrations and quality assessment. Sex hormones were normalized, the values lower than the lowest limit of detection (LOD) were imputed, and different batches were sex-specifically calibrated.

T2D was self-reported which was validated by a physician or medical chart review, or as self-reported current use of glucose-lowering medication. Oral glucose tolerance test (OGTT) was used for the diagnosis of T2D in participants without known T2D. In participants undergoing an OGTT, newly diagnosed diabetes was defined based on the 1999 World Health Organization diagnostic criteria (i.e., fasting glucose > 6.9 mmol/L and/or 2h glucose > 11.0 mmol/L) (15). Incident diabetes at follow-up was determined by combining clinically diagnosed diabetes during the follow-up period with newly diagnosed diabetes based on OGTT data at FF4 among those who did not have diabetes at baseline. Fasting glucose levels were measured in fresh serum using hexokinase-G6PD (GLUFlex; Dade Behring, USA). Fasting insulin levels were measured in thawed serum using an electrochemiluminescence immunoassay (Cobas e602 Immunoassay Analyzer; Roche Diagnostics GmbH, Germany)."

Assessment of covariates at baseline

Data on baseline characteristics, including age, sex, medication use (lipid-lowering medications (yes/ no)), smoking status (never smokers/ ex-smokers/ irregular smokers/ regular smokers (smoking≥1 cigarette a day), alcohol consumption (gr/day), and physical activity (inactive/ active), were collected by trained medical staff using a standardized interview (24). Body weight and waist circumference were measured

according to the standard guidelines. Total cholesterol was measured in serum samples by enzymatic methods (CHOL Flex).

Statistical analysis

Continuous data are presented as mean ± SD for normally distributed variables and median (IQR) for non-normally distributed variables. Largely skewed variables (fasting insulin levels and HOMA-IR) were log-transformed prior to further analysis. Independent-sample t-test or Mann-Whitney U-test for continuous variables, and the chi-squared test for categorical variables were used to perform sex-stratified analysis for baseline characteristics.

Multivariable linear regression models were used to investigate the association of sex with each of androgens (exposure-mediator) and glucose- and insulin-related traits (fasting glucose levels, 2h-glucose levels, fasting insulin levels, and HOMA-IR) (exposure-outcome) as well as androgens with glucose- and insulin-related traits (mediator-outcome). In addition, the association of sex and androgens with the incidence of T2D were assessed using logistic regression analysis. All analyses were adjusted for age (model 1) and additionally, for physical activity, alcohol consumption, and smoking (model 2). These two models were selected as potential confounders based on previous literature and were considered as main models in the mediation analysis.

We focused on measured androgens (TT, DHEA, DHEAs) as potential mediators and excluded calculated variables such as free testosterone (FT). This decision was due to the lack of data on free testosterone in KORA and the inaccuracy of various equations for calculating FT (e.g., the free androgen index), particularly in women (25, 26). We performed the mediation analysis to determine whether each of the androgens (TT, DHEA, and DHEAs) could be a potential mediator of the association of sex (women vs men) and glucose homeostasis and incidence of T2D. The hypothesized causal structure of the association between sex (the exposure) and the outcome (glucose control and incidence of T2D), with androgens as a mediator, are shown as a Directed Acyclic Graph (DAG) in **Supplementary figures 1-3**. The regression-based approach in a counterfactual framework was used to assess the direct effect (DE), indirect effect (IE), total effect (TE), and proportion mediated (PM) (27). Non-parametric bootstrapping (200 times) was used to estimate 95% CI and P values. The proportion mediated (%) was estimated as $OR^{Direct} \times (OR^{Indirect} - 1)/ (OR^{Direct} \times OR^{Indirect} - 1) \times 100$ in the case of a binary outcome, or as $(\beta^{Indirect}/\beta^{Total}) \times 100$ in the case of a continuous outcome (28). The DE represents the effect of the exposure on the outcome at a specific level of the mediator variable, differing from the TE, which signifies the

overall effect of the exposure on the outcome. The IE represents the effect on the outcome due to variations in the exposure resulting from different levels of the mediator (TT/ DHEA/ DHEAs). It is important to note that DE and IE should operate in the same direction for the PM to yield a meaningful summary (29). PM is not valid when DE and mediated effects (IE) have opposite signs which is sometimes referred as inconsistent mediation (29).

Additionally, to test the robustness of our findings, we performed several sensitivity analyses. i) First, we defined another model with additional adjustments for total cholesterol and lipid-lowering mediations (model 3). ii) Second, to investigate the role of age, we divided the participants into two groups based on the median age of 53 and repeated the analyses. 3) Third, to account for the effect of weight, we repeated the mediation analysis for a subset of individuals with normal weight (BMI < 25) and with overweight and obesity (BMI ≥ 25). The second and third sensitivity analyses were run only for glucose- and insulin-related traits as an outcome, and we were not able to rerun the analyses for the incidence of T2D due to the limited number of cases.

Missing values on confounders were treated using multiple imputations. Statistical analyses were performed using R statistical software, version 4.2.2 with CMAverse package (30). All results were considered statistically significant at a p value < 0.05.

Results

Baseline characteristics of the whole population and the stratified analysis based on sex are provided in **Table 1**. In the cross-sectional analysis, 1975 individuals (1155 men, 820 women) with a mean age of 54.1 ± 12.9 and median BMI of 26.6 kg/m^2 (IQR; 24.2, 29.6) were included. The median (IQR) of TT, DHEA and DHEAs were (14.9 (11.8, 18.9), 9.6 (5.9, 14.8), 3368.1 (1927.7, 5305.00) nmol/l for men and 0.7 (0.5, 0.9), 9.6 (6.4, 15), 2280 (1303.5, 3462.5) nmol/l for women. Fasting glucose levels, 2h-glucose levels, fasting insulin levels, and HOMA-IR were significantly higher in women compared to men ($p < 0.05$). No significant differences were observed for total cholesterol and physical activity levels in men and women. Alcohol consumption, the proportion of smokers, and the use of lipid-lowering drugs were higher in men than in women. For the longitudinal analysis, over a median follow-up of 6.5 years, 100 incident T2D cases (70 men, 30 women) were recorded.

Table 1. Baseline characteristics of study participants of KORA F4 in the overall population and stratified by sex.

Characteristic	Total sample (n= 1975)	Men (n= 1155)	Women (n= 820)	p-value
Age	54.1 ± 12.9	54.8 ± 12.9	53.1 ± 12.8	0.004
Body weight (kg)	79.4 ± 15.5	85.4 ± 13.9	70.8 ± 13.5	< 0.001
BMI (kg/m^2)	26.6 (24.2, 29.6)	27.00 (24.9, 29.6)	25.5 (23, 29.5)	< 0.001
WC (cm)	93.2 (84.4, 102.1)	97.2 (90.7, 105.2)	84.5 (76.7, 94.5)	< 0.001
Fasting glucose levels (mg/dl)	94.2 ± 9.42	96.6 ± 8.98	90.9 ± 9.04	< 0.001
2h-glucose levels (mg/dl)	107 ± 30.1	108 ± 30.3	104 ± 29.7	0.003
Fasting insulin levels (µU/ml)	8.5 (6.1, 12)	8.9 (6.5, 12)	7.8 (5.7, 11)	< 0.001
HOMA-IR	1.9 (1.3, 2.8)	2.11 (1.5, 3)	1.7 (1.2, 2.5)	< 0.001
Testosterone (nmol/l)	10.1 (0.7, 15.9)	14.9 (11.8, 18.9)	0.7 (0.5, 0.9)	< 0.001
DHEA (nmol/l)	9.6 (6.2, 15)	9.6 (5.9, 14.8)	9.6 (6.4, 15)	0.44
DHEAs (nmol/l)	2800.2 (1600.6, 4602.5)	3368.1 (1927.7, 5305.)	2280 (1303.5, 3462.5)	< 0.001
Alcohol consumption (g/day)	8.5 (0, 22.8)	15.4 (2.8, 31.4)	2.86 (0, 12.3)	< 0.001
Physically active n, (%)	1112 (56.3)	647 (56%)	465 (56.7%)	0.82
Smoking				
Regular smoker	334 (16.9%)	209 (18.1%)	125 (15.2%)	< 0.001
Irregular smoker	53 (2.7%)	30 (2.6%)	23 (2.8%)	
Ex-smoker	799 (40.5%)	546 (47.3%)	253 (30.9%)	
Never-smoker	786 (39.8%)	368 (31.9%)	418 (51%)	
Total cholesterol (mg/dl)	216 ± 38.6	215 ± 37.9	216 ± 39.5	0.58
Use of lipid lowering medications	199 (10.1%)	134 (11.6%)	65 (7.9%)	0.009

Data are presented as mean ± SD for normally distributed continuous variables, median (IQR) for non-normally distributed variables or numbers (percentage) for categorical variables. P-values were generated by independent-sample t-test or Mann-Whitney U-test for continuous variables and chi-square test for categorical variables. P-values < 0.05 are shown in bold.

Abbreviations: BMI, body mass index; WC, waist circumference; HOMA-IR, homeostatic model assessment for insulin resistance; DHEA, Dehydroepiandrosterone; DHEAs, dehydroepiandrosterone sulfate.

Sex, androgens, glucose- and insulin-related traits and T2D

Supplementary Table 1 presents the association of sex (women vs men) with sex hormones and glucose- and insulin-related traits. TT and DHEAs levels were significantly lower in women than in men, while no significant sex differences were observed for DHEA. Women had lower fasting glucose, 2h-glucose, fasting insulin and HOMA-IR levels in both model 1 and 2. The association of sex hormones and glucose- and insulin-related traits among male participants are presented is **Supplementary Table 2**. An inverse association was found between TT and fasting glucose, 2h-glucose, fasting insulin and HOMA-IR levels in both models. No significant association was observed between DHEA or DHEAs and glucose- and insulin-related traits in men. **Supplementary Table 3** shows the association of sex hormones and glucose- and insulin-related traits among females. Only a positive association was found between TT and fasting glucose levels in women. No significant associations were found between either DHEA or DHEAs and glucose- and insulin-related traits in women.

No sex differences were observed for the incidence of T2D (**Supplementary Table 4)**. While an inverse association was seen between TT and T2D in both models, no associations were observed between DHEA or DHEAs and T2D incidence (**Supplementary Table 4)**.

Mediation analysis

Based on the above-mentioned results, mediation analysis was performed only for TT, to assess whether, and to what extent, the sex differences (women vs men) in glucose- and insulin-related traits and incidence of T2D were mediated by TT levels. The results of the mediation analysis of TT on the association between sex and glucose- and insulin-related traits are shown in **Table 2**. The mediation analysis showed an inconsistent mediatory role of TT (opposing DE and IE) on the association between sex and all glucose- and insulin-related traits, highlighting the complex role of TT, suggesting that TT's effects are not uniform and can lead to differing outcomes in metabolic health across sexes. The Integration of DE and IE within the DAGs, illustrating the association between sex and all glucose- and insulin-related traits through TT as a potential mediator, is provided in **Supplementary Figures 4A-4D**.

Based on model 2, the DE for fasting glucose, fasting insulin, 2h-glucose, and HOMA-IR were (β= -8.05, 95% CI: -9.62, -6.50), (β= -0.52, 95% CI: -0.62, -0.42), (β= -15.67, 95% CI: -20.63, -11.15) and (β= -0.60, 95% CI: -0.71, -0.51), while the IE

were (β= 3.08, 95% CI: 2.04, 4.30), (β= 0.39, 95% CI: 0.30, 0.47), (β= 12.77, 95% CI: 9.01, 16.03) and, (β= 0.41, 95% CI: 0.33, 0.50), respectively.

Table 2. Mediation analysis of testosterone on the association between sex (women vs men [Reference]) and glucose- and insulin-related traits among participants of KORA F4.

Effects	ß (95% CI) model 1	p-value	ß (95% CI) model 2	p-value
Fasting glucose levels				
DE	-8.43 (-10.01, -7.11)	<0.001	-8.05 (-9.62, -6.50)	<0.001
IE	3.16 (2.20, 4.36)	<0.001	3.08 (2.04, 4.30)	<0.001
TE	-5.27 (-6.08, -4.4)	<0.001	-4.95 (-5.63, -4.11)	<0.001
PM	-	-	-	-
Fasting insulin levels				
DE	-0.50 (-0.60, -0.42)	<0.001	-0.52 (-0.62, -0.42)	<0.001
IE	0.40 (0.32, 0.47)	<0.001	0.39 (0.30, 0.47)	<0.001
TE	-0.11 (-0.15, -0.05)	<0.001	-0.13 (-0.18, -0.08)	<0.001
PM	-	-	-	-
2h-glucose levels				
DE	-16.15 (-20.95, -10.76)	<0.001	-15.67 (-20.63, -11.15)	<0.001
IE	13.57 (9.50, 17.40)	<0.001	12.77 (9.01, 16.03)	<0.001
TE	-2.57 (-5.31, -0.01)	<0.001	-2.90 (-5.64, 0.03)	0.06
PM	-	-		
HOMA-IR				
DE	-0.59 (-0.69, -0.52)	<0.001	-0.60 (-0.71, -0.51)	<0.001
IE	0.43 (0.36, 0.52)	<0.001	0.41 (0.33, 0.50)	<0.001
TE	-0.16 (-0.21, -0.12)	<0.001	-0.18 (-0.24, -0.13)	<0.001
PM	-	-	-	-

Model 1: Age.
Model 2: Model 1+ smoking+ alcohol consumption+ physical activity.
fasting insulin levels and HOMA-IR are log-transformed.
Abbreviations:DE, direct effect; IE, indirect effect; TE, total effect; PM, proportion mediated; HOMA-IR, homeostatic model assessment for insulin resistance.

The mediation analysis of TT on the association between sex and incidence of T2D is presented in **Table 3**. The DE and IE were in opposite directions (**Supplementary Figure 4E**). The DE was (OR: 0.14, 95% CI: 0.07, 0.28) and (OR: 0.12, 95% CI: 0.06, 0.20), while the IE was (OR: 7.75, 95% CI: 3.72, 17.87) and (OR: 7.60, 95% CI: 3.43, 24.54) in models 1 and 2, respectively.

Table 3. Mediation analysis of testosterone on the association between sex (women vs men [Reference]) and incidence of T2D between KORA F4 and FF4.

Effects	OR (95% CI) model 1	p-value	OR (95% CI) model 2	p-value
DE	0.14 (0.07, 0.28)	<0.001	0.12 (0.06, 0.20)	<0.001
IE	7.75 (3.72, 17.87)	<0.001	7.60 (3.43, 24.54)	<0.001
TE	0.88 (0.56, 1.38)	0.54	0.75 (0.45, 1.22)	0.3
PM	-	-	-	-

Model 1: adjusted for age.
Model 2: Model 1+ smoking+ alcohol consumption+ physical activity.
Abbreviations: OR, odds ratio; T2D, type 2 diabetes; DE, direct effect; IE, indirect effect; TE, total effect; PM, proportion mediated.

The findings were robust in the sensitivity analysis adding total cholesterol and lipid-lowering medications to model 2 (model 3) (**Supplementary Tables 5 and 6**). The DE and IE were in opposite directions for fasting glucose, fasting insulin, 2h-glucose, HOMA-IR (**Supplementary Table 5)** and incidence of T2D **(Supplementary Table 6)**. The results of sensitivity analysis based on median age were also robust with larger effect estimates in individuals younger than 53 years **(Supplementary Table 7)**. Another sensitivity analysis based on BMI categories (BMI >= 25 kg/m^2 and BMI < 25 kg/m^2) supported the same results with larger effect estimates in overweight and obese individuals **(Supplementary Table 8)**.

Discussion

To our best knowledge, the current study is the first investigation examining the mediatory role of androgens in explaining sex differences in glucose metabolism and the incidence of T2D. While previous studies have independently examined the sex-specific association of androgens with glucose homeostasis and the incidence of T2D in men and women, we utilized mediation analysis to consider the complex combined effects of sex, sex hormones, and glucose homeostasis/ incidence of T2D. Our results indicated an inconsistent mediatory role of TT on sex differences in glucose- and insulin-related traits and incidence of T2D which shows that TT affects glucose metabolism and T2D risk differently in men and women. In men, higher TT levels are generally beneficial for glucose metabolism and lower the risk of T2D, while in women, higher TT levels can be detrimental. In addition, no mediatory role was observed for DHEA and DHEAs on the association of sex with glucose- and insulin-related traits or incidence of T2D.

Our findings on sex differences for androgens and glucose homeostasis were in line with previous research, reporting lower levels of TT (8), DHEA (31), and DHEAs (31) and also, lower levels of fasting glucose (4, 32), insulin (4), and HOMA-IR (33) in women compared to men. While some studies reported sex differences for T2D risk factors, incidence and related complications (2, 34), we found no association of sex differences for incidence of T2D.

We found that TT levels may have an inconsistent mediatory role in sex differences for glucose homeostasis and the development of T2D. Specifically, higher levels of TT in men were associated with decreased levels of fasting glucose, fasting insulin, and HOMA-IR along with a lower odds of T2D, while in women, only an inverse association was observed for TT and fasting glucose levels. In accordance with these findings, O'Reilly et al. (35) found some evidence of a sexually dimorphic feature for TT levels in mediating the incidence of T2D; hence, testosterone deficiency and excess may be independent risk factors for T2D in men and women, respectively. Moreover, Goodman-Gruen et al. (36) reported sex differences in the link between TT levels and glucose tolerance in adults, in which men with higher fasting glucose, high glucose tolerance, and T2D had lower levels of TT, and in contrast, women with T2D had higher bioavailable testosterone levels. Similarly, Oh et al. showed that low TT levels in men and high levels of bioavailable testosterone in women are associated with the incidence of T2D (8). A randomized follow-up study involving 38 women with PCOS reported that higher TT levels were associated with a higher insulin resistance index (37).

A systematic review and meta-analysis of observational studies previously demonstrated that men with higher TT levels (15.6–21.0 nmol/L) had a 42% lower risk of T2D, while higher TT increased T2D risk in women (11). Gyawali et al. (38) reported similar findings, showing that TT levels were inversely associated with T2D incidence in middle-aged to elderly men, independent of established risk factors. Yao et al. (39), in a meta-analysis of cohort studies, suggested that higher TT levels were significantly associated with lower T2D risk in men. Our findings are also supported by a Mendelian randomization study, which demonstrated that higher bioavailable testosterone in men was associated with lower fasting glucose and favorable effects on glucose metabolism (13). Another Mendelian randomization study reported evidence of the beneficial impacts of higher testosterone levels on T2D incidence and related traits in men (40).

Moreover, there are some clinical trials, supporting our findings. Canguven et al. (41) found that testosterone therapy may reduce glycated hemoglobin levels in elderly

hypogonadal men, which might lower the risk of T2D in those participants. A recent systematic review and meta-analysis reported that testosterone replacement therapy can improve glycemic control and hormone levels in hypogonadal men with T2D (42). Additionally, Jenkins et al. (43) showed that the highest adherence to testosterone replacement therapy is associated with improved HbA1c and glycaemic homeostasis over time in men with hypogonadism and T2D.

In our study, we also performed the subset analysis based on median age, showing that the mediatory role of TT in all glucose- and insulin-related traits was stronger in participants < 53 years of age compared to those aged 53 and over. These findings are consistent with a meta-analysis of cohort studies, primarily involving postmenopausal women, which indicated an increased risk of T2D associated with higher levels of TT and FT, but the results were not statistically significant (44). This finding might be due to a reduction in both adrenal and testicular function with aging, leading to a decrease in TT levels in men (45). In women, there is a decline in TT levels with age which is steepest in the early reproductive years. This decline then stabilizes in midlife and tends to slightly increase in the later years (46). Thus, the variation between men and women becomes narrower in older ages. Furthermore, stratification of mediation analysis based on BMI categories revealed that the mediatory role of TT remains stronger in participants with overweight and obesity. These findings are in line with previous evidence showing that androgen balance is different in people with obesity based on their sex. While in men TT decreases with increasing body weight, women with abdominal obesity may experience functional hyperandrogenism (47).

The specific biological mechanisms underlying the link between TT levels and glucose homeostasis, and the development of T2D in a sex-specific manner remain unclear. Evidence suggests that testosterone regulates the expression of key regulatory enzymes involved in glucose and lipid metabolism in major insulin-responsive tissues, including the liver, adipose tissue, and skeletal muscle (48). Additionally, some sex differences in inflammatory cytokine production may predict the risk of T2D (49). The differences in how testosterone affects glucose metabolism and insulin sensitivity may be due to varying interactions with other hormones and differences in body composition between men and women (50). One possible hypothesis is that testosterone deficiency in men leads to abdominal obesity, contributing to T2D (51). Fan et al. (52) found that androgen receptors (AR) significantly affect fat accumulation in male mice. AR knockout reduces the effect of testosterone, leading to obesity, which could contribute to the development of T2D. Testosterone may affect glucose control by increasing lean body mass in

men (53, 54). In women, however, higher testosterone levels can lead to increased body fat, insulin resistance, and higher blood glucose concentrations (55). Additionally, evidence has shown that testosterone treatment in men might reduce the levels of tumor necrosis factor (TNF-a) (56, 57). TNF-a is an inflammatory cytokine that can induce insulin resistance in both muscle and adipose tissues (58-60) and is associated with a higher T2D risk (61, 62). It also has been demonstrated that men with low levels of testosterone, including participants with reduced glucose tolerance and T2D, have manifested lower oxidative phosphorylation in muscle mitochondria, contributing to the increase of insulin resistance (63). On the other hand, a growing body of evidence has shown that testosterone increases lipogenesis in visceral fat in women, whereas, in men, it stimulates adipose tissue lipolysis (64).

In contrast to the sexually dimorphic role of TT, we did not find any significant mediatory role for DHEA and DHEAs in glucose- and insulin-related traits and T2D risk. There have been conflicting findings concerning the link between DHEA and DHEAs and glucose metabolism in the general population. Similar to our findings, a prospective study on postmenopausal women found no association between DHEA and the risk of T2D (65). Additionally, Ding et al. (17) showed that DHEAs was not associated with T2D risk in postmenopausal women. However, another prospective cohort study found that serum levels of DHEA were inversely associated with T2D risk, with no observed sex differences in this association (66). Moreover, a study by Veronese et al. (16) reported no link between DHEAs and T2D whereas, after stratifying by sex, higher levels of DHEAs were inversely associated with T2D risk in men, but not in women.

The current study was performed, both cross-sectionally and longitudinally, on a well-characterized population-based study and adjustments for a wide range of potential confounding factors. The availability of OGTT data at both baseline and follow-up enabled us to assess not only the development of clinically diagnosed T2D but also of early disorders in glucose metabolism and newly OGTT-diagnosed T2D. Similar to prior research on KORA study focusing on sex hormones (67-69), our study benefits from comprehensive data regarding menopausal surgeries (such as oophorectomy or hysterectomy) and various hormonal treatments (including anti-estrogens, estrogens, progestins, hormone replacement therapy, androgens, antiandrogens, enzyme inhibitors, gonadotropin-releasing hormones, and anabolic steroids). This wealth of information allowed us to account for several significant variables in our eligibility criteria. Due to the observational design, the present study could not establish a causal association. Moreover, data on women with

PCOS was not available in the dataset. Even in postmenopausal women, PCOS symptoms can still lead to changes in sex hormone levels and, consequently, in metabolic processes. The lack of precise time-to-event information does not allow us to perform more advanced modelling such as Cox regression analysis. The study's sample size limited our ability to conduct additional sensitivity analyses based on different menopausal status groups. Additionally, we could not stratify our sensitivity analyses for incidence of T2D by categories of age and BMI due to the limited number of incident cases. Lastly, as most of the participants in our study were Europeans, the findings cannot be generalized to other ethnic groups. Thus, we recommend that future mediation analyses on sex hormones and metabolic diseases address these limitations by using larger, more diverse sample sizes and taking menopausal status in women into account.

These findings emphasize the need for a comprehensive and personalized approach to assessing and managing T2D risk that considers both direct and indirect effects of sex and hormones like TT. A simple focus on hormone levels without considering their broader metabolic context might be insufficient. Therefore, understanding the sex-specific effects of testosterone is crucial for developing targeted treatments for diabetes. For men with low testosterone, testosterone replacement therapy might be beneficial in improving metabolic health and reducing diabetes risk. For women, especially those with conditions like PCOS, managing elevated testosterone levels through lifestyle changes or medication might help in mitigating the risk of diabetes. Additionally, the lack of a mediatory role for DHEA and DHEAs suggests that these hormones do not significantly influence the sex differences observed in glucose metabolism and T2D risk. More research is needed to fully understand the mechanisms by which TT influences metabolic health differently in men and women and to explore other potential mediators of sex differences in T2D risk.

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Supplementary materials: The mediatory role of androgens on sex differences in glucose homeostasis and incidence of type 2 diabetes: the KORA study

Supplementary Table 1. Association of sex (women vs men [Reference]) with sex hormones and glucose- and insulin-related traits among participants of KORA F4.

Variable	ß (95 % CI) Model 1	ß (95 % CI) Model 2
Sex hormones		
Testosterone (nmol/l)	-14.97 (-15.38, -14.57)	-15.05 (-15.48, -14.62)
DHEA (nmol/l)	-0.18 (-0.85, 0.49)	-0.29 (-1.00, 0.42)
DHEAs (nmol/l)	-1387.66 (-1559.06, -1216,26)	-1339.36 (-1521.52, -1157,20)
Glucose- and insulin-related traits		
Fasting glucose levels (mg/dl)	-5.27 (-6.04, -4.50)	-4.96 (-5.78, -4.14)
Fasting insulin levels (µU/ml)	-0.11 (-0.15, -0.06)	-0.13 (-0.18, -0.08)
2h-glucose levels (mg/dl)	-2.57 (-5.08, -0.07)	-2.91 (-5.58, -0.24)
HOMA-IR	-0.16 (-0.21, -0.12)	-0.18 (-0.24, -0.13)

Model 1: Age.
Model 2: Model 1 + smoking + alcohol consumption + physical activity.
Fasting insulin levels and HOMA-IR are log-transformed.
Abbreviations: DHEA, dehydroepiandrosterone; DHEAs, dehydroepiandrosterone-sulfate; HOMA-IR, homeostatic model assessment for insulin resistance.

Supplementary Table 2. Association of sex hormones with glucose- and insulin-related traits among male participants of KORA F4.

Variable	ß (95 % CI) model 1	ß (95 % CI) model 2
Testosterone (nmol/l)		
Fasting glucose levels (mg/dl)	-0.22 (-0.30, -0.14)	-0.21 (-0.30, -0.13)
Fasting insulin levels (µU/ml)	-0.02 (-0.03, -0.02)	-0.02 (-0.03, -0.02)
2h-glucose levels (mg/dl)	-0.09 (-1.2, -0.65)	-0.08 (-1.12, -0.57)
HOMA-IR	-0.03 (-0.03, -0.02)	-0.03 (-0.03, -0.02)
DHEA (nmol/l)		
Fasting glucose levels (mg/dl)	0.07 (0.00, 0.14)	0.00 (0.00, 0.14)
Fasting insulin levels (µU/ml)	-0.00 (-0.01, 0.00)	-0.00 (-0.01, 0.00)
2h-glucose levels (mg/dl)	0.08 (-0.14, 0.32)	0.11 (-0.12, 0.34)
HOMA-IR	-0.00 (-0.01, -0.00)	-0.00 (-0.01, -0.00)
DHEAs (nmol/l)		
Fasting glucose levels (mg/dl)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
Fasting insulin levels (µU/ml)	-0.00 (-0.01, 0.00)	-0.00 (-0.01, 0.00)
2h-glucose levels (mg/dl)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
HOMA-IR	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)

Model 1: Age.
Model 2: Model 1+ smoking+ alcohol consumption+ physical activity.
Fasting insulin levels and HOMA-IR are log-transformed.
Abbreviations: DHEA, dehydroepiandrosterone; DHEAs, dehydroepiandrosterone-sulfate; HOMA-IR, homeostatic model assessment for insulin resistance.

Supplementary Table 3. Association of sex hormones with glucose- and insulin-related traits among female participants of KORA F4.

Variable	ß (95 % CI) model 1	ß (95 % CI) model 2
Testosterone (nmol/l)		
Fasting glucose levels (mg/dl)	1.36 (0.32, 2.40)	1.35 (0.29, 2.4)
Fasting insulin levels (µU/ml)	0.02 (-0.03, 0.08)	0 (-0.0, 0.06)
2h-glucose levels (mg/dl)	1.5 (-1.93, 4.94)	1.94 (-1.50, 5.4)
HOMA-IR	0.03 (-0.03, 0.1)	0.01 (-0.04, 0.08)
DHEA (nmol/l)		
Fasting glucose levels (mg/dl)	0.02 (-0.04, 0.09)	0.02 (-0.04, 0.10)
Fasting insulin levels (µU/ml)	-0.00 (-0.00, 0.00)	-0.00 (-0.00, 0.00)
2h-glucose levels (mg/dl)	-0.04 (-0.27, 0.2)	-0.06 (-0.29, 0.17)
HOMA-IR	-0.00 (-0.00, 0.00)	-0.00 (-0.00, 0.00)
DHEAs (nmol/l)		
Fasting glucose levels (mg/dl)	0.00 (-0.00, 0.00)	0.00 (-0.00, 0.00)
Fasting insulin levels (µU/ml)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
2h-glucose levels (mg/dl)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
HOMA-IR	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)

Model 1: Age.
Model 2: Model 1+ smoking+ alcohol consumption+ physical activity.
Fasting insulin levels and HOMA-IR are log-transformed.
Abbreviations: DHEA, dehydroepiandrosterone; DHEAs, dehydroepiandrosterone-sulfate; HOMA-IR, homeostatic model assessment for insulin resistance.

Supplementary Table 4. Association of sex (women vs men [Reference]) and sex hormones with incidence of T2D between KORA F4 and FF4.

Variable	OR (95% CI) model 1	OR (95% CI) model 2
Sex (women vs men [Reference])	0.70 (0.43, 1.08)	0.58 (0.43, 1.08)
Testosterone (nmol/l)	0.86 (0.82, 0.92)	0.87 (0.82, 0.92)
DHEA (nmol/l)	0.96 (0.92, 1.00)	0.96 (0.92, 1.00)
DHEAs (nmol/l)	1.00 (0.99, 1.00)	1.00 (0.99, 1.00)

Model 1: Age.
Model 2: Model 1+ smoking+ alcohol consumption+ physical activity.
Abbreviations: DHEA, dehydroepiandrosterone; DHEAs, dehydroepiandrosterone-sulfate; OR, odds ratio; T2D; type 2 diabetes.

Supplementary Table 5. Mediation analysis of total testosterone on the association between (women vs men [Reference]) and glucose- and insulin-related traits among participants of KORA F4 (model 3).

Effects	ß (95% CI) model 3	p-value
Fasting glucose levels		
DE	-7.98 (-9.51, -6.42)	<0.001
IE	2.97 (1.81, 4.13)	<0.001
TE	-5.01 (-5.76, -4.12)	<0.001
PM	-	-
Fasting insulin levels		
DE	-0.50 (-0.60, -0.40)	<0.001
IE	0.37 (0.28, 0.46)	<0.001
TE	-0.13 (-0.18, -0.08)	<0.001
PM	-	-
2h-glucose levels		
DE	-15.52 (-20.54, -10.74)	<0.001
IE	12.42 (8.91, 16.96)	<0.001
TE	-3.01 (-5.53, -0.16)	0.05
PM	-	-
HOMA-IR		
DE	-0.59 (-0.69, -0.49)	<0.001
IE	0.40 (0.31, 0.49)	<0.001
TE	-0.18 (-0.24, -0.13)	<0.001
PM	-	-

Model 1: Age.
Model 2: Model 1+ smoking+ alcohol consumption+ physical activity.
Model 3: Model 2+ total cholesterol+ lipid lowering medications.
fasting insulin levels and HOMA-IR are log-transformed.
Abbreviations:DE, direct effect; IE, indirect effect; TE, total effect; PM, proportion mediated; HOMA-IR, homeostatic model assessment for insulin resistance.

Supplementary Table 6. Mediation analysis of total testosterone on the association between sex (women vs men [Reference]) and incidence of T2D between KORA F4 and FF4 (model 3).

Effects	OR (95% CI) model 3	p-value
DE	0.10 (0.04, 0.22)	<0.001
IE	7.08 (3.21, 17.46)	<0.001
TE	0.69 (0.47, 1.05)	0.15
PM	-	-

Model 1: Age.
Model 2: Model 1+ smoking+ alcohol consumption+ physical activity.
Model 3: Model 2+ total cholesterol+ lipid lowering medications.
Abbreviations: OR, odds ratio; T2D, type 2 diabetes; DE, direct effect; IE, indirect effect; TE, total effect; PM, proportion mediated.

Supplementary Table 7. Mediation analysis of total testosterone on the association between (women vs men [Reference]) and glucose- and insulin-related traits stratified by median age (53 years).

Fasting glucose levels (mg/dl)				
Mediation analysis for individuals >= 53 years				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-10.20 (-12.23, -8.02)	<0.001	-9.78 (-12.46, -7.84)	<0.001
IE	3.93 (2.20, 5.72)	<0.001	3.78 (2.02, 5.95)	<0.001
TE	-6.27 (-7.15, -5.31)	<0.001	-6.00 (-6.88, -4.97)	<0.001
PM	-	-	-	-
Mediation analysis for individuals >= 53 years				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-6.75 (-8.86, -4.30)	<0.001	-6.55 (-9.05, -4.35)	<0.001
IE	2.50 (0.62, 4.40)	<0.001	2.36 (0.35, 4.34)	0.04
TE	-4.27 (-5.40, -2.98)	<0.001	-4.16 (-5.34, -3.08)	<0.001
PM	-	-	-	-
Fasting insulin levels (µU/ml)				
Mediation analysis for individuals < 53 years				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-0.65 (-0.80, -0.53)	<0.001	-0.65 (-0.75, -0.53)	<0.001
IE	0.46 (0.35, 0.57)	<0.001	0.43 (0.32, 0.53)	<0.001
TE	-0.18 (-0.24, -0.12)	<0.001	-0.22 (-0.27, -0.16)	<0.001
PM	-	-	-	-
Mediation analysis for individuals >= 53 years				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-0.38 (-0.50, -0.25)	<0.001	-0.40 (-0.53, -0.27)	<0.001
IE	0.35 (0.23, 0.46)	<0.001	0.35 (0.23, 0.47)	<0.001
TE	-0.03 (-0.10, 0.04)	0.39	-0.05 (-0.13, 0.00)	0.14
PM	-	-	-	-
2h-glucose levels (mg/dl)				
Mediation analysis for individuals < 53 years				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-19.62 (-26.04, -13.75)	<0.001	-18.51 (-24.73, -11.67)	<0.001
IE	17.00 (12.21, 23.35)	<0.001	15.92 (9.96, 21.44)	<0.001
TE	-2.64 (-5.74, 0.04)	0.06	-2.59 (-5.87, -0.03)	0.05
PM	-	-	-	-

Supplementary Table 7. Continued

Fasting glucose levels (mg/dl)				
Mediation analysis for individuals >= 53 years				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-13.59 (-20,15, -6.28)	<0.001	-14.72 (-21.35, -7.18)	<0.001
IE	11.09 (5.57, 15.75)	<0.001	10.67 (5.02, 16.44)	<0.001
TE	-2.49 (-5.99, 1.37)	0.26	-4.04 (-8.06, 0.23)	0.07
PM	-	-	-	-
HOMA-IR				
Mediation analysis for individuals < 53 years				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-0.75 (-0.89, -0.64)	<0.001	-0.75 (-0.90, -0.60)	<0.001
IE	0.50 (0.38, 0.64)	<0.001	0.47 (0.33, 0.60)	<0.001
TE	-0.25 (-0.32, -0.18)	<0.001	-0.28 (-0.35, -0.21)	<0.001
PM	-	-	-	-
Mediation analysis for individuals >= 53 years				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-0.45 (-0.57, -0.30)	<0.001	-0.46 (-0.60, -0.35)	<0.001
IE	0.37 (0.25, 0.50)	<0.001	0.37 (0.27, 0.49)	<0.001
TE	-0.07 (-0.15, -0.00)	0.05	-0.09 (-0.17, -0.02)	<0.001
PM	-	-	-	-

Model 1: Age.
Model 2: Model 1+ smoking + alcohol consumption + physical activity.
SHBG is log-transformed.
Abbreviations: DE, direct effect; IE, indirect effect; TE, total effect; PM, proportion mediated; HOMA-IR, homeostatic model assessment for insulin resistance.

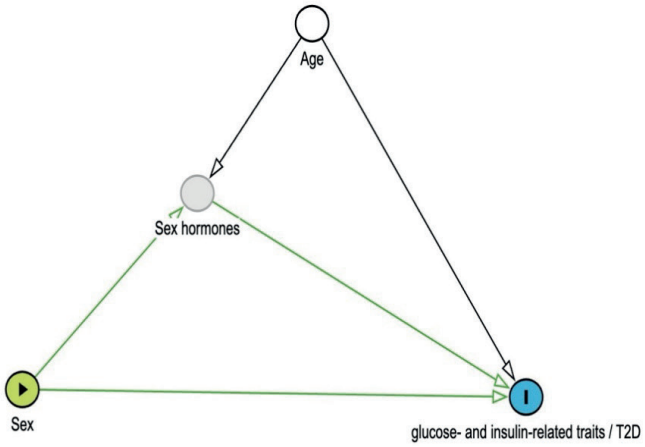
Supplementary Table 8. Mediation analysis of total testosterone on the association between (women vs men [Reference]) and glucose- and insulin-related traits stratified stratified by BMI categories.

Fasting glucose levels (mg/dl)				
Mediation analysis for individuals with normal body weight (BMI < 25 kg/m²)				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-5.50 (-8.13, -2.56)	<0.001	-5.45 (-8.03, -2.40)	<0.001
IE	1.04 (-1.44, 3.07)	0.42	1.16 (-1.32, 3.10)	0.32
TE	-4.46 (-5.7, -3.06)	<0.001	-4.29 (-5.68, -3.04)	<0.001
PM				
Mediation analysis for overweight and obese individuals (BMI >= 25 kg/m²)				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-7.67 (-9.90, -5.80)	<0.001	-7.17 (-9.14, -5.26)	<0.001
IE	2.88 (1.43, 4.82)	<0.001	2.78 (1.03, 4.09)	<0.001
TE	-4.78 (-5.73, -3.66)	<0.001	-4.40 (-5.42, -3.21)	<0.001
PM	-	-	-	-
Fasting insulin levels (µU/ml)				
Mediation analysis for individuals with normal body weight (BMI < 25 kg/m²)				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-0.14 (-0.29, -0.03)	<0.001	-0.19 (-0.34, -0.06)	<0.001
IE	0.12 (0.02, 0.26)	0.01	0.14 (0.04, 0.29)	0.03
TE	-0.01 (-0.07, 0.04)	0.62	-0.04 (-0.11, 0.04)	0.10
PM	-	-	-	-
Mediation analysis for overweight and obese individuals (BMI >= 25 kg/m²)				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-0.41 (-0.51, -0.30)	<0.001	-0.42 (-0.52, -0.33)	<0.001
IE	0.37 (0.28, 0.46)	<0.001	0.35 (0.28, 0.45)	<0.001
TE	-0.09 (-0.02, 0.01)	0.19	-0.06 (-0.12, -0.01)	0.02
PM	-	-	-	-
2h-glucose levels (mg/dl)				
Mediation analysis for individuals with normal body weight (BMI < 25 kg/m²)				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-11.10 (-20.02, -2.16)	0.02	-10.91 (-19.71, -2.45)	0.01
IE	7.09 (-0.79, 14.23)	0.06	6.69 (-0.98, 13.69)	0.12
TE	-4.05 (-8.02, -0.16)	0.04	-4.21 (-7.70, -0.52)	0.02
PM	-	-	-	-

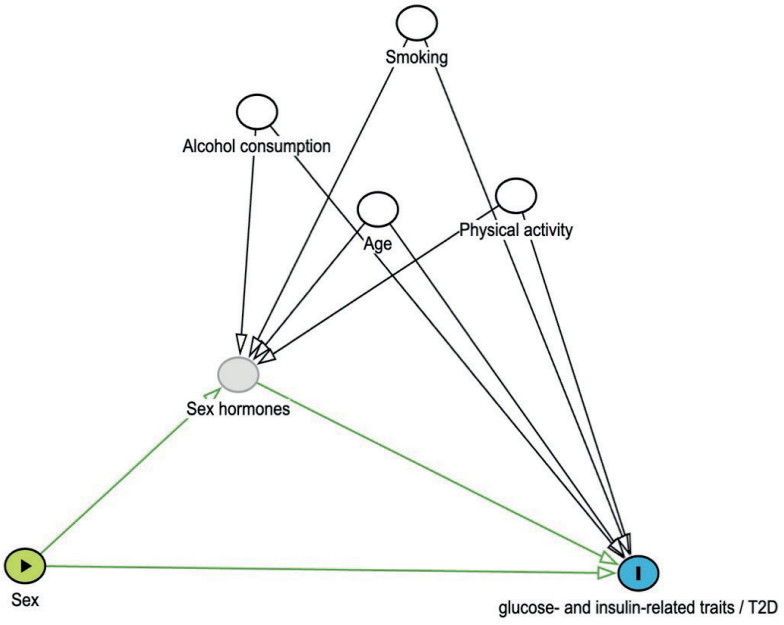
Supplementary Table 8. Continued

Mediation analysis for overweight and obese individuals (BMI >= 25 kg/m²)				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-13.80 (-20.26, -7.95)	<0.001	-13.41 (-19.51, -7.28)	<0.001
IE	14.49 (10.27, 19.45)	<0.001	13.62 (8.48, 18.35)	<0.001
TE	0.69 (-3.24, 4.70)	0.74	0.21 (-3.03, 3.75)	0.79
PM	-	-	-	-
HOMA-IR				
Mediation analysis for individuals with normal body weight (BMI < 25 kg/m²)				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-0.20 (-0.34, -0.08)	<0.001	-0.25 (-0.41, -0.11)	<0.001
IE	0.13 (0.02, 0.26)	<0.001	0.16 (0.04, 0.29)	0.01
TE	-0.06 (-0.13, 0.00)	0.09	-0.09 (-0.16, -0.03)	<0.001
PM	-	-	-	-
Mediation analysis for overweight and obese individuals (BMI >= 25 kg/m²)				
Effects	**ß (95% CI) model 1**	**p-value**	**ß (95% CI) model 2**	**p-value**
DE	-0.49 (-0.62, -0.39)	<0.001	-0.49 (-0.62, -0.39)	<0.001
IE	0.40 (0.31, 0.50)	<0.001	0.38 (0.28, 0.46)	<0.001
TE	-0.09 (-0.14, -0.03)	<0.001	-0.11 (-0.19, -0.04)	<0.001
PM	-	-	-	-

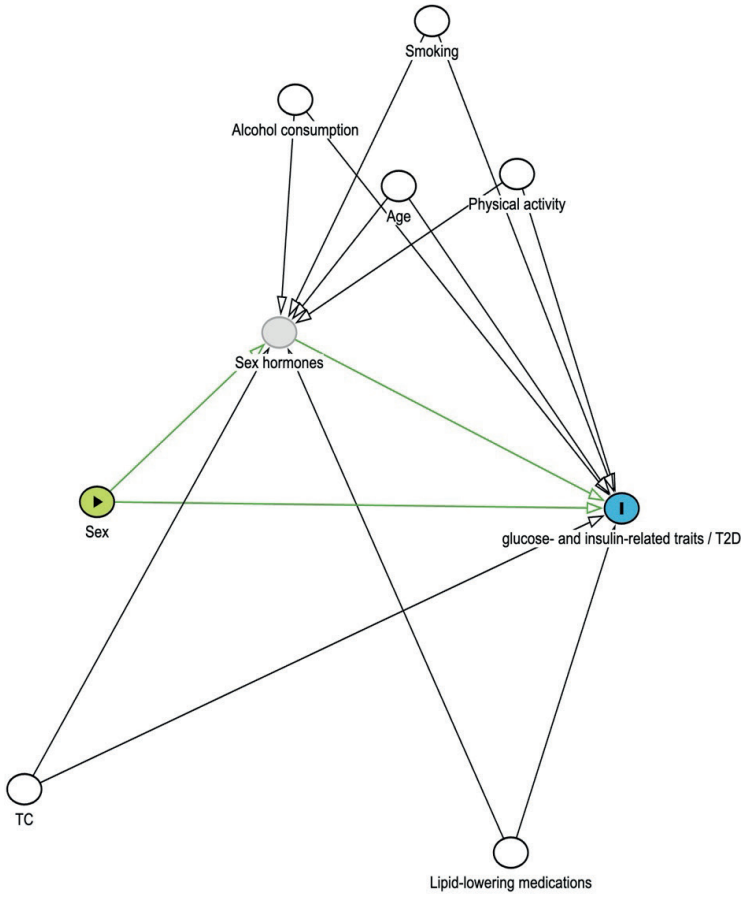
Model 1: Age.
Model 2: Model 1+ smoking + alcohol consumption + physical activity.
SHBG is log-transformed.
Abbreviations: DE, direct effect; IE, indirect effect; TE, total effect; PM, proportion mediated; HOMA-IR, homeostatic model assessment for insulin resistance.



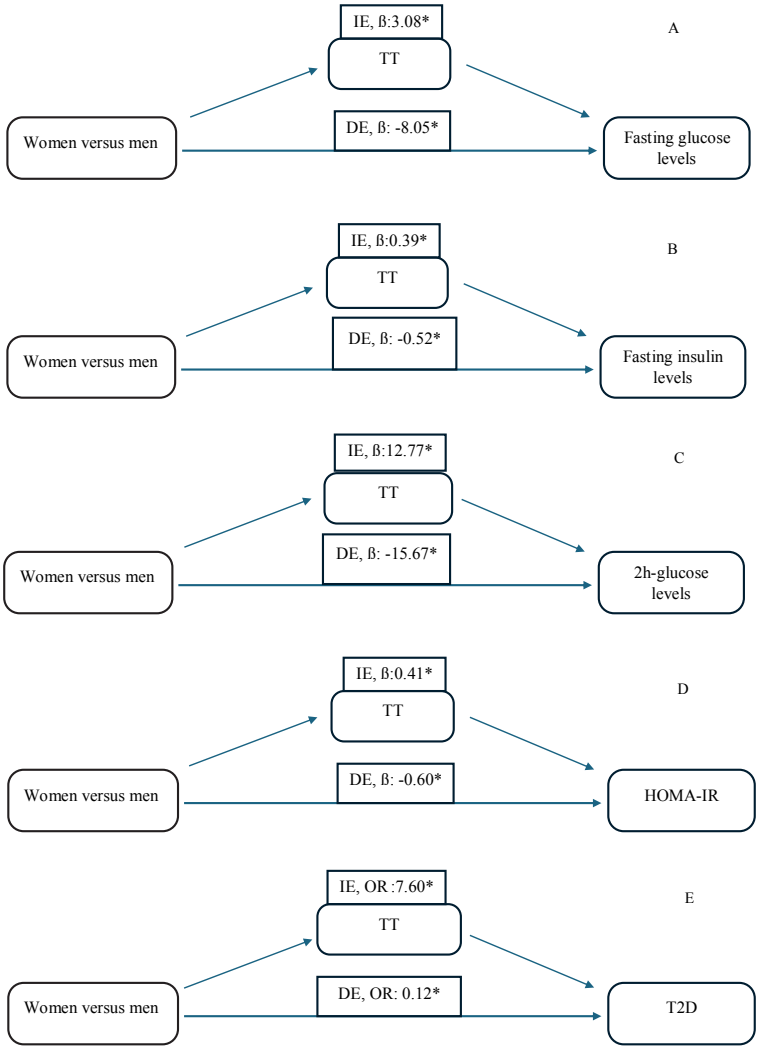
Supplementary Figure 1. Directed acyclic graph (DAG) for the association of sex through, sex hormones (total testosterone, dehydroepiandrosterone, dehydroepiandrosterone-sulfate) on glucose- and insulin-related traits / type 2 diabetes (T2D). Study DAG for model 1, exposure = sex, mediator = sex hormones, outcome = glucose- and insulin-related traits / T2D, adjustment = age.



Supplementary Figure 2. Directed acyclic graph (DAG) for the association of sex through, sex hormones (total testosterone, dehydroepiandrosterone, dehydroepiandrosterone-sulfate) on glucose- and insulin-related traits / type 2 diabetes (T2D). Study DAG for model 2, exposure = sex, mediator = sex hormones, outcome = glucose- and insulin-related traits / T2D, adjustment variables = age + alcohol consumption + smoking + physical activity.



Supplementary Figure 3. Directed acyclic graph (DAG) for the association of sex through, sex hormones (total testosterone, dehydroepiandrosterone, dehydroepiandrosterone-sulfate) on glucose- and insulin-related traits / type 2 diabetes (T2D). Study DAG for model 3, exposure = sex, mediator = sex hormones, outcome = glucose- and insulin-related traits / T2D, adjustment variables = age + alcohol consumption + smoking, physical activity + lipid lowering medications + total cholesterol.



Supplementary Figures 4A-4E. Directed acyclic graphs (DAGs) for the association of sex through total testosterone as potential mediator on glucose- and insulin-related traits / type 2 diabetes (T2D). Study DAG for model 2 (adjustment variables = age + alcohol consumption + smoking, physical activity), exposure = sex, mediator = total testosterone, outcome = A: fasting glucose levels; B: fasting insulin levels; C: 2h-glucose levels; D: HOMA-IR; E: T2D.

Abbreviations: DE, direct effect; IE, indirect effect; OR, odds ratio; HOMA-IR, homeostatic model assessment for insulin resistance; T2D, type 2 diabetes.



Chapter 5

Do androgens and sex hormone-binding globulin mediate sex differences in the risk of stroke and myocardial infarction? The KORA Study

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Abstract

Background: Sex differences are well documented in the risk of cardiovascular disease (CVD), including stroke and myocardial infarction (MI). Evidence suggests that androgens and sex hormone-binding globulin (SHBG) may contribute to CVD development in a sex-specific manner. Accordingly, we aimed to investigate whether circulating levels of androgens and SHBG help explain the observed sex differences in stroke and MI risk.

Methods: Observational data from the German population-based KORA F4 study (n = 1,970; mean age 54 years; 58% men) was used as baseline examination. Incident stroke and incident MI cases were identified through regular follow-ups until the end of 2016. Multivariable linear and Cox regression models were employed to explore associations. Mediation analyses were performed to assess direct effects (DE), indirect effects (IE), and the mediating role of androgens (total testosterone (TT), dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), and dehydroepiandrosterone-sulfate (DHEAs)) and SHBG in the association between sex (women vs. men) and risk of stroke/MI.

Results: During a median follow-up of 8.6 years, 63 MI occurred over 16,626 person-years, and 71 strokes occurred over 16,332 person-years. After adjustment for confounders (model 1: age; model 2: model 1 + smoking, alcohol consumption, and physical activity), women had lower levels of TT, DHT, and DHEAs compared to men. An inverse association was observed for TT and DHT with risk of stroke in men, while no association was found for androgens or SHBG with risk of stroke in women. No associations were observed between sex hormones or SHBG and risk of MI in either men or women. Sex differences were observed for risk of MI, but not for risk of stroke. An inconsistent mediatory role was observed for TT and DHT in the association between sex and risk of stroke. The DE were 0.42 (95% CI: 0.18, 0.86) and 0.40 (95 % CI: 0.17, 0.82) and IE were 2.48 (95% CI: 1.27, 6.17) and 2.90 (95% CI: 1.67, 6.35) for TT and DHT, respectively. No mediating role was observed for other androgens or SHBG in relation to stroke, and neither androgens nor SHBG mediated the risk of MI.

Conclusions: Our findings suggest that both TT and DHT may have dimorphic mediating role with stroke risk, and that these associations may vary between men and women. Future studies are warranted to explore the potential mediatory roles of TT, DHT, and other sex hormones across a broader spectrum of cardiovascular outcome.

Background

Cardiovascular diseases (CVD), especially ischemic heart disease and stroke are the leading cause of mortality in the world (1). Substantial sex differences exist in the incidence, presentation, and outcomes of CVD, including myocardial infarction (MI) and stroke (2). While these differences are partly explained by socio-cultural factors, biological mechanisms are thought to play a central role (3). Among these, endogenous sex hormones and sex hormone-binding globulin (SHBG) have been proposed as key contributors due to their effects on vascular biology and cardiometabolic regulation (4, 5). Understanding whether sex hormones mediate sex differences in CVD risk is essential to identify biologically relevant pathways that may enable sex-specific prevention and treatment strategies.

Epidemiological evidence suggests that the relationship between sex hormones and CVD risk is sex-specific and complex. In men, low testosterone levels have been associated with higher CVD risk (6-8), whereas non-linear associations have been reported for all-cause mortality (9, 10). In contrast, studies on women have found that high serum total TT and low SHBG concentrations have been associated with higher burden of CVD risk factors and a higher incidence of CVD (11-15). The findings from a limited number of studies on dihydrotestosterone (DHT) and CVD risk have primarily focused on men and have reported associations with stroke (16), incident CVD (17), and all-cause or CVD-specific mortality (10).

Experimental and clinical evidence suggests that testosterone influences cardiovascular risk through pathways including endothelial function, vascular tone, lipid metabolism, inflammation, and insulin sensitivity (18, 19). Notably, these effects appear to differ by sex: while lower testosterone in men is associated with increased adiposity, insulin resistance, and pro-inflammatory states, elevated androgen levels in women have been linked to adverse cardiometabolic risk profiles (19, 20). In addition, androgens may affect coagulation and atherosclerotic processes, providing biological plausibility for their role in CVD development (21). These observations support the hypothesis that sex hormones may contribute differently to CVD risk in women and men.

A sex-specific mendelian randomization (MR) study found that genetically predicated higher serum SHBG levels are associated with decreased risk of ischemic stroke in males and females (14). Findings of another MR study using UK biobank data indicated that endogenous testosterone was positively associated with thromboembolism, heart failure (HF) and MI which were stronger in men than

women (22). However, another MR study found that both SHBG and TT had no direct causal effect with coronary heart disease (CHD) in men (23).

Evidence on other androgens, such as dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S), remains limited. Findings of a prospective cohort study on older men and women found that low DHEA-S is associated with increased risk for HF and mortality but not CHD in both sexes (24).

Despite these advances, an important gap remains. Prior studies have largely focused on associations or causal effects of sex hormones within sexes but have not examined whether these hormones/ SHBG explain (mediate) the observed differences in CVD risk between women and men. Addressing this question is essential to move beyond descriptive sex differences toward a mechanistic understanding of CVD. Thus, we aimed to assess the potential mediating role of androgens and SHBG and, if any, to what extent they mediate the association between sex and the incidence of stroke/ MI in a population-based setting of middle-aged and elderly adults.

Methods

Setting and study population

This study was conducted among participants of the prospective population-based Cooperative Health Research in the Region of Augsburg (KORA) study, selected from population registries in the city of Augsburg (Germany) and two surrounding counties between 1999 and 2001 (baseline visit, KORA S4). Follow-up examinations were performed between 2006-2008 (KORA F4) and 2013-2014 (KORA FF4). The KORA F4 study enrolled 3080 participants which were used as the baseline sample for the current analysis. All study participants have provided written informed consent. The study was approved by the Ethics Committees of the Bavarian Chamber of Physicians (Ethical Approval Number 06068) adhering to the declaration of Helsinki. The study design has been described in detail elsewhere (25).

The following participants were excluded from the analysis: participants who withdrew consent (n=3), participants with missing information on fasting status and non-fasting participants (n=21), participants diagnosed with stroke or MI at baseline (n=146), participants taking sex hormones (e.g. anti-estrogens, estrogens, progestin, hormone replacement therapy, androgens, antiandrogens, enzyme inhibitors, gonadotropin releasing hormones, and anabolic steroids) (n=271),

women with gynecological surgeries such as hysterectomy and oophorectomy or missing information on these surgeries (n=271), participants with missing information on sex hormones (TT, DHT, DHEA, and DHEAs) and SHBG (n=246) and participants with missing information on incident stroke/ MI during follow-up (n=152) which resulted in 1970 participants. The study flowchart of included participants is shown in **Figure 1**. The present analysis includes data from the KORA F4 as baseline and follow-up information from various sources until the end of 2016.

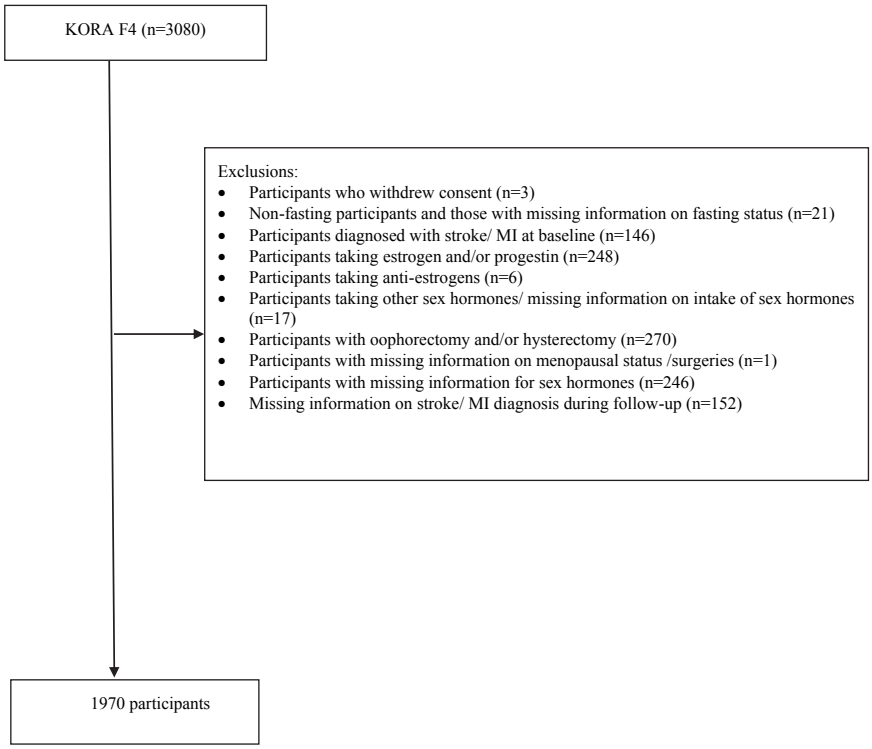


Figure 1. The study flowchart of included participants.

Androgens, and SHBG measurements

Sex hormones (TT, DHT, DHEA and DHEAs) were measured in plasma or serum samples which were stored at -80 °C until being assayed. The detailed assessment procedures have been described previously (26). Quantification of sex hormones was performed using the AbsoluteIDQ™ Stero17 Kit in combination with electrospray ionization liquid chromatography–tandem mass spectrometry (ESI-LC-MS/MS). The acquisition and quantification protocols of the Stero17 Kit comply with the European Medicines Agency's guideline on bioanalytical method validation (July 21, 2011).

Measurements of SHBG in serum were performed using the ARCHITECT SHBG assay, a chemiluminescent microparticle immunoassay (CMIA) for absolute quantification of SHBG (Abbott Diagnostics). Sample preparation and LC-MS/MS measurements were carried out following the manufacturer's manual (UM-STERO17) (27). Mass spectrometric analysis was performed using a QTRAP 5500 triple quadrupole mass spectrometer (Sciex Deutschland GmbH, Darmstadt, Germany), coupled with a 1260 Series HPLC system (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland), operated via Analyst 1.6.2 software. Data evaluation, quantification of hormone concentrations, and quality control were conducted using MultiQuant 3.0.1 and the MetIDQ™ software package (Biocrates Life Sciences AG, Innsbruck, Austria). Hormone concentrations were normalized; values below the lower limit of detection (LOD) were imputed, and batch effects were adjusted through sex-specific calibration.

Assessment of Covariates

Baseline data on age, sex, and medication use (antihypertensive mediations and lipid-lowering medications (yes/ no)) were obtained via standardized interviews conducted by trained medical staff. Participants were asked to bring all medication packages to the study center and exact preparations were extracted from a medication database and subsequently ATC-coded. Additional lifestyle factors such as smoking status (categorized as never, ex-smoker, irregular smoker, or regular smoker [≥1 cigarette/ day]), alcohol consumption (g/day), and physical activity (inactive/active) were also collected (28).

Anthropometric measurements, including body weight, waist circumference (WC) and body mass index (BMI), and blood pressure measurements were taken according to standardized protocols. Baseline T2D was initially assessed by self-report and subsequently validated by contacting the treating physician or medical chart review. Participants only reporting current use of glucose-lowering medication were also classified as having T2D. High C-reactive protein (CRP) was quantified in plasma using a high-sensitivity latex-enhanced nephelometric assay (BN II Analyzer, Dade Behring). Thyroid-stimulating Hormone (TSH) was measured using electrochemiluminescent methods (Dimension Vista Systems; Siemens, Germany). Total cholesterol was measured in fresh serum by enzymatic methods (CHOL Flex).

Ascertainment of myocardial infarction (MI) and stroke

Information on self-reported stroke or myocardial infarction (MI) was collected through regular postal follow-up questionnaires or during follow-up examinations involving face-to-face interviews. Vital status was periodically verified across

Germany via population registries and, more recently, through, the " Anstalt für Kommunale Datenverarbeitung in Bayern" (http://www.zemaonline.de/). Death certificates were obtained from the district health departments for all those who died in Bavaria and were coded for the underlying causes of death according to the International Classification of Diseases (ICD-9 and ICD 10).

The following sources were used to validate incidental cases of MI:

First, we used data from the KORA Myocardial Infarction Registry in the Augsburg region of Germany to identify individuals with a first MI. The case definition for both non-fatal and fatal events (coronary death or fatal MI) followed the criteria of the WHO MONICA protocol (29, 30). Additional non-fatal events have been diagnosed according to the European Society of Cardiology and American College of Cardiology criteria since 2001 (31, 32) . All cases occurring in individuals aged 25–84 years (25–74 years until 2009) with residence and event within the study region were validated through this registry (33).

Second, all self-reported non-fatal MIs obtained from postal follow-up questionnaires or face-to-face interviews that were not covered by the KORA Myocardial Infarction Registry were additionally validated using hospital discharge letters or information provided by the participants' treating physicians. Third, all fatal cases (coronary death or fatal MI) not included in the KORA MI registry population were validated based on coded death certificates.

Incidental strokes were validated analogously to MI, as described above under second and third. Stroke-related deaths were classified using the three-digit codes 433, 434, and 435 from the German modification of the International Classification of Diseases, Ninth Revision (ICD-9).

Statistical analysis

Continuous data are presented as mean ± SD for normally disturbed variables and median (IQR) for non-normally distributed variables. Largely skewed variables were log-transformed prior to further analysis. Independent-sample t-test or Mann-Whitney U-test for continuous variables, and the chi-squared test for categorical variables were used to perform sex-stratified analysis for baseline characteristics.

Multivariable linear regression models were used to investigate the association of sex with each of androgens/ SHBG (exposure-mediator). Multivariable Cox proportional hazards regression models were used to assess the association of sex

with the risk of stroke and MI (exposure–outcome) and of androgens/SHBG with the risk of these outcomes (mediator–outcome). All analyses were adjusted for age (model 1) and additionally, for physical activity, alcohol consumption, and smoking (model 2). These two models were selected based on variables defined as potential confounders derived from previous literature and were considered as main models in the mediation analysis as well. We then performed mediation analyses to investigate whether circulating androgens (TT, DHT, DHEA, and DHEAs) or SHBG mediate the association between sex (women vs. men) and the incidence of stroke/ MI. The hypothesized causal pathway is illustrated in **Supplementary Figure 1**.

The regression-based approach in a counterfactual framework was used to assess the direct effect (DE), indirect effect (IE), total effect (TE) and proportion mediated (PM) (34). Non-parametric bootstrapping (200 times) was used to estimate 95% confidence intervals (CI) and P values. Given the absence of significant interaction between sex and the mediators, a two-way decomposition model was applied. Cox proportional hazards regression models were used to estimate hazard ratios (HRs) and 95% CIs for the effects. The DE represents the effect of the exposure (sex) on the outcome (risk of stroke/ MI) at a specific level of the mediator variable (sex hormones/ SHBG), differing from the TE, which signifies the overall effect of the exposure (sex) on the outcome (risk of stroke/ MI). The IE represents the effect on the outcome (risk of stroke/ MI) due to variations in the exposure (sex) resulting from different levels of the mediator (TT/ DHT/ DHEA/ DHEAs and SHBG). It is important to note that DE and IE should operate in the same direction for the PM to yield a meaningful result (35). The mediation models adjusted for relevant confounders including age, smoking status, alcohol consumption and physical activity. The mediator was modelled continuously, and the outcome was time to incident stroke/ MI, accounting for censoring. We applied non-parametric bootstrapping with 200 resamples to obtain robust estimates and 95% CIs for DE, IE, and TE. Missing data on covariates were addressed using multiple imputation.

Additionally, to test the robustness of our findings, we performed a sensitivity analysis. We defined another model with additional adjustments for WC, systolic blood pressure (SBP), antihypertensive medications, lipid lowering medications, total cholesterol, CRP, TSH and baseline diabetes (model 3). We did not consider model 3 as one of our primary models because the covariates in this model are more likely to serve as intermediaries in the pathways of the investigated associations rather than as confounders. Therefore, the findings from the third model should be interpreted with caution. As noted by VanderWeele (36), the confounding assumptions in mediation analysis are critical, and violations of these assumptions

can lead to misleading results. All statistical analyses were performed in R version 4.2.2 using the CMAverse package (37). All results were considered statistically significant at a p value < 0.05.

Results

Baseline characteristics of the whole population and the stratified analysis based on sex are provided in **Table 1**. Overall, 1970 individuals (1140 men, 830 women) with a mean age of 54.7 ± 13 and mean BMI of 27.4 ± 4.6 kg/m^2 were included. The median (IQR) of TT, DHT DHEA, DHEAs and SHBG were 14.8 (11.5, 18.8), 1.3 (0.90, 1.70), 9.2 (5.7, 14.7), 3330.0 (1832.1, 5309.0), 47.9 (34.7, 65.3) nmol/l for men and 0.7 (0.5, 1.0), 0.2 (0.1, 0.4), 9.6 (6.3, 14.9), 2239.4 (1240.1, 3461.1), 75.4 (53.8, 100.4) nmol/L for women. No significant differences were observed for prevalent diabetes, TSH, CRP, total cholesterol, lipid lowering medications and physical activity levels between men and women. Alcohol consumption, the proportion of smokers, and SBP levels were higher in men than in women. During a median follow-up of 8.6 years, 71 strokes occurred over 16,332 person-years (47 in men and 24 in women) and 63 MIs occurred over 16,626 person-years (54 in men and 9 in women).

Sex, androgens, SHBG and risk of stroke/ MI

In line with our previous publications (38, 39), **Supplementary Table 1** presents the association of sex (women vs. men) with sex hormones and SHBG. TT, DHT and DHEAs levels were significantly lower in women than in men, while no significant sex differences were observed for SHBG and DHEA in both models 1 and 2. The associations of sex hormones and SHBG with risk of stroke among male and female participants are presented in **Supplementary Table 2**. An inverse association was found between TT and DHT with risk of stroke in both models. No significant association was observed for SHBG, DHEA or DHEAs with risk of stroke in men. No significant associations were observed for sex hormones and SHBG with risk of stroke in women. No sex differences were observed for risk of stroke (**Supplementary Table 3**).

The association of sex hormones and SHBG with risk of MI among male and female participants are presented is **Supplementary Table 4**. No significant associations were observed for either SHBG or sex hormones with risk of MI in both men and women, while sex differences were observed for risk of MI (**Supplementary Table 5**).

Table 1. Baseline characteristics of study participants of KORA F4 in the overall population and stratified by sex.

Variable	Overall (n=1970)	Male (n=1140)	Female (n=830)	p-value
Age (years)	54.7 ± 13.0	55.5 ± 13.0	53.6 ± 12.8	0.001
Weight (kg)	79.6 ± 15.5	85.8 ± 13.8	71.3 ± 13.9	<0.001
BMI (kg/m^2)	27.4 ± 4.6	27.7 ± 4.1	26.9 ± 5.2	<0.001
WC (cm)	93.5 (84.7, 102.5)	97.8 (91.2, 105.7)	85.1 (77.0, 94.9)	<0.001
Physically active, n (%)	1111 (56.4)	639 (56.1)	472 (56.9)	0.787
Smoking status, n (%)				<0.001
Regular smoker	320 (16.3)	204 (17.9)	116 (14.0)	
Irregular smoker	53 (2.7)	29 (2.5)	24 (2.9)	
Ex-smoker	812 (41.3)	553 (48.6)	259 (31.2)	
Never-smoker	782 (39.8)	352 (30.9)	430 (51.9)	
Alcohol consumption (gr/day)	16.1 (20.7)	22.0 (23.7)	8.1 (11.6)	<0.001
TT (nmol/L)	9.9 (0.8, 15.6)	14.8 (11.5, 18.8)	0.7 (0.5, 1.0)	<0.001
DHT (nmol/L)	0.8 (0.3, 1.4)	1.3 (0.9, 1.7)	0.2 (0.1, 0.4)	<0.001
DHEA (nmol/L)	9.4 (6.0, 14.8)	9.2 (5.7, 14.7)	9.6 (6.3, 14.9)	0.256
DHEAs (nmol/L)	2734.9 (1522.5, 4565.6)	3330.0 (1832.1, 5309.0)	2239.4 (1240.1, 3461.1)	<0.001
SHBG (nmol/L)	57.9 (39.7, 81.0)	47.9 (34.7, 65.3)	75.4 (53.8, 100.4)	<0.001
Without prevalent diabetes, n (%)	1438 (73.0)	800 (70.2)	638 (76.9)	0.001
Lipid lowering medications, n (%)	190 (9.6)	118 (10.4)	72 (8.7)	0.1
Antihypertensive medications, n (%)	519 (26.3)	320 (28.1)	199 (24.0%)	0.047
Total cholesterol (mg/dL)	214.0 (188.0, 239.0)	214.0 (189.0, 238.0)	214.0 (187.2, 241.0)	0.617
SBP (mmHg)	121.0 (110.0, 133.5)	125.5 (116.1, 137.0)	112.5 (102.5, 125.0)	<0.001
TSH (mIU/L)	1.3 (0.8, 1.9)	1.3 (0.9, 1.9)	1.3 (0.8, 1.9)	0.277
C-reactive protein (mg/L)	1.0 (0.5, 2.2)	1.0 (0.5, 2.3)	1.0 (0.5, 2.2)	0.281

Data are presented as mean ± SD for normally distributed continuous variables, median (IQR) for non-normally distributed variables or numbers (percentage) for categorical variables. P-values were generated by independent-sample t-test or Mann-Whitney U-test for continuous variables and chi-square test for categorical variables. P-values < 0.05 are shown in bold.

Abbreviations: BMI, body mass index; WC, waist circumference; TT, total testosterone; DHT, dihydrotestosterone; DHEA, dehydroepiandrosterone; DHEAs, dehydroepiandrosterone-sulfate; SHBG, sex hormone-binding globulin; SBP, systolic blood pressure; TSH, thyroid stimulating hormone.

Mediation analysis

Based on the above-mentioned results, mediation analyses were performed for TT and DHT to assess whether, and to what extent, the sex differences (women vs. men) in risk of stroke mediated by TT or DHT. The results of the mediation analysis of TT and DHT on the association between sex and risk of stroke are shown in **Table 2**. The mediation analysis showed an inconsistent mediatory role of TT (opposing DE and IE) on the association between sex and risk of stroke, highlighting the complex role of TT, suggesting that TT's effects are not uniform among men and women and can lead to differing outcomes in metabolic health across sexes. The DE was HR: 0.31 (95% CI: 0.13, 0.61) and 0.42 (95% CI: 0.18, 0.86), while the IE was 2.62 (95% CI:1.53, 5.50) and 2.48 (95% CI:1.27, 6.17) in model 1 and 2, respectively. To understand the decomposition of the effect of sex on stroke risk through TT, the forest plot of the TE, DE, and IE is provided in **Supplementary Figure 2, panel A**, indicating that DE and IE act in opposite directions (inconsistent mediation). The mediation analysis on DHT on the association between sex and risk of stroke showed the same pattern (**Table 2**). The DE was HR: 0.30 (95% CI: 0.14, 0.56) and 0.40 (95% CI: 0.17, 0.82), while the IE was 2.87 (95% CI:1.76, 5.08) and 2.90 (95% CI:1.67, 6.35) in model 1 and 2, respectively. The forest plot of the decomposition of the effect of sex on stroke risk through DHT is provided in **Supplementary Figure 2, panel B.** The findings of the sensitivity analysis (model 3) were comparable to the main model findings, the DE were HR: 0.59 (95% CI:0.22, 1.50) and 0.50 (95% CI:0.20, 0.97) and the IE were 2.02 (95% CI:1.05, 4.73) and 2.50 (95% CI:1.50, 5.85) for TT and DHT, respectively (**Supplementary Table 6**).

Table 2. Mediation analysis of total testosterone and dihydrotestosterone on the association between sex (women vs men (reference)) and risk of stroke.

Effects	HR (95% CI) model 1	p-value	HR (95% CI) model 2	p-value
TT				
DE	0.31 (0.13, 0.61)	P<0.001	0.42 (0.18, 0.86)	0.03
IE	2.62 (1.53, 5.50)	P<0.001	2.48 (1.27, 6.17)	0.01
TE	0.81 (0.50, 1.25)	0.36	1.06 (0.62, 1.75)	0.94
PM	-	-	-	-
DHT				
DE	0.30 (0.14, 0.56)	P<0.001	0.40 (0.17, 0.82)	0.01
IE	2.87 (1.76, 5.08)	P<0.001	2.90 (1.67, 6.35)	P<0.001
TE	0.85 (0.50, 1.40)	0.6	1.13 (0.61, 1.94)	0.68
PM	-	-	-	-

Model 1: adjusted for age.

Model 2: Model 1+ smoking+ alcohol consumption+ physical activity.

Abbreviations: HR, hazard ratio; TT, total testosterone; DHT, dihydrotestosterone; DE, direct effect; IE, indirect effect; TE, total effect; PM, proportion mediated.

Discussion

To our best knowledge, the current study is the first one investigating the mediatory role of androgens and SHBG on explaining sex differences for risk of stroke/ MI. While we found no association between TT and DHT with stroke risk in women, our mediation analysis findings revealed an inconsistent mediating role of TT and DHT in explaining sex differences in stroke risk, suggesting that TT and DHT impact stroke risk differently in men and women. Specifically, higher TT and DHT levels were associated with a lower risk of stroke in men, whereas in women, elevated TT and DHT levels may have adverse effects. No mediating role was observed for other androgens or SHBG in relation to stroke, and neither androgens nor SHBG mediated the risk of MI.

In line with our findings, results of a prospective cohort study including 3,690 community-dwelling men aged 70-89 with a mean follow-up of 6.6 years found that higher plasma TT or DHT is a biomarker for reduced risk of stroke, but not MI (16). Similar findings were reported in another analysis from a comparable cohort of older men (n= 3,443; mean follow-up 3.5 years), in which lower TT levels were associated with an increased incidence of stroke or transient ischemic attack (40). Another recent prospective cohort study using UK biobank data including 479,797 participants (55.1% women) with median follow-up of over 12.5 years also found no association between TT and risk of MI in either men or women (41). Additionally, in the population-based FINRISK97 study of 7,671 individuals with a median follow-up of 13.8 years, low testosterone levels were not found to be predictive of CHD in either men or women (42). Findings of another prospective cohort study on 1,032 older men with a median follow-up of 10 years indicated that DHT but not TT, is associated with risk of stroke (43). This is consistent with our results, as we observed stronger effect estimates for DHT.

We found that TT and DHT were associated with stroke risk in men, but not in women. While our study focused on the mediating role of sex hormones in sex differences in stroke risk, a recent mediation analysis of 6,175 adults investigated blood pressure as a potential mediator of the association between testosterone and stroke. That study reported that blood pressure partially mediated the testosterone–stroke association in men, but not in women (29), highlighting differences in mediation pathways compared with our approach (44). The results of a meta-analysis study accompanied by a large prospective cohort study including 4,615 adult men and 4,724 women with a follow-up of 29 years found that extremely low endogenous testosterone levels are associated with higher risk of ischemic

stroke in men, while no association was observed for women (45). They also found that the risk of ischemic stroke is mediated by BMI and hypertension (45). The results of our sensitivity analysis, which additionally adjusted for other potential confounders (model 3), were consistent with the main findings and indicated inconsistent mediation of TT and DHT. However, these results should be interpreted with caution, as this sensitivity analysis also adjusted for variables that may lie on the causal pathway from sex to the risk of stroke.

The sex-specific associations between testosterone and stroke risk may be driven by distinct physiological and pathophysiological mechanisms in men and women. In men, endogenous testosterone appears to have neuroprotective and vasoprotective effects that may lower the risk of stroke (46). Experimental studies suggest that testosterone promotes vasodilation by enhancing nitric oxide synthesis in cerebral arteries, which improves cerebral blood flow and reduces the risk of ischemic events (47). Additionally, testosterone has been shown to attenuate oxidative stress and exert anti-inflammatory effects in the central nervous system (48, 49), while also enhancing antioxidant defence mechanisms, increasing brain-derived neurotrophic factor (BDNF) levels, and promoting neuronal cell proliferation (50), factors that may collectively mitigate vascular damage and neuronal injury following an ischemic insult. In contrast, the role of testosterone in cardiovascular health of women is still debatable (51). Higher androgen levels in women have been associated with adverse cardiometabolic profiles, including increased insulin resistance, dyslipidemia, and hypertension, which are established risk factors for CVD (20). Research has indicated that low testosterone level might be protective for risk of atrial fibrillation or ischemic stroke in women (52). These sex-specific differences suggest that testosterone may influence stroke risk through distinct and potentially opposing pathways in men and women, which may help explain the contrasting associations observed in our study.

There are limited sex-specific MR studies on the causal role of sex hormones on risk of CVD. A large MR study investigated the association of genetic predictors of increased testosterone (variants in two gene regions (SHBG and JMJD1C) with cardiovascular risk factors and risk of CAD and ischemic stroke using CARDIoGRAMplusC4D consortium and UK biobank data. They found that none of these two gene variants were associated with risk of CAD in either men or women. However, one of these variants was associated with risk of ischemic stroke in men but not in women (53). On the contrary to our findings, the results a sex-specific MR study reported that genetically predicted high SHBG is causally associated with risk of ischemic stroke in both men and women (54). The results of a prospective

cohort study including 13,192 postmenopausal women with a median follow-up of 11.6 years found that SHBG levels are inversely associated with risk of ischemic stroke (55). Another large prospective cohort study using UK biobank data on 479,797 participants reported that higher SHBG are associated with a lower risk of MI in men but not women (41).

There is limited evidence on the association of DHEA or DHEA-S and risk of stroke/ MI in men or women and most of the evidence focuses on other forms of CVDs. A nested case-control study of women (461 cases and 461 controls) found that lower DHEA-S are associated with greater risk of ischemic stroke (56). Another nested-case control study using the Nurses' Health Study cohort found a positive association between plasma concentrations of DHEA and DHEA-S with the risk of subsequent MI among predominantly postmenopausal women (57). The findings of a meta-analysis of 14 case-control studies found that DHEA-S might be associated with CHD risk but not with arteriosclerosis (58). Additionally, no association was found between DHEA levels and the risk of CHD (58). Also, genetic studies in general show no causal impact of DHEA on CVD or major risk factors such as diabetes, suggesting that DHEA could be a marker rather than a causal factor.

Our study has several notable strengths. While previous research has examined the associations of sex, androgens, and SHBG with the risk of stroke/ MI independently, our use of mediation analysis allowed us to investigate the more complex interrelationships between sex, sex hormones, and risk of stroke/ MI in combination. Consistent with prior findings from the KORA study on sex hormones (38, 39, 59), our study also benefits from rich data on menopausal surgeries (e.g., oophorectomy and hysterectomy) and a wide range of hormonal treatments, including anti-estrogens, estrogens, progestins, hormone replacement therapy, androgens, anti-androgens, enzyme inhibitors, gonadotropin-releasing hormones, and anabolic steroids. This extensive information enabled us to tailor our eligibility criteria to align with our research question. However, due to its observational design, our study cannot establish causal relationships.

The time-to-event analysis enabled us to appropriately account for censored observations using Cox regression, which was further integrated into the mediation analysis to provide more robust evidence. This study has several limitations that should be acknowledged. The overall sample size and relatively low number of events may have limited the statistical power of our analyses, particularly for mediation effects, potentially leading to underestimation or non-detection of some associations. In addition, the sample size constrained our ability to perform further

sensitivity analyses across different menopausal status groups, and we were unable to stratify analyses by stroke subtype to explore the potential mediating role of TT and DHT in relation to other types of strokes. Finally, as the majority of participants in our study were of European descent, the generalizability of our findings to other ethnic groups may be limited. Future studies with larger sample sizes and a higher number of events are warranted to provide more robust and precise estimates and to confirm the potential mediating role of sex hormones and SHBG in explaining sex differences in cardiovascular disease.

Our findings may provide complementary insights to the recently published TRAVERSE trial, which enrolled 5,246 men aged 45 to 80 years with preexisting or high cardiovascular risk and hypogonadal symptoms (60). The primary cardiovascular safety endpoint was the first occurrence of death from cardiovascular causes, nonfatal MI, or nonfatal stroke. TRAVERSE demonstrated that testosterone-replacement therapy was noninferior to placebo for this composite endpoint (60), providing reassurance regarding the overall cardiovascular safety of testosterone therapy in men.

Our mediation findings mirror earlier evidence showing that TT, DHT influences glucose homeostasis and T2D risk in a sex-specific manner but are established here within a different statistical framework that enables a direct comparison between women and men. Whether these differences are due to different biological effects or different levels of hormones in men and women needs further exploration. These findings highlight the need to address such discrepancies, for example, through the application of sex-stratified genetic instruments and analyses in future MR studies. Critically, current genome-wide association studies (GWAS) of sex hormones are limited by their predominant use of mixed-sex cohorts, which mask sex-specific signals and yield limited power in female subgroups (61). Recent genetic studies have confirmed these limitations, showing that the overlap between variants associated with sex hormone traits in men and women is minimal, except for SHBG, and that even shared loci often exert divergent effects across sexes (62). This low cross-sex replication rate not only undermines the reliability of genetic instruments for causal inference but also perpetuates a male-biased understanding of hormone-metabolism interactions, as evidenced by underpowered female strata in landmark studies (62).

In addition, we observed inconsistent mediating roles of TT and DHT in relation to stroke risk, but not MI risk. These findings indicate that endogenous sex hormones may have disease-specific effects rather than acting as general mediators across all CVD outcomes. Moreover, the role of specific hormones may differ by sex; for

example, TT and DHT may exert divergent or even opposite effects on stroke risk. Taken together, these findings emphasize the importance of sex-specific MR studies of endogenous sex hormones to clarify their causal and potentially divergent roles in cardiometabolic disease, both overall and in relation to specific condition.

Finally, a better understanding of the sex-specific effects of endogenous hormones may have important clinical implications. If confirmed, these findings could inform the development of sex-specific prevention strategies or therapeutic targets for cardiovascular disease.

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Supplementary materials: Do androgens and sex hormone-binding globulin mediate sex differences in the risk of stroke and myocardial infarction? The KORA Study

Supplementary Table 1. Association of sex (women vs men [reference]) with sex hormones and SHBG among participants of KORA F4.

Variable	ß (95 % CI) Model 1	ß (95 % CI) Model 2
SHBG	0.45 (0.41, 0.49)	0.45 (0.40, 0.49)
Sex hormones		
TT (nmol/L)	-14.82 (-15.22, -14.42)	-14.87 (-15.30, -14,44)
DHT (nmol/L)	-1.15 (-1.22, -1.08)	-1.16 (-1.23, -1.09)
DHEA (nmol/L)	-0.22 (-0.89, 0.45)	-0.35 (-1.08, 0.36)
DHEA-S (nmol/L)	-1368.06 (-1539.69, -1196.42)	-1326.52 (-1509.85, -1143.20)

Model 1: Age.
Model 2: Model 1 + smoking + alcohol consumption + physical activity.
SHBG is log-transformed.
Abbreviations: SHBG, sex hormone-binding globulin; TT, total testosterone; DHT, dihydrotestosterone; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone-sulfate.

Supplementary Table 2. Associations of sex hormones and SHBG (per 1unit increase) with risk of stroke among male and female participants KORA F4 and FF4.

Variable	HR (95% CI) model 1	HR (95% CI) model 2
Male adults		
SHBG (nmol/L)	0.82 (0.41, 1.65)	0.83 (0.41, 1.68)
Sex hormones		
TT (nmol/L)	0.93 (0.88, 0.98)	0.94 (0.88, 0.99)
DHT (nmol/L)	0.40 (0.23, 0.69)	0.39 (0.22, 0.68)
DHEA (nmol/L)	0.96 (0.89, 1.04)	0.96 (0.89, 1.04)
DHEA-S (nmol/L)	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)
Female adults		
SHBG (nmol/L)	1.21 (0.52, 2.82)	1.25 (0.55, 2.93)
Sex hormones		
Testosterone (nmol/L)	1.17 (0.71, 1.92)	1.11 (0.65, 1.88)
DHT (nmol/L)	0.26 (0.01, 4.31)	0.32 (0.02, 5.02)
DHEA (nmol/L)	0.93 (0.84, 1.03)	0.93 (0.84, 1.03)
DHEA-S (nmol/L)	1.00 (1.00, 1.00)	0.95 (0.89, 1.01)

Model 1: Age.
Model 2: Model 1 + smoking + alcohol consumption + physical activity.
SHBG is log-transformed.
Abbreviations: HR, hazard ratio; SHBG, sex hormone-binding globulin; TT, total testosterone; DHT, dihydrotestosterone; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone-sulfate.

Supplementary Table 3. Association of sex (women vs men [reference]) with risk of stroke between KORA F4 and FF4.

Variable	HR (95% CI) model 1	HR (95% CI) model 2
Sex (women vs men [reference])	0.76 (0.47, 1.25)	1.01 (0.58, 1.76)

Model 1: Age.
Model 2: Model 1+ smoking+ alcohol consumption+ physical activity.
Abbreviations: HR, hazard ratio.

Supplementary Table 4. Associations of sex hormones and SHBG (per 1 unit increase) with risk of MI among male and female participants KORA F4 and FF4.

Variable	HR (95% CI) model 1	HR (95% CI) model 2
Male adults		
SHBG (nmol/L)	0.91 (0.48, 1.73)	0.83 (0.43, 1.60)
Sex hormones		
TT (nmol/L)	0.99 (0.95, 1.04)	0.99 (0.95, 1.04)
DHT (nmol/L)	0.97 (0.72, 1.31)	0.98 (0.72, 1.35)
DHEA (nmol/L)	0.97 (0.92, 1.03)	0.97 (0.92, 1.03)
DHEA-S (nmol/L)	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)
Female adults		
SHBG (nmol/L)	0.33 (0.09, 1.20)	0.31 (0.08, 1.21)
Sex hormones		
Testosterone (nmol/L)	0.47 (0.08, 2.59)	0.52 (0.09, 3.02)
DHT (nmol/L)	1.35 (0.04, 38.3)	1.40 (0.06, 32.4)
DHEA (nmol/L)	1.00 (0.87, 1.15)	1.00 (0.87, 1.15)
DHEA-S (nmol/L)	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)

Model 1: Age.
Model 2: Model 1 + smoking + alcohol consumption + physical activity.
SHBG is log-transformed.
Abbreviations: MI, myocardial infarction; HR, hazard ratio; SHBG, sex hormone-binding globulin; TT, total testosterone; DHT, dihydrotestosterone; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone-sulfate.

Supplementary Table 5. Association of sex (women vs men [reference]) with risk of MI between KORA F4 and FF4.

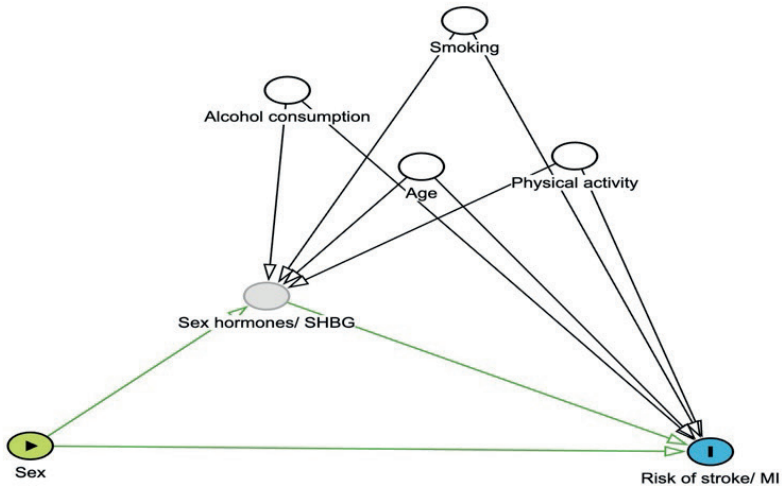
Variable	HR (95% CI) model 1	HR (95% CI) model 2
Sex (women vs men [reference])	0.24 (0.12, 0.50)	0.22 (0.10, 0.46)

Model 1: Age.
Model 2: Model 1+ smoking+ alcohol consumption+ physical activity.
Abbreviations: HR, hazard ratio.

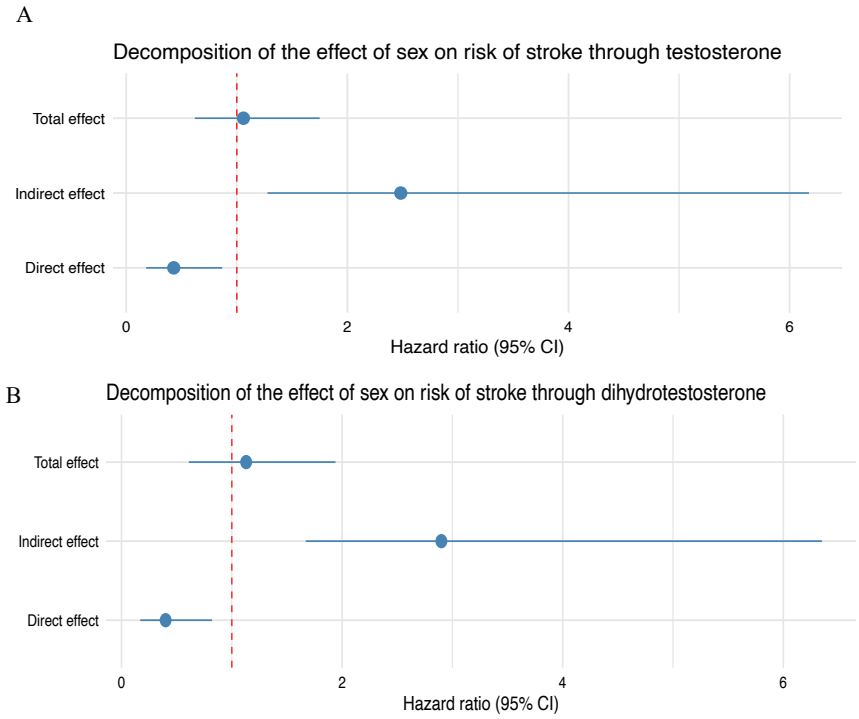
Supplementary Table 6. Mediation analysis of total testosterone and dihydrotestosterone on the association between (women vs men [reference]) and risk of stroke between KORA F4 and FF4 (model 3).

Effects	HR (95% CI) model 3	p-value
TT		
DE	0.59 (0.22, 1.50)	0.24
IE	2.02 (1.05, 4.73)	0.04
TE	1.19 (0.59, 2.11)	0.58
PM	-	-
DHT		
DE	0.50 (0.20, 0.97)	0.05
IE	2.50 (1.50, 5.85)	P<0.001
TE	1.26 (0.70, 2.21)	0.48
PM	-	-

Model 3: Model 2+ waist circumference + SBP + antihypertensive medications + lipid lowering medications + total cholesterol + CRP + TSH and baseline diabetes.
Abbreviations: HR, hazard ratio; SBP, systolic blood pressure; CRP, C-reactive protein; TSH, thyroid stimulating hormone; TT, total testosterone; DHT, dihydrotestosterone; DE, direct effect; IE, indirect effect; TE, total effect; PM, proportion mediated.



Supplementary Figure 1. Directed acyclic graph (DAG) for the association of sex through, sex hormones (total testosterone, dihydrotestosterone, dehydroepiandrosterone, dehydroepiandrosterone-sulfate) or SHBG on risk of stroke/ myocardial infarction. Study DAG for model 2, exposure = sex, mediator = sex hormones/ SHBG, outcome = stroke/ myocardial infarction, adjustment variables = age + alcohol consumption + smoking + physical activity. Abbreviations: SHBG, sex hormone-binding globulin.



Supplementary Figure 2. Forest plot showing the decomposition of the effect of sex on the risk of stroke into total, direct, and indirect effects.

Total Effect: Overall effect of sex on stroke risk, including both direct and mediator-driven pathways. Direct Effect: Effect of sex on stroke risk not explained by the mediators.
Indirect Effect: Effect of sex on stroke risk that is mediated through sex hormones**.**
Panel A: total testosterone (TT).
Panel B: dihydrotestosterone (DHT).
Model adjustments: age+ smoking+ alcohol consumption+ physical activity. Effect estimates are shown as hazard ratio with 95% confidence intervals.



Chapter 6

Endogenous sex steroid hormones, sex hormone-binding globulin, and risk of all-cause and cause-specific mortality: A systematic review and dose-response meta-analysis of prospective cohort studies

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Abstract

Background: While abundant research suggests a sex-specific role of endogenous sex steroid hormones in chronic diseases, research on mortality remains inconclusive. We quantified the sex-specific associations of endogenous sex steroid hormones including total testosterone (TT), free testosterone, bioavailable testosterone, estradiol (E2), dehydroepiandrosterone, and dehydroepiandrosterone-sulphate and sex hormone binding globulin (SHBG) with risk of all-cause and cause-specific mortality in the general population.

Methods: Embase, Medline, Web of Science, and Cochrane Central were searched and population-based cohort studies investigating the association of interested were included. The risk of bias was assessed using the ROBINS-E tool. The certainty of evidence was evaluated using GRADE framework. Pooled hazard ratios (HRs) and 95% confidence intervals (CI) were calculated using a random effects model for the top versus bottom tertile of sex hormones and risk of mortality.

Results: 53 publications with 359,047 participants were included in the systematic review. A significant association was observed between higher level of TT and risk of all-cause mortality (HR (95%CI): 0.89 (0.83 to 0.97), n=19 studies) in men, while no association was found in women. Dose-response analysis suggested a significant U-shaped association between TT and all-cause mortality in men and a J-shaped association in women. Higher level of SHBG was significantly associated with higher risk of all-cause mortality in women (1.25 (1.13 to 1.39), n=3) and no association was observed in men. Additionally, higher DHEAs levels were associated with lower risk of all-cause mortality in men (0.72 (0.57 to 0.91), n=6) and no association was observed in women.

Conclusions: This meta-analysis reveals a dose-response link between endogenous sex steroid hormones and mortality, highlighting the need for sex-specific studies on hormone modulation's impact on mortality and longevity.

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PROSPERO registration code: CRD42022329605

Keywords: Androgens, Sex differences; Testosterone; SHBG; Estradiol; Death.

Introduction

Abundant evidence suggests a role of endogenous sex steroid hormones in development of chronic diseases and the associated risk factors, which might be sex-specific (1). For instance, hypoandrogenism in men and hyperandrogenic conditions in women have been linked to obesity, insulin resistance, and incidence of type 2 diabetes and cardiovascular disease (CVD) (2-8).

While endogenous sex steroid hormones have implications for chronic diseases, findings on mortality are inconsistent. Several studies have shown the association between lower levels of total testosterone (TT) and higher risk of either all-cause or cause-specific mortality in men (9-11) while in women studies are indicative of either no association (12, 13) or higher risk of all-cause mortality (14). While majority of studies showed no association between sex hormone binding globulin (SHBG) and risk of all-cause and cause-specific mortality (10, 15-17), findings of a large population-based study reported a higher risk of all-cause mortality in higher level of SHBG in both men and women (14) and cancer and CVD mortality in men. The association of Estradiol (E2) and risk of mortality has been majorly studied in men which the findings are controversial particularly for all-cause and cancer mortality (10, 11). Some studies have found an association between E2 and risk of all-cause (11) and cause-specific mortality (18), others found no significant association with mortality risk (19). Similar controversy has been explored also with menopausal therapy, with the largest clinical trial showing no impact on mortality (20).

Additionally, some studies reported protective role of dehydroepiandrosterone sulfate (DHEAs) against all-cause or CVD mortality in men (21, 22) and women (23), on the contrary other studies found no association either in men (23, 24) or in women (25, 26).

Although a few meta-analyses have been performed on the association of endogenous sex steroid hormones and risk of mortality; they only included studies on men, examined a single endogenous sex steroid hormone (mainly TT), and investigated the association only with all-cause mortality (27-29). Furthermore, none of these studies investigated the shape of association.

Therefore, we systematically summarized and quantified the available evidence on the sex-specific associations of endogenous sex steroid hormones including TT, bioavailable testosterone (BT), free testosterone (FT), DHEAs, E2 and dehydroepiandrosterone

(DHEA), as well as SHBG with risk of all-cause and cause-specific mortality, including CVD, cancer, and other causes in the general population.

Methods

This systematic review was designed and conducted in accordance with two recent systematic review guidelines and is reported following the PRISMA recommendations (30-32). The study protocol was registered in the PROSPERO database in May 2022 (registration number: CRD42022329605).

Data Sources and Search Strategy

We combined endogenous sex steroid hormones and mortality-related keywords to find potentially relevant studies. Embase.com, Medline ALL (Ovid), Web of Science Core Collection, and Cochrane Central were searched until March 21, 2024. Furthermore, filters to exclude conference abstracts, case-report studies, non-adult populations, and animal studies were applied. The search strategy was developed by an expert research librarian (BM). The search results were imported in EndNote and de-duplicated with the method as described by Bramer et al (33). The details of the search strategy and keywords are presented in **Table S1** (34). To complete our search, the references of the included studies and published reviews were manually reviewed.

Eligibility Criteria

We selected original published studies with aggregate data if (i) they had a prospective design (case-cohort, nested-case control and cohort studies); (ii) were conducted in a general adult population (≥18 years); (iii) measured at least one of the following endogenous sex steroid hormones as exposure at baseline: TT, BT, FT, DHEA, DHEAs, and E2 or SHBG; (iv) reported associations with all-cause or cause-specific mortality: CVD, cancer, and other causes; and (v) reported associations as adjusted effect estimates (relative risk, hazard ratio, or odds ratio) with 95% confidence interval (95%CI). Among the reported models, we included the one with the most adjustments. For publications reporting results from the same study and the same exposure and outcome, those with the largest number of participants, or the most recent publication, were included in the analysis; in case they had different publications on non-overlapping sex hormones and risk of mortality, we included the additional endogenous sex steroid hormones from the other publications conducted on the identical cohort.

Retrospective studies, conference abstracts, ecological studies, case reports, case-series, letters to the editor, conference proceedings, narrative reviews, systematic reviews or meta-analyses as well as studies conducted in animals, children, or adolescents were excluded. Also, studies conducted solely on patients were excluded. We did not include non-English language articles.

Study Selection and data extraction

All titles/abstracts were screened in duplicate by two independent groups of researchers (HRD, MA and SB together with HQ, FA and FKH) according to the eligibility criteria. Afterwards, all selected full-texts were reviewed again in duplicate (by HRD, MA and SB). The data from the included studies were extracted based on a predesigned form (by HRD, MA and SB) and both steps were conducted with EndNote 20. We extracted the first author's name, study design, study name, publication year, location, number of participants, sex distribution of the population, age, follow-up duration, level of sex steroid hormones, sex steroid hormones assessment method, number of deaths, causes of mortality, adjustments, and hazard/ risk/odds ratios.

Risk of bias assessment and certainty of evidence

Risk of bias assessments were conducted in duplicate by independent reviewers (HRD, MA and SB) and disagreements were resolved by consensus. We evaluated the risk of bias of the cohort studies with the recent version of the ROBINS-E tool (Risk Of Bias In Non-randomized Studies - of Exposures) (35). The certainty of evidence was assessed for all associations with the updated Grading of Recommendations Assessment, Development, and Evaluation (GRADE) tool by investigators independently (HRD, MA and SB) (36). More information on these tools is provided in **Supplementary information 1** (34).

Data analysis

We used the DerSimonian-Laird random effects model to calculate pooled hazard ratios (HR) and 95% confidence intervals (CI) (37). For analyses with a small number of studies (e.g., ≤5), we applied the Hartung-Knapp method for random effects to calculate pooled HR and 95% CI (38). Relative risk was treated as HR and for studies reporting odds ratios, only if the OR was between 0.5 and 2.5, the values were included and treated as HR (39). We extracted the risk estimates of the most adjusted model and stratified by sex. To enable a consistent and standardized approach in the meta-analysis, all risk estimates for the association between endogenous sex steroid hormones and mortality were transformed to the top versus bottom third of sex hormones distribution in each study (40). Based

on this method, we need to use conversion factors driving based on the ratio of expected differences in mean levels of the standardized exposure, for the target comparison versus reported comparison. Examples of the risk conversion methods are described in **Supplementary information 2** (34). For studies that used other categories than the first one as the reference category, we changed the reference category to the lowest one and recalculated the risk estimates using the method developed by Hamling (41). We calculated standard errors (SE) using a method developed by Greenland (42) for studies that did not report CI or SE. Heterogeneity between studies was assessed using the I^2 (43). We conducted subgroup analyses for each exposure and specific outcomes when 10 or more studies were included in the meta-analysis (44). Subgroup analyses were performed by: follow-up duration (<10 or ≥ 10 years), geographical region (Australia, Europe, North America), and whether studies adjusted for some risk factors such as alcohol consumption, lipid markers, blood pressure, physical activity, history of hypertension, T2D, and hyperlipidemia. Sensitivity analyses were also performed to explore if the results were robust using leave-one-out analysis, excluding each study at a time from the analysis. Publication bias was assessed when at least 10 studies were available with Egger's test and by visually exploring funnel plots for asymmetry (45, 46). For the linear dose-response meta-analyses, we estimated summary HRs and 95% CI for each prospective cohort study according to the method developed by Crippa et al (47). Additionally, one stage weighted mixed effects meta-analysis was used to explore curvilinear dose-response associations (47). Further information on these analyses is explained in the supplements.

We performed the statistical analyses of our meta-analysis in R (Version 4.0.5) using meta, dmetar, and dosresmeta packages and Stata (Version 16.0). P <0.05 was considered significant.

Results

Eligible studies

Among 11,193 references, 230 full-text articles were assessed for eligibility. Of those, 177 references were excluded, leaving 53 publications, based on 34 unique studies, to be included in the qualitative analysis. Due to insufficient data to perform risk conversions (48-55) or an OR lower than 0.5 (56), 9 of these were not qualified to be part of the quantitative synthesis. Eventually, 44 publications, based on 28 studies, were included in the meta-analysis. **Figure-1** represents the study selection procedure.

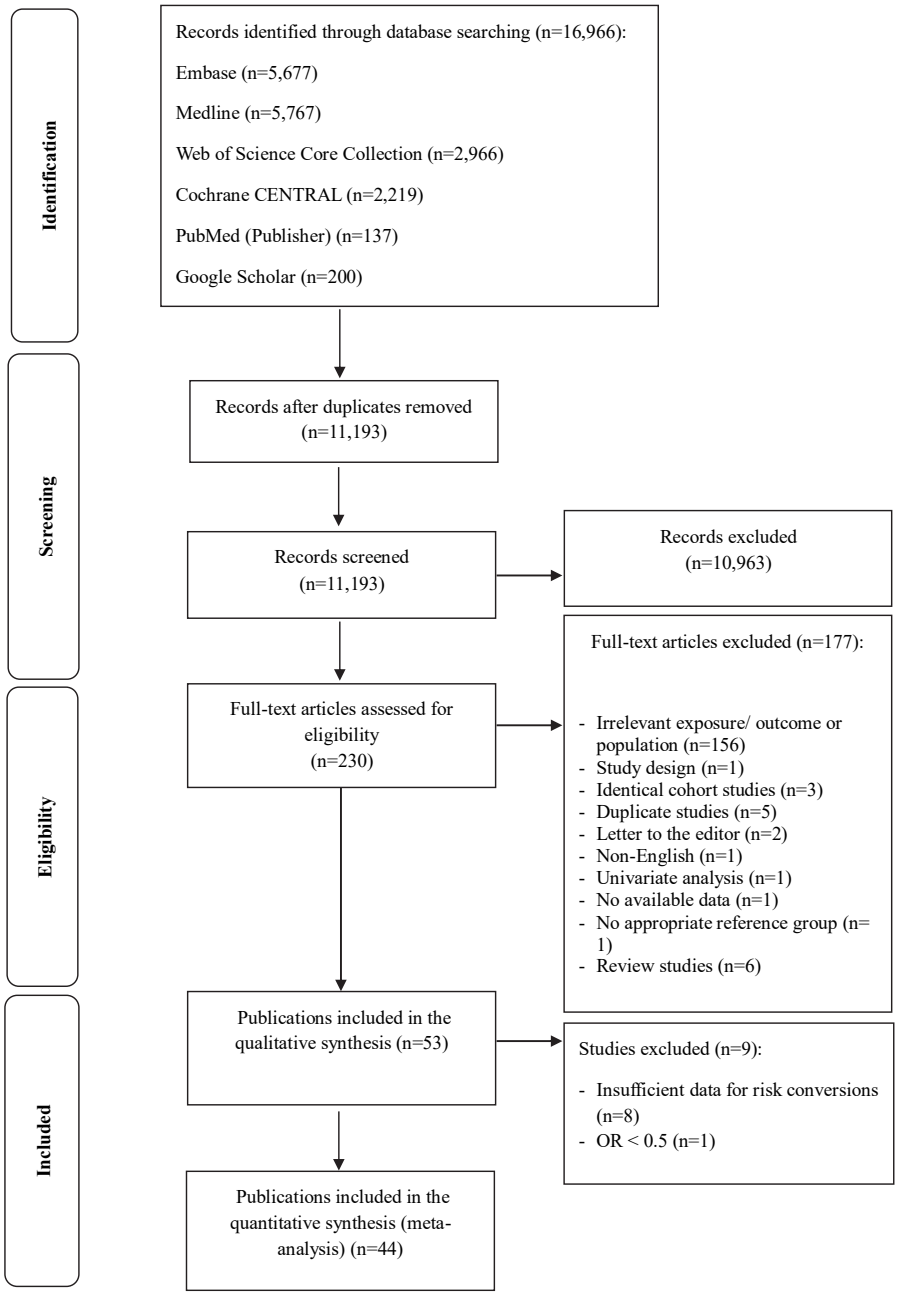


Figure 1. Study selection procedure.

Study characteristics

Characteristics of the included publications are presented in more detail in **Table 1**. Briefly, the publication date varied between 1986 and 2024. Overall, there were 359,047 participants included and 28,212 all-cause mortality events, 7,512 CVD mortality, 6,895 cancer mortality, and 2,550 death events due to the other causes. Among these studies, the median of follow-up duration was 10 years with an interquartile range of 7.4 and 14.9 years. The Majority of the studies was conducted in European countries (n=28) followed by North American countries (n=17), Australia (n=6), and Asia (n=2). All except 3 studies (57-59) reported results stratified by sex. The assessment methods for hormones and SHBG reported in each study are summarized in **Table S2** (34). Based on the ROBINS-E tool, 11 publications (20.7%) rated as low risk of bias, 16 (30.2%) as having some concerns, and 26 (49.05%) were judged to be at high risk of bias (**Table S3**) (34).

Table 1. Characteristics of the included studies.

Study characteristics					Participants' characteristics			Exposure and outcomes					
									No. of death				
Author, year	**Country**	**Study name**	**Design**	**Follow-up[1]**	**Sex**	**Age[2]**	**No. of Participants**	**Sex hormones**	**All-cause**	**CVD**	**Cancer**	**Other**	**Adjustments**
Araujo et al, 2007 (17)	US	Massachusetts Male Aging Study	Prospective cohort	15.3	M	40-70	1686	TT, FT, SHBG	395	101	127	-	Age, BMI, WC, HDL, SBP, race (white vs other), alcohol consumption, calories expended in PA, ever smoking, self-assessed health (excellent, very good, good, or fair/poor), self-reported chronic disease (heart disease, HTN, or DM).
Antonio et al, 2022 (60)	Multiple countries*	European Male Aging Study	Prospective cohort	12.6	M	59.4 ±10.8	1788	SHBG, E2	420	-	-	-	Study Centre, BMI, alcohol intake, smoking status, PA, number of comorbidities at baseline
Appiah et al, 2022 (18)	USA	NHANES (1988–1991)	Prospective cohort	25.2	M	35.7 ± 11.6	954	E2	40	-	-	-	age. Race, ethnicity, and education. BMI, smoking status, SBP, alcohol intake, PA, HDL, TC. CRP. TT
Barrett-Connor et al, 1995 (26)	US	Rancho Bernardo Study	Prospective cohort	19	F	65.5	942	DHEAs	-	192	-	-	Age, cholesterol, BP, smoking, estrogen replacement therapy, obesity, FPG, family history of heart disease
Barrett-Connor et al, 1986 (22)	US	Rancho Bernardo Study	Prospective cohort	12	M	50-79	208	DHEAs	65	38	-	-	Age, obesity, SBP, TC, FPG, current smoking status.
Baylis et al, 2012 (59)	UK	Hertfordshire Ageing Study	Prospective cohort	10	B	65-70	254	TT, SHBG, DHEAs	NM	-	-	-	Age, gender, baseline height, weight for height, smoking, alcohol, social class and waking speed, baseline number of systems medicated as a marker of comorbidity.
Belladelli et al, 2021 (19)	US	NHANES (1999-2004)	Prospective cohort	9.5	M	43.1 ± 20.78	1109	E2	237	44	63	-	Age, race, education, BMI, smoking status, alcohol use, comorbidities.
Benn et al, 2015 (52)	Denmark	Copenhagen City Heart Study	Nested case-control**	19	F	59 (49-65)	4716 (TT), 4600 (E2)	TT, E2	2716	659	531	-	Age, BMI, TC, HDL, menopausal status, age at menopause, HTN, DM, current smoking, alcohol intake.

Table 1. Continued

Study characteristics					Participants' characteristics			Exposure and outcomes					
									No. of death				
Author, year	**Country**	**Study name**	**Design**	**Follow-up[1]**	**Sex**	**Age[2]**	**No. of Participants**	**Sex hormones**	**All-cause**	**CVD**	**Cancer**	**Other**	**Adjustments**
Cappola et al, 2009 (58)	US	Cardiovascular Health Study	Prospective cohort	17	B	72.85 ± 5.41	950	DHEAs	NM	-	-	-	Age, race, cardiovascular disease, pulmonary disease, DM, cancer, depression.
Chan et al, 2016 (61)	Australia	Busselton Health Study	Prospective cohort	14.9	M	50.3 ± 16.8	1804	TT, FT, DHEA, E2	319	141	-	-	Age, smoking, vigorous exercise, alcohol, BMI, DM, CVD history, COPD history, non-skin cancer history, SBP, HTN, lipids medication, cholesterol, HDL, TG, CRP, creatinine
Cummings-Vaughn et al, 2011 (62)	US	NM	Prospective cohort	9	M	78.89 ± 6.9	190	TT, BT	41	-	-	-	Age, comorbidities (arthritis, HTN, cardiopulmonary conditions, cerebrovascular disease, DM, cancer [malignant], and kidney disease), lower body functional limitations, physician visits.
Enomoto et al, 2008 (53)	Japan	Tanushimaru Study	Prospective cohort	27	M	51.1 ± 16.4	396	DHEAs	95	-	-	-	Age
Enomoto et al, 2008 (53)	Japan	Tanushimaru Study	Prospective cohort	27	F	50.4 ± 15.6	544	DHEAs	124	-	-	-	Age
Forti et al, 2012 (57)	Italy	Conselice Study of Brain Aging	Prospective cohort	8	B	74.19 ± 6.65	920	DHEAs	248	-	-	-	Age, sex, smoking, BMI, IHD, preexisting major diseases, disability, frailty, Geriatric Depression Scale, the Mini-Mental State Examination, high serum CRP.
Feldman et al, 2000 (24)	US	Massachusetts Male Aging Study	Prospective cohort	9	M	40-70	1167	DHEAs	-	22	-	-	Age.
Guadalupe-Grau et al, 2017 (56)	Spain	Toledo Study for Healthy Aging	Prospective cohort	5.5	F	NM	796	TT, DHEA, DHEAs, E2	92	-	-	-	Age, PASE, hip and waist perimeters, and Charlson index for each sex
Guadalupe-Grau et al, 2017 (56)	Spain	Toledo Study for Healthy Aging	Prospective cohort	5.5	M	NM	566	TT, DHEA, DHEAs, E2	110	-	-	-	Age, PASE, hip and waist perimeters, and Charlson index for each sex

Table 1. Continued

Study characteristics					Participants' characteristics			Exposure and outcomes					
									No. of death				
Author, year	**Country**	**Study name**	**Design**	**Follow-up[1]**	**Sex**	**Age[2]**	**No. of Participants**	**Sex hormones**	**All-cause**	**CVD**	**Cancer**	**Other**	**Adjustments**
Goodman-Gruen et al, 1995 (54)	USA	Rancho Bernardo study	Prospective cohort	19	F	66.7 ± 7.1	624	SHBG	-	153	-	-	Age, BP, TC, HDL, fasting plasma glucose, smoking, alcohol, BMI.
Goodman-Gruen et al, 1995 (54)	USA	Rancho Bernardo study	Prospective cohort	19	M	65.8 ± 7.2	760	SHBG	-	235	-	-	Age, BP, TC, HDL, fasting plasma glucose, smoking, alcohol, BMI.
Gyawali et al, 2019 (63)	Australia	MAILES	Prospective cohort	4.9	M	54.2 ± 11.1	1492	TT, SHBG	-	56	-	-	Age, sex, smoking, BMI, IHD, preexisting major diseases, disability, frailty, Geriatric, Depression Scale, the Mini-Mental State Examination, high serum CRP.
Haring et al, 2010 (64)	Germany	SHIP	Prospective cohort	7.2	M	20-79	1954	TT	-	-	73	40	Age, WC, smoking, high-risk alcohol use, PA, renal insufficiency, and DHEAS.
Holmboe et al, 2015 (11)	Denmark	MONICA I–III and Inter99	Prospective cohort	18.5	M	30-70	5323	TT, FT, SHBG, E2	1533	374	480	-	BMI, study, alcohol consumption, PA.
Hyde et al, 2011 (65)	Australia	Health in Men Study	Prospective cohort	5.1	M	70-88	3637	SHBG	-	160	-	-	Age, WHR, prevalent IHD, HTN, dyslipidemia, smoking status, DM, Charlson's weighted co-morbidity index.
Haring et al, 2012 (66)	Germany	SHIP	Prospective cohort	10	M	52 (37.4, 65.5)	2039	TT	321	157	-	-	Age, BMI, smoking status, exercise, alcohol consumption, dietary pattern, education level.
Haring et al, 2013 (50)	USA	Framingham Heart Study	Prospective cohort	10	M	75.5 ± 5.4	254	TT, DHEAs, E2	104	-	-	-	Age, BMI, smoking, TC, HDL, DM, SBP, antihypertensive medication.
Hyde et al, 2012 (51)	Australia	Health In Men Study	Prospective cohort	5.1	M	70 - 88	3637	TT, FT, SHBG	605	207	231	-	Age, WHR, HTN, dyslipidemia, DM, smoking status, Charlson's weighted comorbidity index, prevalent cardiovascular disease, number of cancer diagnoses.

Table 1. Continued

Study characteristics					Participants' characteristics			Exposure and outcomes					
									No. of death				
Author, year	**Country**	**Study name**	**Design**	**Follow-up[1]**	**Sex**	**Age[2]**	**No. of Participants**	**Sex hormones**	**All-cause**	**CVD**	**Cancer**	**Other**	**Adjustments**
Islam et al, 2022 (67)	Australia	ASPREE trial	Follow-up of a clinical trial	4.6	F	74 (71.7, 77.7)	5535	TT, DHEA, SHBG	200	-	-	-	Age, BMI, smoking status, alcohol consumption, DM, dyslipidaemia, HTN, impaired renal function, treatment allocation (aspirin vs placebo).
Jia et al, 2020 (23)	US	ARIC study	Prospective cohort	18	M	62.8 ± 5.7	3650	DHEAs	1402	624	-	-	Age, race, TC, HDL, SBP, antihypertensive medication use, current smoking, DM, BMI, lipid-lowering medication use, eGFR, testosterone, SHBG, plus NT-proBNP.
Jia et al, 2020 (23)	US	ARIC study	Prospective cohort	18	F	74.5 ± 5.1	4042	DHEAs	1420	639	-	-	Age, race, TC, HDL, SBP, antihypertensive medication use, current smoking, DM, BMI, lipid-lowering medication use, estimated glomerular filtration rate, testosterone, and SHBG, plus NT-proBNP.
Kalme et al, 2005 (15)	Finland	Seven Countries Study	Prospective cohort	8	M	70 - 89	335	SHBG	NM	NM	-	-	Age, BMI.
Khaw et al, 2007 (9)	UK	EPIC-Norfolk	Nested case-control**	7	M	42 - 78	2314	TT	825	369	304	-	Age, date of visit, BMI, SBP, TC, cigarette smoking, DM, alcohol intake, PA, social class, education, DHEAs, androstanediol glucuronide, SHBG.
Lopez et al, 2022 (68)	US	NHANES (1988–1991,1999–2004,2011–2014)	Prospective cohort	7.6	M	46.64	5379	TT, FT	666	160	156		Age, race and ethnicity, smoking status, history of HTN, PA, alcohol consumption, BMI, sex steroid hormones, hypercholesterolemia.
Laouali et al, 2020 (69)	France	Three-City study	Prospective cohort	12	M	> 65	338	TT, BT	-	30	45	55	Center, smoking, alcohol drinking, education level, history of CV disease, HTN, hypercholesterolemia, DM, BMI.
Laouali et al, 2018 (70)	France	Three-City study	Prospective cohort	12	M	73.6 ± 5.1	444	TT, BT	166	-	-	-	Mass index, sex steroid hormones, hypercholesterolemia.

Table 1. Continued

Study characteristics					Participants' characteristics			Exposure and outcomes					
									No. of death				
Author, year	**Country**	**Study name**	**Design**	**Follow-up[1]**	**Sex**	**Age[2]**	**No. of Participants**	**Sex hormones**	**All-cause**	**CVD**	**Cancer**	**Other**	**Adjustments**
Laouali et al, 2018 (71)	France	Three-City study	Prospective cohort	12	M	73.9± 5.4	472	E2	183	44	57	82	Age, date of visit, BMI, WHR, SBP, cholesterol, history of DM, history of HTN, history of high TC, aspirin use, alcohol intake, cigarette smoking status, PA, social class, education level, SHBG, DHEAs, androstanediol glucuronide.
Laughlin et al, 2008 (72)	US	Rancho Bernardo study	Prospective cohort	11.8	M	63.6–78.9	794	TT, BT	538	-	-	-	Age, BMI, WHR, alcohol use, current smoking, exercise.
Lin et al, 2011 (49)	USA	NHANES III (1988–1991)	Prospective cohort	15.6	M		409	FT	152	33	NM	NM	Age, cigarette smoking.
Li et al, 2024 (73)	China	GBCS	Prospective cohort	10.5	M	66.9 ± 6.7	3948	TT, FT, BT, SHBG	949	312	-	-	Age, education, occupation, personal annual income, smoking status, PA, BMI, hypertension, dyslipidemia, diabetes, and self-reported cardiovascular disease (except for cardiovascular disease mortality)
Menke et al, 2010 (74)	US	NHANES (1988-1991)	Prospective cohort	16	M	40	1114	E2, BT, SHBG	NM	-	-	-	Age, race-ethnicity, smoking status, pack-years of smoking, household income, education, alcohol consumption, exercise, percent body fat, testosterone, estradiol, SHBG.
Maggio et al, 2009 (75)	Italy	In-CHIANTI	Prospective cohort	9	F	65-102	5009	E2	135	-	-	-	Age, WHR, CRP, education, cognitive function, PA, caloric intake, smoking, DM, cardiovascular disease, cancer, COPD, TT.

Table 1. Continued

Study characteristics					Participants' characteristics			Exposure and outcomes					
									No. of death				
Author, year	**Country**	**Study name**	**Design**	**Follow-up[1]**	**Sex**	**Age[2]**	**No. of Participants**	**Sex hormones**	**All-cause**	**CVD**	**Cancer**	**Other**	**Adjustments**
Maggio et al, 2007 (76)	Italy	In-CHIANTI	Prospective cohort	6	M	65-92	410	BT, DHEAs	126	-	-	-	Age, BMI, cancer, interleukin 6, education, cognitive function, depression, PA, caloric and alcohol intake, smoking, coronary heart disease (angina and MI), congestive heart failure, stroke, DM, HTN, Parkinson disease, peripheral artery disease, asthma, cancer, COPD.
Mukama et al, 2023 (77)	Germany	EPIC-Heidelberg	Nested case cohort	11.7	M	40.3 - 65.4	1607	DHEAs	314	96	125	-	age at blood draw. time at blood collection, smoking status, BMI, waist-to-hip ratio, PA, self-reported diabetes
Mukama et al, 2023 (77)	Germany	EPIC-Heidelberg	Nested case cohort	12.2	F	35.2 - 66	800	DHEAs	151	38	73	-	age at blood draw. time at blood collection, smoking status, BMI, waist-to-hip ratio, PA, self-reported diabetes age at blood draw. time at blood collection, smoking status, BMI, waist-to-hip ratio, PA, self-reported diabetes, use of oral contraceptives at blood collection.
Ohlsson et al, 2010 (21)	Sweden	MrOS Sweden cohort	Prospective cohort	4.5	M	75.5± 3.2	2644	DHEA, DHEAs	328	-	-	-	Age.
Pye et al, 2014 (78)	Multiple countrie*	European Male Aging Study	Prospective cohort	4.3	M	59.49±10.48	2599	TT, FT	147	56	60	-	Age, center, BMI, current smoking, poor general health.
Szulc et al, 2009 (79)	France	MINOS	Prospective cohort	10	M	50-85	782	TT	182	-	-	-	Age, BMI, smoking, PA, physical performance, health status, vitamin D supplementation.
Srinath et al, 2015 (80)	US	ARIC study	Prospective cohort	12.8	M	63.1 ± 5.6	1588	TT	347	92	-	-	Age, race/center, BMI, WC, cigarette smoking, DM, HTN, LDL, and HDL.

Table 1. Continued

Study characteristics					Participants' characteristics			Exposure and outcomes					
									No. of death				
Author, year	**Country**	**Study name**	**Design**	**Follow-up[1]**	**Sex**	**Age[2]**	**No. of Participants**	**Sex hormones**	**All-cause**	**CVD**	**Cancer**	**Other**	**Adjustments**
Shores et al, 2014 (81)	US	Cardiovascular Health Study	Prospective cohort	8.9	M	76	1032	TT, FT	777	-	-	-	Age, race, clinic site, smoking status, alcohol consumption, SBP, antihypertensive use, HDL, BMI, WC, SHBG.
Schaffrath et al, 2015 (16)	Germany	SHIP	Prospective cohort	10.9	F	49 ± 16.1	1638 (TT), 1525 (FT), 1970 (SHBG)	TT, FT, SHBG	NM	NM	-	-	Age, smoking status, WC, PA, alcohol consumption.
Sievers et al, 2010 (12)	Germany	DETECT study	Prospective cohort	4.5	F	57.96 ± 14.37	2914	TT	103	-	-	-	age, BMI, smoking.
Schederecker et al, 2020 (48)	Germany	KORA-F4	Prospective cohort	8.7	M	61.65	1006	SHBG, TT, FT	128	60	34	34	Age, batch, SBP, LDL, lipid lowering medication, smoking status, antihypertensive medication, BMI, education years, PA, alcohol consumption, eGFR, HsCRP prevalent cancer/ myocardial infarction/ stroke/DM, TT, E2
Schederecker et al, 2020 (48)	Germany	KORA-F4	Prospective cohort	8.7	F	63.08	709	SHBG, TT, FT	70	25	26	19	Age, batch, SBP, LDL, lipid lowering medication, smoking status, antihypertensive medication, BMI, education years, PA, alcohol consumption, eGFR, HsCRP prevalent cancer/ myocardial infarction/ stroke/DM, TT, E2
Smith et al, 2005 (55)	UK	Caerphilly Study	Prospective cohort	16.5	M	45 - 59	2515	TT	482	192	-	290	Smoking status, adult social class, alcohol consumption, height, FEV1/height2, fibrinogen, white cell count, SBP, TG, TC, HDL BMI, FPG, fasting insulin.
Trivedi et al, 2001 (25)	England	Cambridge General Practice Study	Prospective cohort	7.4	M	65 - 76	963	DHEAs	117	104	-	-	Age, BP, BMI, TC, current smokers, steroid use, past history of cardiovascular disease or cancer.

Table 1. Continued

Study characteristics					Participants' characteristics			Exposure and outcomes					
									No. of death				
Author, year	**Country**	**Study name**	**Design**	**Follow-up[1]**	**Sex**	**Age[2]**	**No. of Participants**	**Sex hormones**	**All-cause**	**CVD**	**Cancer**	**Other**	**Adjustments**
Trivedi et al, 2001 (25)	England	Cambridge General Practice Study	Prospective cohort	7.4	F	65 - 76	1171	DHEAs	119	55	-	-	Age, BP, BMI, TC, current smokers, steroid use, past history of cardiovascular disease or cancer.
Tivesten et al, 2009 (10)	Sweden	MrOS Sweden cohort	Prospective cohort	4.5	M	75.4 ± 3.2	2639 (TT, E2), 2618 (FT), 2625 (SHBG)	TT, FT, E2, SHBG	383	-	-	-	Age, MrOS site, BMI, PA, current smoking.
Vikan et al, 2009 (82)	Norway	Tromsø Study	Prospective cohort	11.2 (All-cause), 10 (CVD)	M	59 ± 10.2	1568	TT, E2, FT	395	130	-	-	Age, SBP, HDL/TC, self-reported DM, current smoking, WHR.
Wang et al, 2021 (14)	UK	UK Biobank	Prospective cohort	8.9	F	≥ 30	93314	TT, FT, SHBG	2435	346	1583	506	Age at blood draw, ethnicity, fasting status, college or university degree, BMI, smoking status, alcohol consumption, summed metabolic equivalent of task-hours per week for all activity, family history of CVD or cancer, FPG, HDL, LDL, hormone replacement therapy, SHBG.
Wang et al, 2021 (14)	UK	UK Biobank	Prospective cohort	8.9	M	≥ 30	154962	TT, FT, SHBG	5754	1243	2987	1524	Age at blood draw, ethnicity, fasting status; college or university degree, BMI , smoking status, alcohol consumption, summed metabolic equivalent of task-hours per week for all activity, family history of CVD or cancer, FPG, HDL, LDL, TT.
Yeap et al, 2014 (83)	Australia	Health In Men Study	Prospective cohort	6.7	M	70 - 89	3690	TT, FT, E2	974	325	-	649	Age, education, smoking, BMI, WHR, HTN, dyslipidemia, DM, creatinine, prevalent CVD, cancer.
Zeller et al, 2019 (13)	Finland	FINRISK97 study	Prospective cohort	13.8	F	46.9 ± 21	3961	TT	269	-	-	-	Age, geographical region, TC, HDL, SBP, known HTN, known DM, smoking status, time period of blood drawn.

Table 1. Continued

Study characteristics					Participants' characteristics			Exposure and outcomes					
									No. of death				
Author, year	**Country**	**Study name**	**Design**	**Follow-up[1]**	**Sex**	**Age[2]**	**No. of Participants**	**Sex hormones**	**All-cause**	**CVD**	**Cancer**	**Other**	**Adjustments**
Zeller et al, 2019 (13)	Finland	FINRISK97 study	Prospective cohort	13.8	M	48.2 ± 22.6	3710	TT	510	-	-	-	Age, geographical region, TC, HDL, SBP, known HTN, known DM, smoking status, time period of blood drawn.

TT: Total testosterone; FT: Free testosterone; SHBG: Sex hormone binding globulin ; DHEA: Dehydroepiandrosterone; DHEAs: Dehydroepiandrosterone sulfate; E2: Estradiol; HTN: Hypertension; BP: Blood pressure; DM: diabetes; BMI: body mass index; HDL: high-density lipoprotein cholesterol; FPG: fasting plasma glucose; eGFR: estimated glomerular filtration rate; LDL: low-density lipoprotein cholesterol; TC: Total cholesterol; TG: triglycerides; CRP: C-reactive protein; MI: myocardial infarction; IHD: Ischemic heart diseases; SBP: Systolic blood pressure; WHR: Waist/Hip ratio; WC: Waist circumferences; PA: physical activity; NM: Not mentioned; NHANES: National Health and Nutrition Examination Survey; MAILES: Men Androgen Inflammation Lifestyle Environment and Stress cohort study; ARIC: Atherosclerosis Risk in Communities study; SHIP: Study of Health in Pomerania; DETECT: Diabetes Cardiovascular Risk-Evaluation: Targets and Essential Data for Commitment of Treatment.
* Florence, Italy; Leuven, Belgium; Malmö, Sweden; Manchester, United Kingdom; Santiago de Compostela, Spain; Lódz, Poland; Szeged, Hungary; Tartu, Estonia.
** prospective population study

Meta-analyses and dose-response analyses

A summary of the hazard ratios of mortality outcomes for top versus bottom third of each of the endogenous sex steroid hormones and SHBG are presented in **Figures 2-3** and **Figures S1-S4** (34). Findings of the dose-response analyses are presented in **Figure 4, Figures S10-S12** (34), and **Table S4** (34). The GRADE evidence for these associations is summarized in **Tables S5-S8** (34).

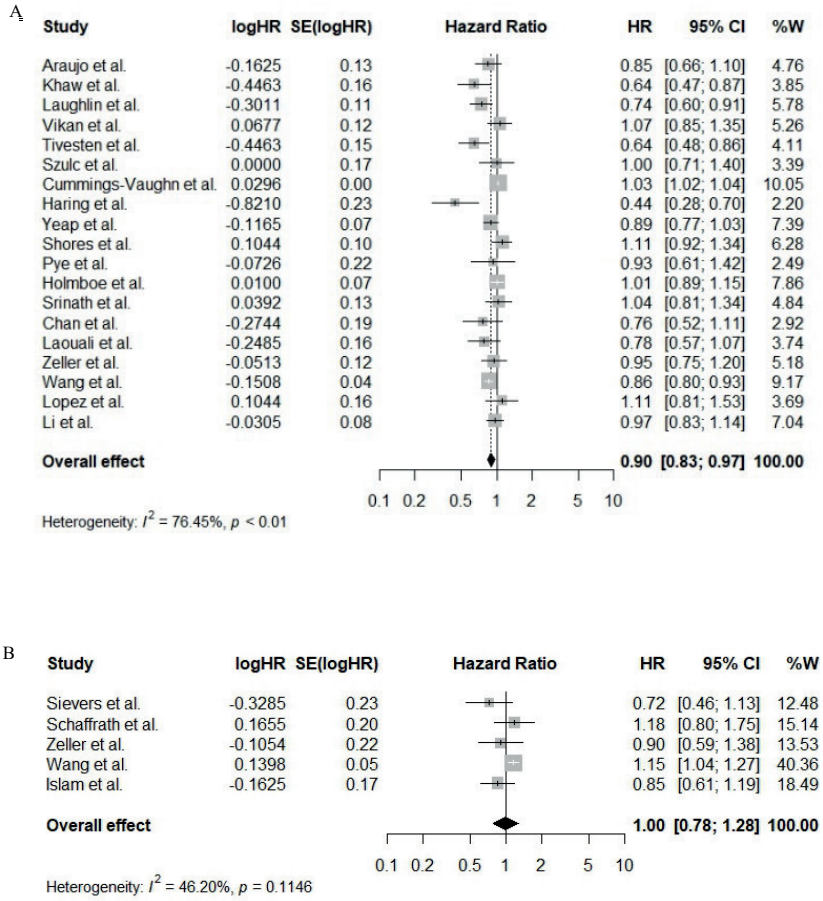


Figure 2. Summary hazard ratios of all-cause mortality for top versus bottom third of total testosterone (TT) levels in **A**) men and **B**) women.

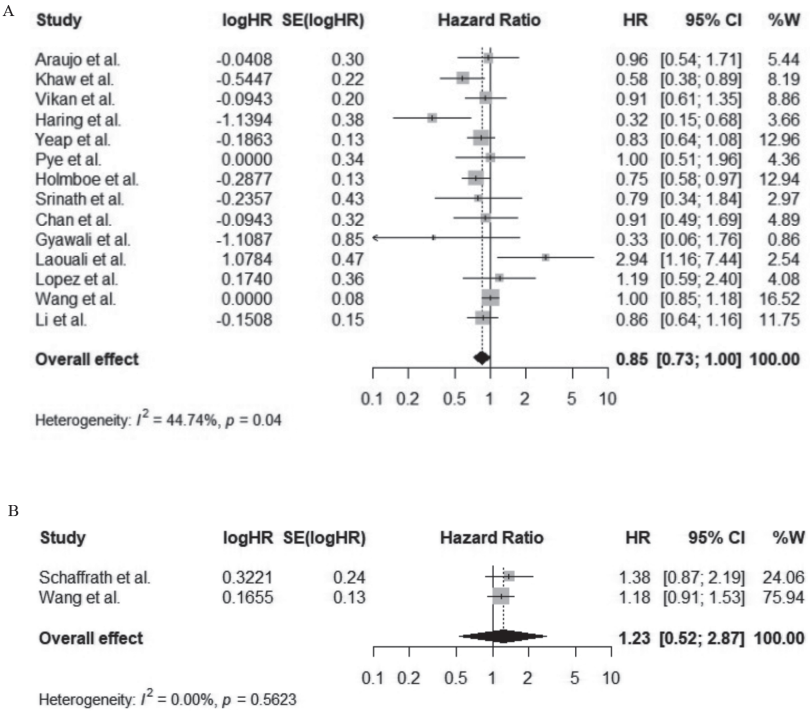


Figure 3. Summary hazard ratios of CVD mortality for top versus bottom third of total testosterone (TT) levels in **A**) Men and **B**) Women.

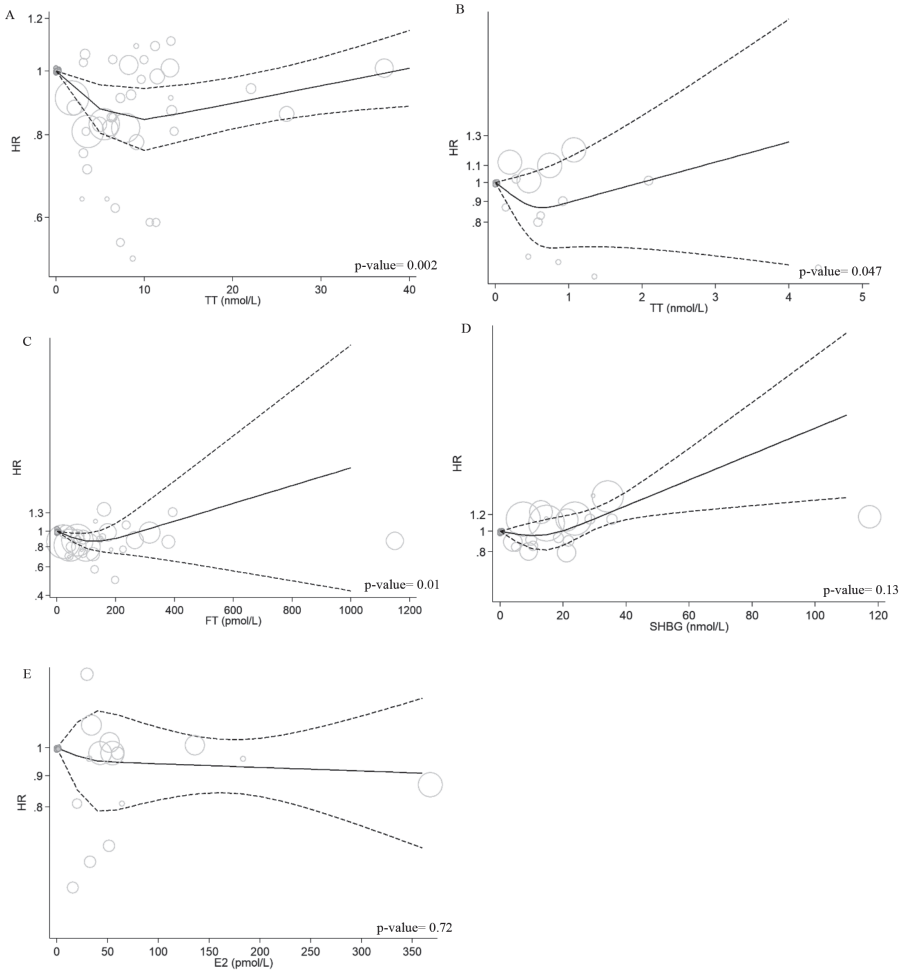


Figure 4. Non-Linear dose response association of sex hormones and all-cause mortality.
A) TT-Men; **B**) TT-Women; **C**) FT-Men; **D**) SHBG-Men; **E**) E2-Men.

Total testosterone

Our meta-analyses showed a lower risk of all-cause mortality in the top versus bottom third of TT in men (HR (95%CI): 0.90 (0.83 to 0.97), n=19, I^2: 76.45%, **Figure-2-A**) but not in women (HR (95%CI): 1.00 (0.78 to 1.28), n=5, I^2: 46.20%, **Figure-2-B**). Non-linear dose-response analysis suggested a U-shaped association in men ($p_{nonlinearity}$: 0.002, **Figure 4-A**) and a similar but slightly more J-shaped association in women ($p_{nonlinearity}$: 0.047, **Figure 4-B**). The certainty of evidence was graded as low for men and high for women (**Table S6** and **Table S7**) (34).

No association was observed between TT levels and risk of CVD mortality in neither men (HR (95%CI): 0.85 (0.71 to 1.00), I^2: 44.74%, n=14, **Figure 3-A**) and nor in women (HR (95%CI): 1.23 (0.52 to 2.87), n=2, **Figure 3-B**). The certainty of evidence was low for men and moderate for women (**Table S6** and **Table S7**) (34). For cancer mortality or death due to the other causes study were only available to be pooled in men, and showed a lower risk of mortality due to other causes (HR (95%CI): 0.82 (0.70 to 0.94), I^2: 0.0%, n=4, **Figure S4-A**) (34) with a high certainty of evidence for this association (**Table S6)** (34) and no statistically significant association for risk of cancer mortality (HR (95%CI): 0.90 (0.68 to 1.18), I^2: 72.6%, n=7, **Figure S3-A**) (34) with a low certainty of evidence (**Table S6**) (34). However, only one study by Wang et al. reported the association of TT and risk of cancer or other causes mortality in women **(Table 1).**

Free testosterone

We observed no association between top versus bottom third of FT level and risk of all-cause mortality in men (HR (95%CI): 0.94 (0.83 to 1.07), I^2: 80.57%, n=11, **Figure S1-A**) (34) or women (HR (95%CI): 1.04 (0.93 to 1.17), n=2, **Figure S1-B**) (34). The dose-response analysis was only feasible in men and showed a J-shaped association ($p_{nonlinearity}$: 0.01, **Figure 4-C**). The certainty of evidence was moderate for men and high for women (**Table S6** and **Table S7**) (34). Similarly, pooled HR showed no association with risk of CVD mortality in either men (HR (95%CI): 1.04 (0.83 to 1.30), n=9, I^2: 70.48%, **Figure S2-A**) (34) or women (HR (95%CI): 1.27 (0.23 to 6.86), n=2, **Figure S2-B**) (34). The certainty of evidence was very low for men and moderate for women (**Table S6** and **Table S7**) (34).

The analysis in men showed no association with risk of cancer mortality (HR (95%CI): 0.94 (0.76 to 1.16), I^2: 17.42%, n=4, **Figure S3-B**) (34) and mortality due to the other causes (HR (95%CI): 0.80 (0.19 to 3.29), I^2: 71.5%, n=2, **Figure S4-B**) (34). The certainty of evidence for these associations was moderate (**Table S6**) (34).

Only one study by Wang et al. reported the association of FT and risk of cancer and other causes mortality in women (**Table 1**).

Bioavailable testosterone

Our analysis showed no association between level of BT with risk of all-cause mortality (HR (95%CI): 0.93 (0.80 to 1.07), I^2: 61.28%, n=6, **Figure S1-C**) (34) in men with moderate certainty of evidence (**Table S6**) (34) and CVD mortality (HR (95% CI): 1.56 (0.69 to 480.12), I^2: 76.30%, n=2, **Figure S2-C**) (34) in this population with low certainty of evidence (**Table S6**) (34).

One study by Laouali et al. reported the association of BT and risk of cancer and other causes of mortality in men (**Table 1**).

Sex-hormone binding-globulin

Our meta-analysis showed no association between SHBG and risk of all-cause mortality in men (HR (95%CI): 1.10 (0.94 to 1.28), I^2: 80.46%, n=8, **Figure S1-D**) (34) but a higher risk in women (HR (95%CI): 1.25 (1.13 to 1.39), I^2: 0.0%, n=3, **Figure S1-E**) (34). For men, the certainty of evidence was low while it was high for women (**Table S6** and **Table S7**) (34). No association was observed for risk of CVD mortality either in men (HR (95%CI): 0.93 (0.67 to 1.29), I^2: 80.3%, n=6, **Figure S2-D**) (34) or women (HR (95%CI): 1.02 (0.75 to 1.39), I^2: 0.0%, n=2, **Figure S2-E**) (34). The certainty of evidence was moderate for men and high for women (**Table S6** and **Table S7**) (34). Additionally, no association with the risk death due to cancer in men was observed (HR (95%CI): 1.21 (0.99 to 1.48), I^2: 0.0%, n=3, **Figure S3-C**) (34), with a moderate certainty of evidence (**Table S5**) (34).

One study by Wang et al. presented the association of SHBG with risk of cancer and other causes of mortality in women (**Table 1**).

Estradiol

Studies examined the association between E2 and risk of all-cause and cause specific mortality in men and only one study by Maggio et al. investigated this association in women (**Table 1**). Pooled effect estimates indicated no association between top versus bottom third of E2 and risk of all-cause mortality (HR (95%CI): 0.99 (0.88 to 1.11), n=9, I^2: 48.09%, **Figure S1-F**) (34) with a moderate certainty of evidence (**Table S6**) (34), CVD mortality (HR (95%CI): 1.02 (0.75 to 1.39), n=6, I^2: 53.64%, **Figure S2-F**) (34) with a low certainty of evidence (**Table S6**) (34), with risk of death due to the other causes (HR (95%CI): 0.93 (0.68 to 1.28), I^2: 0.0%, n=3, **Figure S4-C**) (34) with low certainty of evidence in men (**Table S6**) (34), or cancer mortality (HR (95%CI): 1.28 (0.97 to 1.68), I^2: 0.0%, n=3, **Figure S3-D**) (34) with a low certainty of evidence (**Table S6**) (34).

Dehydroepiandrosterone-sulfate and Dehydroepiandrosterone

We observed a significant association between top versus bottom third level of DHEAs and lower risk of all-cause mortality in men (HR (95%CI): 0.72 (0.57 to 0.91), I^2: 73.99%, n=6, **Figure S1-G**) (34) but not in women (HR (95%CI): 0.84 (0.57 to 1.24), I^2: 17.03%, n=3, **Figure S1-H**) (34). The certainty of evidence was very low for men and moderate for women (**Table S6** and **Table S7**) (34). Three studies reported the association of DHEAs and risk of all-cause mortality in men and women combined;

meta-analysis of these studies also showed no association (HR (95%CI): 0.90 (0.51 to 1.57), **Figure S1-I**) (34) with low certainty of evidence (**Table S8**) (34).

Similarly, we found no association between DHEAs levels and risk of CVD death in men (HR (95%CI): 0.76 (0.41 to 1.41), I^2: 58.35%, n=5, **Figure S2-G**) (34) or women (HR (95%CI): 0.83 (0.52 to 1.30), I^2: 73.47%, n=4, **Figure S2-H**) (34) with low certainty of evidence (**Table S6** and **Table S7**) (34).

Pooled effect estimates of 2 studies showed no association between DHEA and risk of all-cause mortality in men (HR (95%CI): 0.73 (0.12 to 4.55), **Figure S1-J**) (34).

Only one study by Mukama et al. presented the association of DHEAs and risk of cancer mortality in men and women. One study by Islam et al. reported the association of DHEA and all-cause mortality in women (**Table 1**).

Additional analyses

Findings of additional analyses including leave-one-out, publication bias, and subgroup analysis are reported in **Figure S5-S8, Figure S9, Table S4,** and **Supplementary information 3** (34).

Discussion

To the best of our knowledge, this study is the first comprehensive systematic review and meta-analysis that also implemented a dose-response analysis on the association between endogenous sex steroid hormones and SHBG with risk of all-cause and cause-specific mortality. Our analysis indicated a 10% lower risk of all-cause mortality and an 18% lower risk of death due to other causes for the top versus bottom third of TT levels in men, while no associations were observed in women. Also, SHBG levels were associated with an increased risk of all-cause mortality by 25% in women. We found a lower risk of all-cause mortality for the top versus bottom third of DHEAs level in men, while no associations were seen in women.

We found a U-shaped association between TT levels and risk of all-cause mortality in men, and a J-shaped association in women. Our findings suggested that while we found no evidence of higher all-cause mortality within increasing TT levels to the highest normal ranges (40 nmol/l) in men, a steeper increase in the risk of all-cause mortality for elevated values of TT in women was observed.

Our results challenge the previous concept that lower TT is associated with increased mortality in a linear fashion. A limited number of individual studies have reported the dose-response associations between TT and mortality, which were consistent with our observations. The Swedish Osteoporotic Fractures in Men study (MrOS), including 3104 men with 4.5 years of follow-up, found that men with TT in the second quartile versus the fourth quartile had a lower risk of mortality than those with TT in the first quartile (10). Findings of another study by Yeap et al. (83), including 3690 men with a median follow-up of 6.7 years, showed the lowest risk of mortality with an optimal range of TT (9.8-15.8 nmol/l), which is close to our results (5-15 nmol/l).

A previous meta-analysis on the association of testosterone levels and risk of mortality in men, published more than 10 years ago and based on 11 studies (27), in line with our results revealed that low endogenous TT levels were associated with an increased risk of all-cause mortality.

A recent individual participant data (IPD) meta-analysis of nine studies in men investigated the association between testosterone and related hormones and the risk of mortality. Owing to the nature of the study, the authors applied more rigorous eligibility criteria, including community-dwelling men with sex hormone levels measured using mass spectrometry and at least five years of follow-up. They accounted for a comprehensive set of potential confounders in their statistical models, including age, body mass index, marital status, alcohol consumption, smoking, physical activity, hypertension, diabetes, creatinine concentration, the ratio of total to high-density lipoprotein cholesterol, and lipid-lowering medication use. They found no association between low versus high levels of TT and risk of all-cause or CVD mortality (84). Our meta-analysis complements the IPD meta-analysis by Yeap et al. (84) by applying broader eligibility criteria, including studies on women, additional sex hormones such as DHEA and DHEAs, and a wider range of mortality outcomes, including cancer and other causes.

The results of another more recent systematic review and meta-analysis of observational studies among men indicated a higher risk of overall mortality and CVD mortality with lower TT levels (28). However, this review suffers from methodological issues, e.g. by pooling results of different patient groups; not including certain eligible studies performed on general population (11, 61, 83); and pooling the lowest versus highest estimates such as quintile, quartile, and tertile without standardizing them first. Neither of the above-mentioned aggregated meta-analyses evaluated the shape of the dose-response associations nor summarized the associations of BT and FT with the risk of mortality (27, 28).

Most of the evidence regarding the potential protective effects of testosterone have been attributed to its effect on cardiovascular system. For instance, a large body of literature suggests a direct vasodilator effect of TT on many vascular districts, including the coronary arteries, in addition to a role in endothelium repair (85). Furthermore, studies carried out in hypogonadal subjects demonstrated that testosterone therapy is able to decrease the production of inflammatory cytokines, and to increase the level of the anti-atherogenic interleukin-10 (86). It has been shown that testosterone replacement therapy decreased the occurrence of typical atherosclerotic processes such as neointima formation and fatty streak accumulation (87). Strong evidence supports it that androgens could decrease arterial stiffness probably by inhibiting vascular smooth muscle calcification (88).

For women, the association of endogenous sex steroid hormones and risk of all-cause and cause-specific mortality has been poorly investigated. Many available studies have been performed on women with a wide age range without any information on the menopausal status and had relatively small sample sizes (12, 13, 16), leading to conflicting results. However, among the included studies on women, only a recent study done by Wang et al. (89) investigated the association of endogenous sex steroid hormones with the risk of all-cause and cause-specific mortality on a large number of postmenopausal women (93,314 individuals). This study found a higher risk of all-cause mortality with elevated TT levels in women. Consistent with our findings, the results of a cohort study with a long-term follow-up (up to 30 years) and a relatively large sample size including 4716 women, not receiving oral contraceptives or hormonal replacement therapy, found that extremely high concentrations of TT were associated with increased risk of mortality (90). It is worth mentioning that testosterone levels can be aromatized to E2 in endothelial cells in women (91). Thus, it seems an important factor which needs to be controlled to explore an unbiased association between testosterone levels and mortality. Almost none of the included studies in the current meta-analysis have adjusted their models based on E2 levels.

According to our results, higher SHBG levels were associated with an increased risk of all-cause mortality in women which were independent of free or bioavailable testosterone levels. Consistent with the IPD meta-analysis by Yeap et al. (84), we observed a similar direction of association between SHBG levels and the risk of all-cause mortality in men; however, our results did not reach statistical significance. In line with our observation, there is evidence supporting the direct effect of SHBG independent of sex steroids (92, 93). It has been shown that SHBG may mediate cell-surface signalling, cellular delivery, and biological action of endogenous sex steroid

hormones via activation of a specific plasma receptor directly (94, 95). Among the included studies, only the study done by Wang et al. adjusted their models for TT levels and found increased risk of all-cause mortality with higher SHBG levels in both men and women (89).

In agreement with most of the previous literature, our results were indicative of no association of E2 with risk of all-cause, CVD mortality and other cause of mortality in men. In addition, findings from a recent IPD meta-analysis reported no association between E2 levels and the risk of all-cause mortality, CVD mortality, or incident CVD events (84). This is consistent with other evidence which showed that E2 was not associated with prevalent CVD or intermittent claudication (96, 97). Findings of a meta-analysis on the association of E2 and CVD-related outcomes in men failed to establish any associations (98). Studies on the association of E2 and risk of mortality in women are lacking and more evidence is needed to explore these associations.

We found a protective association of DHEAs, but not DHEA (top versus bottom third of distribution) with the risk of all-cause mortality in men. Evidence regarding the association of DHEA and DHEAs and risk of mortality is limited and conflicting. Some studies were in support of our findings (21-23), while others found no associations (25, 61). Physiological functions of DHEA (s) in CVD as well as noncancer and con-CVD disease defence may justify these findings. DHEA(s) has been shown to be an effective activator of peroxisome proliferator-activated receptor- α (99, 100), which may contribute as a modulator of immune functions (101, 102) and oxidative stress (99) as well as atherosclerosis (103). Moreover, low DHEA(s) has been recognized as general marker of poor health hence an epiphenomenon of subclinical diseases (100).

Strengths and limitations

We included all evidence on the association of endogenous sex steroid hormones and SHBG with the risk of all-cause and cause-specific mortality and reported the sex-specific associations which has not been done before. In order to have a consistent approach to conducting the meta-analysis, we transformed the estimates which often reported differently by each study (such as per unit or per–1-SD change or comparing quintiles, quartiles, tertiles, etc.) to top vs bottom third of endogenous sex steroid hormones distribution, which is applied by the previous meta-analysis concerning the association of biomarkers and risk of chronic diseases and mortality (104-106).

Residual confounding is inevitable and should be considered due to the nature of the included study designs (observational cohort studies). We pooled the results

of multiple cohort studies with different methods of hormonal assessment, sample sizes, follow-up duration and adjustments in the statistical methods; however, an individual participant data meta-analysis might provide more accurate evidence regarding these associations.

While we included the studies on the general population, the bias due to the pre-existing diseases might stand. Also, our study explores only baseline values of endogenous sex steroid hormones and SHBG with mortality, future research could benefit from incorporating longitudinal, repeated measurements of sex hormones to better understand how changes in these levels over time may influence mortality risk.

Policy implications and future research

Future studies such as large prospective cohort studies or clinical trials should explore dose-response associations and identify sex-specific cut-off levels of endogenous sex steroid hormones and SHBG, that could be critical for both men and women.

Our findings also highlight a gap in understanding the impact of endogenous sex steroid hormones on mortality in different sexes, considering most of the studies were based in males. Our study also provides potential support for future studies such as Mendelian randomization and clinical trials to explore causality separately in men and women. Most included studies investigated the association of endogenous sex steroid hormones and all-cause and CVD mortality and only a limited number of studies have investigated the association of endogenous sex steroid hormones with cancer mortality in both sexes. Sex differences in incidence and mortality of cancer have been highlighted in the last decades (107, 108). Growing evidence shows sex-specific differences in the incidence and mortality of cancers not only for sex-specific organs such as prostate and colorectal cancers in males and breast cancer in females, but also for colon, lung and liver cancers (109, 110). Overall, cancer mortality is greater in men than in women with lung, colorectal and stomach cancers as the leading causes of mortality in men rather than women (109). Thus, future studies on the association between these hormones and the risk of specific cancer mortality may provide more evidence of these sex differences. Additionally, considering the function and relationship between steroid hormones and hormones such as luteinizing hormone (LH) or other factors such as hematocrit (Hct), exploring the association between steroid hormones and the risk of death while accounting for LH and Hct would add valuable insights. LH, as a key regulator of testosterone, could serve as a marker of hormonal dysregulation, while Hct, influenced by testosterone (111), may affect risk of

cardiovascular diseases (112-115). Investigating these interactions could enhance our understanding of the mechanisms linking steroid hormones to mortality.

Most of the studies included in the current study were from North America, Europe and Australia and evidence on this topic is lacking for some ethnic groups like Africans and Asians. Since racial/ethnic differences have been proposed for endogenous sex steroid hormones for either men or women, more studies are needed to be performed on other ethnic groups (116, 117).

Among the included studies only three studies adjusted their statistical models for SHBG (9, 14, 81). As a major carrier of testosterone and E2, SHBG correlates with TT and E2 levels and has been associated with a higher risk of all-cause mortality. Thus, the complex relations between androgens, SHBG and mortality needs further investigation.

It should be noted that sex steroid hormones and SHBG levels were measured using different commercial methods, primarily immunoassays rather than Liquid Chromatography-Tandem Mass Spectrometry (LCMS). This is particularly important for the measurement of TT in women and E2 in postmenopausal women and men since immunoassays are less reliable due to method-specific bias and inaccuracy in low sex hormone concentrations. Moreover, FT and BT are calculated variables that lack certified standards and are dominated by SHBG. While we aimed to investigate the dose-response association between endogenous sex steroid hormones, SHBG, and mortality risk, these methodological limitations should be considered. Our dose-response findings highlight the need for future studies to move beyond the conventional approach of categorizing sex steroid hormones and SHBG into high or low levels when examining their associations with chronic diseases and mortality. Instead, research should focus on dose-response relationships separately in men and women. Future large individual-participant meta-analysis can help explore and define sex hormones cut-offs related to mortality in men and women, which could have clinical and public health implication for introducing prevention strategies.

Conclusion

Our findings provide more evidence on the sex-specific associations of endogenous sex steroid hormones and SHBG with risk of all-cause and cause-specific mortality, which may help to implement sex-specific randomized clinical trials of sex steroid hormones treatments to assess whether modulating these hormones might reduce risk of mortality and improve longevity.

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Supplementary materials: Endogenous sex steroid hormones, sex hormone binding globulin and risk of all-cause and cause-specific mortality: a systematic review and dose-response meta-analysis of prospective cohort studies

Table S1. Search strategy

Database	Search strategy
Embase.com	('sex hormone binding globulin'/de OR 'androgen'/de OR 'androgen blood level'/exp OR 'estradiol'/de OR 'estradiol blood level'/de OR 'testosterone'/de OR 'testosterone blood level'/de OR 'dehydroepiandrosterone'/de OR 'dehydroepiandrosterone sulfate'/de OR (androgen* OR 'sex hormone*' OR 'sex steroid*' OR testosterone* OR 'sexual hormone*' OR shbg OR dehydroepiandrosterone* OR androstenolone* OR dhea OR dheas OR 'dehydroepiandrosterone sulfate' OR 'dhea s' OR estradiol* OR oestradiol* OR prasterone*):ab,ti) AND ('mortality'/exp OR 'death'/exp OR 'mortality risk'/de OR 'mortality risk score'/de OR 'cardiovascular disease'/de OR 'coronary artery disease'/de OR 'heart muscle ischemia'/de OR 'heart infarction'/de OR 'Coronary Disease'/de OR 'cerebrovascular accident'/de OR 'heart failure'/de OR (mortalit* OR death* OR fatal* OR 'cardiovascular disease*' OR 'coronary disease*' OR 'coronary artery disease*' OR 'heart disease*' OR 'coronary heart disease*' OR 'ischemic heart disease*' OR 'ischaemic heart disease*' OR 'myocardial ischemia*' OR 'myocardial ischaemia*' OR 'myocardial infarct*' OR 'heart infarct*' OR stroke* OR 'sudden cardiac death' OR 'heart failure*' OR 'cardiovascular event*' OR 'cardiac event*' OR MACE OR MACEs OR survival* OR CVD OR CHD):ab,ti) AND ('cohort analysis'/de OR 'prospective study'/de OR 'follow up'/de OR 'longitudinal study'/de OR 'hazard ratio'/de OR (((hazard* OR odds OR risk* OR inciden*) NEXT/1 (ratio* OR proportion*)) OR cohort* OR prospectiv* OR longitudinal OR follow-up* OR followup* OR followed-up OR population-based OR case-cohort* OR nested case-control* OR 'relative risk*' OR inciden*):ab,ti) NOT ([animals]/lim NOT [humans]/lim) NOT (animal*:ti OR mice:ti OR rat:ti OR rats:ti OR in-vivo:ti OR in-vitro:ti OR tissue:ti OR tissues:ti OR cell*:ti) NOT (([newborn]/lim OR [infant]/lim OR [child]/lim OR [adolescent]/lim) NOT [adult]/lim) NOT ('pregnancy'/exp OR pregnan*:ti OR fetus:ti OR fetal:ti OR foetus:ti OR foetal:ti OR neonatal*:ti OR perinatal*:ti OR maternal*:ti OR infant*:ti OR child*:ti OR newborn*:ti OR pediatric*:ti OR paediatric*:ti OR juvenile*:ti) NOT ([Conference Abstract]/lim OR [Letter]/lim OR [Note]/lim OR [Editorial]/lim OR [Review]/lim) NOT ('systematic review'/exp OR 'meta analysis'/exp OR 'case report'/de OR 'case study'/de OR 'case report*':ti OR 'case stud*':ti OR 'case series':ti OR review*:ti OR 'meta-analysis':ti)

Table S1. Continued

Database	Search strategy
Medline (Ovid)	(Sex Hormone-Binding Globulin/ OR Androgens/ OR Estradiol/ OR Testosterone/ OR Dehydroepiandrosterone/ OR Dehydroepiandrosterone Sulfate/ OR (androgen* OR sex hormone* OR sex steroid* OR testosterone* OR sexual hormone* OR shbg* OR dehydroepiandrosterone* OR androstenolone* OR dhea OR dheas OR dehydroepiandrosterone sulfate OR dhea-s OR estradiol* OR oestradiol* OR prasterone*).ab,ti.) AND (exp Mortality/ OR exp Death/ OR Cardiovascular Diseases/ OR Coronary Disease/ OR Coronary Artery Disease/ OR Myocardial Ischemia/ OR Myocardial Infarction/ OR exp Stroke/ OR Heart Failure/ OR (mortalit* OR death* OR fatal* OR cardiovascular disease* OR coronary disease* OR coronary artery disease* OR heart disease* OR coronary heart disease* OR ischemic heart disease* OR ischaemic heart disease* OR myocardial ischemia* OR myocardial ischaemia* OR myocardial infarct* OR heart infarct* OR stroke* OR sudden cardiac death* OR heart failure* OR cardiovascular event* OR cardiac event* OR MACE OR MACEs OR survival* OR CVD OR CVDs OR CHD).ab,ti.) AND (Cohort Studies/ OR Prospective Studies/ OR Follow-Up Studies/ OR Longitudinal Studies/ OR (((hazard* OR odds OR risk* OR inciden*) adj1 (ratio* OR proportion*)) OR cohort* OR prospectiv* OR longitudinal OR follow-up* OR followup* OR followed-up OR population-based OR case-cohort* OR nested case-control* OR relative risk* OR (inciden* AND risk*)).ab,ti.) NOT (exp animals/ NOT humans/) NOT (animal*.ti. OR mice.ti. OR rat.ti. OR rats.ti. OR in-vivo.ti. OR in-vitro.ti. OR tissue.ti. OR tissues.ti. OR cell*.ti.) NOT ((exp infant/ OR exp child/ OR adolescent/) NOT (exp adult/)) NOT (exp Pregnancy/ OR pregnan*.ti. OR fetus.ti. OR fetal.ti. OR foetus.ti. OR foetal.ti. OR neonatal*.ti. OR perinatal*.ti. OR maternal*.ti. OR infant*.ti. OR child*.ti. OR newborn*.ti. OR pediatric*.ti. OR paediatric*.ti. OR juvenile*.ti.) NOT (letter* OR news OR comment* OR editorial* OR congres*).pt. NOT ("review"/ OR systematic review/ OR meta-analysis/ OR case reports/ OR case report*.ti. OR case series.ti. OR case stud*.ti. OR review*.ti. OR meta-analysis.ti.) NOT (Hormone Replacement Therapy/ OR replacement.ti OR (hormone adj2 treatment*).ti OR (hormone adj2 therap*).ti)
Cochrane Central Register of Controlled Trials (CENTRAL)	((androgen* OR sex NEXT hormone* OR sex NEXT steroid* OR testosterone* OR sexual NEXT hormone* OR shbg OR dehydroepiandrosterone* OR androstenolone* OR dhea OR dheas OR "dehydroepiandrosterone sulfate" OR "dhea-s" OR estradiol* OR oestradiol* OR prasterone*):ab,ti) AND ((mortalit* OR death* OR fatal* OR cardiovascular NEXT disease* OR coronary NEXT/2 disease* OR heart NEXT disease* OR ischemic NEXT heart NEXT disease* OR ischaemic NEXT heart NEXT disease* OR myocardial NEXT ischemia* OR myocardial NEXT ischaemia* OR myocardial NEXT infarct* OR heart NEXT infarct* OR stroke* OR "sudden cardiac death" OR heart NEXT failure* OR cardiovascular NEXT event* OR cardiac NEXT event* OR MACE OR MACEs OR survival* OR CVD OR CHD):ab,ti) AND ((((hazard* OR odds OR risk* OR inciden*) NEXT/1 (ratio* OR proportion*)) OR cohort* OR prospectiv* OR longitudinal OR follow NEXT up* OR followup* OR followed NEXT up OR population NEXT based OR case NEXT cohort* OR nested NEXT case NEXT control* OR relative NEXT risk* OR inciden*):ab,ti) NOT (replacement:ti OR hormone NEXT/2 treatment*:ti OR hormone NEXT/2 therap*:ti) NOT (pregnan*:ti OR fetus:ti OR fetal:ti OR foetus:ti OR foetal:ti OR neonatal*:ti OR perinatal*:ti OR maternal*:ti OR infant*:ti OR child*:ti OR newborn*:ti OR pediatric*:ti OR paediatric*:ti OR juvenile*:ti)

Table S1. Continued

Database	Search strategy
Web of Science Core Collection	Focus on precision: search terms for sex hormones and CV-mortality, all-cause mortality only in *Title* and *Keyword Plus* TI=((androgen* OR "sex hormone*" OR "sex steroid*" OR testosterone* OR "sexual hormone*" OR shbg OR dehydroepiandrosterone* OR androstenolone* OR dhea OR dheas OR "dehydroepiandrosterone sulfate" OR "dhea-s" OR estradiol* OR oestradiol* OR prasterone*) AND (mortalit* OR death* OR fatal* OR "cardiovascular disease*" OR coronary NEAR/2 disease* OR "heart disease*" OR "ischemic heart disease*" OR "ischaemic heart disease*" OR "myocardial ischemia*" OR "myocardial ischaemia*" OR "myocardial infarct*" OR "heart infarct*" OR stroke* OR "sudden cardiac death*" OR "heart failure*" OR "cardiovascular event*" OR "cardiac event*" OR MACE OR MACEs OR survival* OR CVD OR CHD)) OR KP=((androgen* OR "sex hormone*" OR "sex steroid*" OR testosterone* OR "sexual hormone*" OR shbg OR dehydroepiandrosterone* OR androstenolone* OR dhea OR dheas OR "dehydroepiandrosterone sulfate" OR "dhea-s" OR estradiol* OR oestradiol* OR prasterone*) AND (mortalit* OR death* OR fatal* OR "cardiovascular disease*" OR coronary NEAR/2 disease* OR "heart disease*" OR "ischemic heart disease*" OR "ischaemic heart disease*" OR "myocardial ischemia*" OR "myocardial ischaemia*" OR "myocardial infarct*" OR "heart infarct*" OR stroke* OR "sudden cardiac death*" OR "heart failure*" OR "cardiovascular event*" OR "cardiac event*" OR MACE OR MACEs OR survival* OR CVD OR CHD)) AND TS=(((hazard* OR odds OR risk* OR inciden*) NEAR/1 (ratio* OR proportion*)) OR cohort* OR prospectiv* OR longitudinal OR "follow up*" OR followup* OR "followed up" OR "population based" OR "case cohort*" OR "nested case control*" OR "relative risk*" OR inciden*) NOT TS=((animal* OR plant* OR rat OR rats OR mouse OR mice OR murine OR pigs OR primate* OR nonhuman*) NOT (human* OR patient*)) Exclude Document Types: Review Articles

Table S1. Continued

Database	Search strategy
PubMed (publisher)	(Sex Hormone-Binding Globulin[mh] OR Androgens[mh] OR Estradiol[mh] OR Testosterone[mh] OR Dehydroepiandrosterone[mh] OR Dehydroepiandrosterone Sulfate[mh] OR (androgen*[tiab] OR sex hormone*[tiab] OR sex steroid*[tiab] OR testosterone*[tiab] OR sexual hormone*[tiab] OR shbg*[tiab] OR dehydroepiandrosterone*[tiab] OR androstenolone*[tiab] OR dhea[tiab] OR dheas[tiab] OR dehydroepiandrosterone sulfate[tiab] OR dhea-s[tiab] OR estradiol*[tiab] OR oestradiol*[tiab] OR prasterone*[tiab])) AND (Mortality[mh] OR Death[mh] OR Cardiovascular Diseases[mh] OR Coronary Disease[mh] OR Coronary Artery Disease[mh] OR Myocardial Ischemia[mh] OR Myocardial Infarction[mh] OR Stroke[mh] OR Heart Failure[mh] OR (mortalit*[tiab] OR death*[tiab] OR fatal*[tiab] OR cardiovascular disease*[tiab] OR coronary disease*[tiab] OR coronary artery disease*[tiab] OR heart disease*[tiab] OR coronary heart disease*[tiab] OR ischemic heart disease*[tiab] OR ischaemic heart disease*[tiab] OR myocardial ischemia*[tiab] OR myocardial ischaemia*[tiab] OR myocardial infarct*[tiab] OR heart infarct*[tiab] OR stroke*[tiab] OR sudden cardiac death*[tiab] OR heart failure*[tiab] OR cardiovascular event*[tiab] OR cardiac event*[tiab] OR MACE[tiab] OR MACEs[tiab] OR survival*[tiab] OR CVD[tiab] OR CVDs[tiab] OR CHD[tiab])) AND (Cohort Studies[mh] OR Prospective Studies[mh] OR Follow-Up Studies[mh] OR Longitudinal Studies[mh] OR (((hazard*[tiab] OR odds[tiab] OR risk*[tiab] OR inciden*[tiab]) AND (ratio*[tiab] OR proportion*[tiab])) OR cohort*[tiab] OR prospectiv*[tiab] OR longitudinal[tiab] OR follow-up*[tiab] OR followup*[tiab] OR followed-up[tiab] OR population-based[tiab] OR case-cohort*[tiab] OR nested case-control*[tiab] OR relative risk*[tiab] OR (inciden*[tiab] AND risk*[tiab]))) NOT (animals[mh] NOT humans[mh]) NOT (letter[pt] OR news[pt] OR comment[pt] OR editorial[pt] OR congress[pt]) AND publisher[sb]
Google Scholar	"sex|sexual hormone|hormones|steroids"|androgen|testosterone|estradiol|oestradiol|dehydroepi-androsterone|dhea|dheas mortality|death|fatal|cardiovascular|cardiac|coronary|ischemic|heart|myo-cardial|stroke|survival cohort|prospective|"follow-up"|followup|longitudinal

Supplementary information 1

Quality assessment and certainty of evidence

Quality of the studies were evaluated with the recent version of the ROBINS-E tool. This tool provides a structured approach to assessing the risk of bias in observational epidemiological studies and shares many characteristics with the risk of bias (ROB) 2 tool for randomized clinical trials and the ROBINS-I tool for non-randomized studies of interventions. Indeed, it provides further development of ROBINS-I. The tool includes 7 domains and considers potential biases resulting from confounding, assessment of exposure, selection of participants, post-exposure interventions, missing data, measurement of the outcome, and selective reporting of the results (1). Certainty of evidence was assessed with the updated GRADE tool. According to GRADE, high or moderate certainty of evidence can be interpreted as, it is very likely or probable that the true effect lies close to the estimated finding, and a recommendation can be made, while low or very low certainty of evidence indicates that our confidence in the result is limited or very weak, respectively (2).

Supplementary information 2

Risk conversions

For studies that reported the mortality in tertiles of sex hormones, the hazard ratios (HRs) or risk ratios (RRs) were included as reported. For studies that grouped the exposure in quartile, quintile, high versus low of the marker, or reported the results as continuous, such as per SD or per unit, we converted the HRs or RRs from the original studies to the HR or RR for the third vs. bottom tertile of sex hormones distribution using defined methods (3). Based on this method, to convert log relative risks from the reported scale in the studies to top versus bottom third, we need to use conversion factors driving based on the ratio of expected differences in mean levels of the standardized exposure, for the target comparison versus reported comparison.

For example, a factor of 2.54 is the difference in the means of the upper and lower quartiles and the expected difference in means of the top versus bottom thirds of the standard normal distribution is 2.18. So, by applying a multiplication conversion factor of 2.18/2.54 to the log relative risk and its standard error, we can convert the top versus bottom quartile comparison to correspond to top versus bottom third comparison. Similarly, a conversion factor of 2.18 and 2.18 * SD are used to obtain the risk for the third versus first tertile for the estimations reported per SD and unit increment of exposure, respectively. The multiplicative conversion factors

of 2.18/2.80 and 2.18/1.59 were used to transform the RR for the top versus the bottom quintiles and high versus low levels of hormones. This method has been widely used in previous publications (4-9).

Dose-response analysis

For the linear dose-response meta-analyses, we estimated summary HRs and 95%CI for each prospective cohort study according to the method developed by Crippa et al (10). We used the increment of 5 nmol/l of TT in males and 0.5 nmol/l in females, 50 pmol/l of FT in males, 5 nmol/l of SHBG in males, and 30 pmol/l of E2 in males. We chose these units close to the mean of standard deviations of each hormone in all studies. We then used random-effects models to pool the individual study results. For these analyses, the median levels of sex hormones per each category, number of individuals with the mortality events and participants or person years, and adjusted effect estimates across at least two categories of exposures were extracted from each primary cohort study. For studies reporting exposures as a range in each category, the midpoint of the lower and upper bounds was used as a proxy for the median. The widths of the open-ended categories were considered equal to the adjacent categories.

One stage weighted mixed effects meta-analysis was used to explore curvilinear dose-response associations (10). We modelled the exposures by using restricted cubic splines with three knots, according to Harrell's recommended centiles (10%, 50%, and 90%) of the distribution (11). The correlation within each category of published risk estimates was taken into account and the estimates specific to each study were combined in a one stage weighted mixed effects meta-analysis (10). This method estimates the slope lines specific to each study and combines them to obtain an overall average slope in one stage (12, 13).

Table S2. Assessment methods of hormones and SHBG.

Author, year	Country	Study name	Design	Sex	Sex hormones	Assessment methods
Araujo et al, 2007 (14)	US	Massachusetts Male Aging Study	Prospective cohort	M	TT, FT, SHBG	*TT, SHBG*: Radioimmunoassay. *FT*: Calculated from testosterone and SHBG using the mass action equations.
Barrett-Connor et al, 1995 (15)	US	Rancho Bernardo Study	Prospective cohort	F	DHEAs	*SHBG*: Radioimmunoassay.
Barrett-Connor et al, 1986 (16)	US	Rancho Bernardo Study	Prospective cohort	M	DHEAs	*SHBG*: Radioimmunoassay.
Baylis et al, 2012 (17)	UK	Hertfordshire Ageing Study	Prospective cohort	B	TT, SHBG, DHEAs	*DHEAs*: Enzyme-linked immunosorbent assay. *TT, SHBG*: NM.
Belladelli et al, 2021 (18)	US	NHANES (1999-2004)	Prospective cohort	M	E2	*E2:* Elecsys 2010 system.
Cappola et al, 2009 (19)	US	Cardiovascular Health Study	Prospective cohort	B	DHEAs	*DHEAs*: Competitive enzyme immunoassay kit.
Chan et al, 2016 (20)	Australia	Busselton Health Study	Prospective cohort	M	TT, FT, DHEA, E2	*TT, DHEA, E2*: Mass spectrometry. *FT*: An empirical formulae,
Cummings-Vaughn et al, 2011 (21)	US	NM	Prospective cohort	M	TT, BT	*TT, BT:* Radioimmunoassay.
Forti et al, 2012 (22)	Italy	Conselice Study of Brain Aging	Prospective cohort	B	DHEAs	*DHEAs*: Immunoenzymatic assay.
Gyawali et al, 2019 (23)	Australia	MAILES	Prospective cohort	M	TT, SHBG	*TT*: Stable isotope dilution LC-MS/MS. *SHBG*: Immunoassay.
Haring et al, 2010 (24)	Germany	SHIP	Prospective cohort	M	TT	*TT*: Immunoassay.

Table S2. Continued

Author, year	Country	Study name	Design	Sex	Sex hormones	Assessment methods
Holmboe et al, 2015 (25)	Denmark	MONICA I–III and Inter99	Prospective cohort	M	TT, FT, SHBG, E2	*TT*: Fluoroimmunoassay. *FT*: Calculated from testosterone and SHBG using the mass action equations. *SHBG*, *E2*: Time-resolved immunofluorometric assay.
Hyde et al, 2011 (26)	Australia	Health in Men Study	Prospective cohort	M	SHBG	*SHBG*: Immunoassay.
Jia et al, 2020 (27)	US	ARIC study	Prospective cohort	M	DHEAs	*DHEAs*: Immunoassay.
Jia et al, 2020 (27)	US	ARIC study	Prospective cohort	F	DHEAs	*DHEAs*: Immunoassay
Feldman et al, 2000 (28)	US	Massachusetts Male Aging Study	Prospective cohort	M	DHEAs	*DHEAs*: Radioimmunoassay.
Haring et al, 2012 (29)	Germany	SHIP	Prospective cohort	M	TT	*TT*: Immunoassay.
Islam et al, 2022 (30)	Australia	ASPREE trial	Follow-up of a clinical trial	F	TT, DHEA, SHBG	*TT, DHEA, SHBG*: Immunoassay.
Lopez et al, 2022 (31)	US	NHANES (1988–1991,1999–2004,2011–2014)	Prospective cohort	M	TT, FT	*TT*: Electrochemiluminescence immunoassays. *FT*: Vermeulen formula.
Laouali et al, 2020 (32)	France	Three-City study	Prospective cohort	M	TT, BT	*TT*: Radioimmunoassay (RIA) method with an Orion Diagnostica device. *BT*: An indirect method.
Laouali et al, 2018 (33)	France	Three-City study	Prospective cohort	M	TT, BT	*TT*: Radioimmunoassay (RIA) method with an Orion Diagnostica device. *BT*: An indirect method.
Menke et al, 2010 (34)	US	NHANES (1988-1991)	Prospective cohort	M	E2, BT, SHBG	*E2*, *SHBG*: Competitive electrochemiluminescence immunoassays. *BT*: Vermeulen formula.

Table S2. Continued

Author, year	Country	Study name	Design	Sex	Sex hormones	Assessment methods
Laouali et al, 2018 (35)	France	Three-City study	Prospective cohort	M	E2	*E2*: Sensitive direct radioimmunoassay (RIA) with an Orion Diagnostica device.
Ohlsson et al, 2010 (36)	Sweden	MrOS Sweden cohort	Prospective cohort	M	DHEA, DHEAs	*DHEA, DHEAs*: Liquid chromatography-mass spectrometry.
Maggio et al, 2009 (37)	Italy	In-CHIANTI	Prospective cohort	F	E2	*E2*: Mass action equations.
Maggio et al, 2007 (38)	Italy	In-CHIANTI	Prospective cohort	M	BT, DHEAs	*BT*: Vermeulen formula. *DHEAs*: Radioimmunologic kits.
Laughlin et al, 2008 (39)	US	Rancho Bernardo study	Prospective cohort	M	TT, BT	*TT, BT*: Tremblay and Dube ammoniumsulfate precipitation method.
Szulc et al, 2009 (40)	France	MINOS	Prospective cohort	M	TT	*TT*: Tritiated Radioimmunoassay (RIA).
Zeller et al, 2019 (41)	Finland	FINRISK97 study	Prospective cohort	F	TT	*TT*: Chemiluminescent microparticle immunoassay (CMIA).
Zeller et al, 2019 (41)	Finland	FINRISK97 study	Prospective cohort	M	TT	*TT*: Chemiluminescent microparticle immunoassay (CMIA).
Wang et al, 2021 (42)	UK	UK Biobank	Prospective cohort	F	TT, FT, SHBG	*TT*: Chemiluminescent immunoassays. *FT*: Calculated by a validated algorithm based on TT, SHBG, albumin, and the association constants for the binding of testosterone to SHBG and albumin. *SHBG*: Chemiluminescent immunoassays.
Wang et al, 2021 (42)	UK	UK Biobank	Prospective cohort	M	TT, FT, SHBG	*TT*: Chemiluminescent immunoassays. *FT*: Calculated by a validated algorithm based on TT, SHBG, albumin, and the association constants for the binding of testosterone to SHBG and albumin. *SHBG*: Chemiluminescent immunoassays.

Table S2. Continued

Author, year	Country	Study name	Design	Sex	Sex hormones	Assessment methods
Yeap et al, 2014 (43)	Australia	Health In Men Study	Prospective cohort	M	TT, FT, E2	*TT, E2*: Single LC-MS/MS run without derivatization using atmospheric pressure photoionization in positive mode for androgens and negative mode for estrogens. *FT*: An empirical formula.
Vikan et al, 2009 (44)	Norway	Tromsø Study	Prospective cohort	M	TT, E2, FT	*TT, E2*: Immulite 2000. *FT*: Vermeulen formula.
Trivedi et al, 2001 (45)	England	Cambridge General Practice Study	Prospective cohort	M	DHEAs	*DHEAs*: RIA using a commercial kit.
Trivedi et al, 2001 (45)	England	Cambridge General Practice Study	Prospective cohort	F	DHEA	*DHEAs*: RIA using a commercial kit.
Tivesten et al, 2009 (46)	Sweden	MrOS Sweden cohort	Prospective cohort	M	TT, FT, E2, SHBG	*TT, E2, SHBG*: Specific gas chromatography-mass spectrometry. *FT*: Vermeulen formula.
Srinath et al, 2015 (47)	US	ARIC study	Prospective cohort	M	TT	*TT*: Liquid chromatography mass spectrometry
Shores et al, 2014 (48)	US	Cardiovascular Health Study	Prospective cohort	M	TT, FT	*TT*: Liquid chromatography-tandem mass spectrometry Assay. *FT*: Mazer formula.
Schaffrath et al, 2015 (49)	Germany	SHIP	Prospective cohort	F	TT, FT, SHBG	*TT*: Liquid chromatography–tandem mass spectrometry. *FT*: Vermeulen formula. *SHBG*: Radioimmunoassay.
Pye et al, 2014 (50)	Multiple countrie*	European Male Aging Study	Prospective cohort	M	TT, FT	*TT*: Gas chromatography-mass spectrometry *FT*: Vermeulen formula..
Sievers et al, 2010 (51)	Germany	DETECT study	Prospective cohort	F	TT	*TT*: Immunoassay.

Table S2. Continued

Author, year	Country	Study name	Design	Sex	Sex hormones	Assessment methods
Kalme et al, 2005 (52)	Finland	Seven Countries Study	Prospective cohort	M	SHBG	*SHBG*: Immunofluorometric assay
Khaw et al, 2007 (53)	UK	EPIC-Norfolk	Nested case-control**	M	TT	*TT*: Immulite Chemiluminescent Immunoassay System
Haring et al, 2013 (54)	USA	Framingham Heart Study	Prospective cohort	M	TT, DHEAs, E2	*TT, DHEAs, E2:* Radioimmunoassay
Hyde et al, 2012 (55)	Australia	Health In Men Study	Prospective cohort	M	TT, FT, SHBG	*TT, SHBG*: Chemiluminescent immunoassays. *FT*: Vermeulen formula.
Lin et al, 2011 (56)	USA	NHANES III (1988–1991)	Prospective cohort	M	FT	*FT*: Vermeulen formula.
Benn et al, 2015 (57)	Denmark	Copenhagen City Heart Study	Nested case-control**	F	TT, E2	*TT, E2*: Immunoassay.
Enomoto et al, 2008 (58)	Japan	Tanushimaru Study	Prospective cohort	M	DHEAs	*DHEAs*: Radioimmunoassay.
Enomoto et al, 2008 (58)	Japan	Tanushimaru Study	Prospective cohort	F	DHEAs	*DHEAs*: Radioimmunoassay.
Guadalupe-Grau et al, 2017 (59)	Spain	Toledo Study for Healthy Aging	Prospective cohort	F	TT, DHEA, DHEAs, E2	*TT, DHEA, DHEAs, E2*: Enzyme-linked immunosorbent assay.
Guadalupe-Grau et al, 2017 (59)	Spain	Toledo Study for Healthy Aging	Prospective cohort	M	TT, DHEA, DHEAs, E2	*TT, DHEA, DHEAs, E2*: Enzyme-linked immunosorbent assay.
Goodman-Gruen et al, 1995 (60)	USA	Rancho Bernardo study	Prospective cohort	F	SHBG	*SHBG:* NM.
Goodman-Gruen et al, 1995 (60)	USA	Rancho Bernardo study	Prospective cohort	M	SHBG	*SHBG:* NM
Schederecker et al, 2020 (61)	Germany	KORA-F4	Prospective cohort	M	SHBG, TT, FT	*SHBG*: Immunoassay. *TT*: Mass-spectrometry. *FT*: Vermeulen formula.

Table S2. Continued

Author, year	Country	Study name	Design	Sex	Sex hormones	Assessment methods
Schederecker et al, 2020 (61)	Germany	KORA-F4	Prospective cohort	F	SHBG, TT, FT	*SHBG*: Immunoassay. *TT*: Mass-spectrometry. *FT*: Vermeulen formula.
Smith et al, 2005 (62)	UK	Caerphilly Study	Prospective cohort	M	TT	*TT*: Radioimmunoassay.
Antonio et al, 2022 (63)	Multiple countries*	European Male Aging Study	Prospective cohort	M	SHBG, E2	*SHBG*: Electrochemiluminescence immunoassay. *E2*: Gas Chromatography-tandem mass spectrometry.
Li et al, 2024 (64)	China	GBCS	Prospective cohort	M	TT, FT, BT, SHBG	*TT*: ARCHITECT 2nd Generation Testosterone Reagent Kit. *FT, BT*: Vermeulen formula. *SHBG*: Immulite 2000.
Mukama et al, 2023 (65)	Germany	EPIC-Heidelberg	Nested case cohort	M	DHEAs	*DHEAs*: Radioimmunoassay
Mukama et al, 2023 (65)	Germany	EPIC-Heidelberg	Nested case cohort	F	DHEAs	*DHEAs*: Radioimmunoassay
Appiah et al, 2022 (66)	USA	NHANES (1988–1991)	Prospective cohort	M	E2	*E2*: Electrochemiluminescence immunoassays

TT: Total testosterone; FT: Free testosterone; SHBG: Sex hormone binding globulin ; DHEA: Dehydroepiandrosterone; DHEAs: Dehydroepiandrosterone sulfate; E2: Estradiol; NHANES: National Health and Nutrition Examination Survey; MAILES: Men Androgen Inflammation Lifestyle Environment and Stress cohort study; ARIC: Atherosclerosis Risk in Communities study; SHIP: Study of Health in Pomerania; DETECT: Diabetes Cardiovascular Risk-Evaluation: Targets and Essential Data for Commitment of Treatment; NM: Not mentioned.

* Florence, Italy; Leuven, Belgium; Malmö, Sweden; Manchester, United Kingdom; Santiago de Compostela, Spain; Lódz, Poland; Szeged, Hungary; Tartu, Estonia. ** prospective population study

Table S3. ROBINS-E judgement for each domain and overall.

First Author, year (ref)	Bias due to confounding	Bias due to exposure assessment	Bias due to selection of participants	Bias due to post-exposure interventions	Bias due to missing data	Bias due to measurement of the outcome	Bias due to selective reporting of the results	Overall judgement
Araujo et al, 2007 (14)	Some concerns	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	Some concerns
Barrett-Connor et al, 1995 (15)	High risk	Low risk	Low risk	Low risk	Some concerns	Low risk	High risk	High risk
Barrett-Connor et al, 1987 (16)	High risk	Low risk	Low risk	Low risk	Some concerns	Low risk	High risk	High risk
Baylis et al, 2012 (17)	High risk	Low risk	Low risk	Low risk	High risk	Low risk	Low risk	High risk
Belladelli et al, 2021(18)	High risk	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	High risk
Cappola et al, 2009 (19)	High risk	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	High risk
Chan et al, 2016 (20)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Cummings-Vaughn et al, 2011 (21)	High risk	Low risk	Low risk	Low risk	Some concerns	Low risk	High risk	High risk
Forti et al, 2012 (22)	Some concerns	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	Some concerns
Gyawali et al, 2019 (23)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Haring et al, 2010 (24)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Holmboe et al, 2015 (25)	High risk	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	High risk
Hyde et al, 2011 (26)	High risk	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	High risk
Jia et al, 2020 (27)	Some concerns	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	Some concerns

Table S3. Continued

First Author, year (ref)	Bias due to confounding	Bias due to exposure assessment	Bias due to selection of participants	Bias due to post-exposure interventions	Bias due to missing data	Bias due to measurement of the outcome	Bias due to selective reporting of the results	Overall judgement
Jia et al, 2020 (27)	Some concerns	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	Some concerns
Feldman et al, 2000 (28)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Haring et al, 2012 (29)	Some concerns	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	Some concerns
Islam et al, 2022 (30)	Low risk	Low risk	Low risk	Some concerns	Some concerns	Low risk	Some concerns	Some concerns
Smith et al, 2005 (67)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Lopez et al, 2022 (68)	Some concerns	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Some concerns
Laouali et al, 2020 (69)	Some concerns	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Some concerns
Laouali et al, 2018 (33)	High risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	High risk
Menke et al, 2010 (70)	Some concerns	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	Some concerns
Laouali et al, 2018 (71)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Ohlsson et al, 2010 (72)	High risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	High risk
Maggio et al, 2009 (73)	Some concerns	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Some concerns
Maggio et al, 2007 (74)	Some concerns	Low risk	Low risk	Low risk	Low risk	Low risk	High risk	High risk
Laughlin et al, 2008 (75)	Some concerns	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Some concerns
Szulc et al, 2009 (76)	Some concerns	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Some concerns
Zeller et al, 2019 (41)	Some concerns	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Some concerns

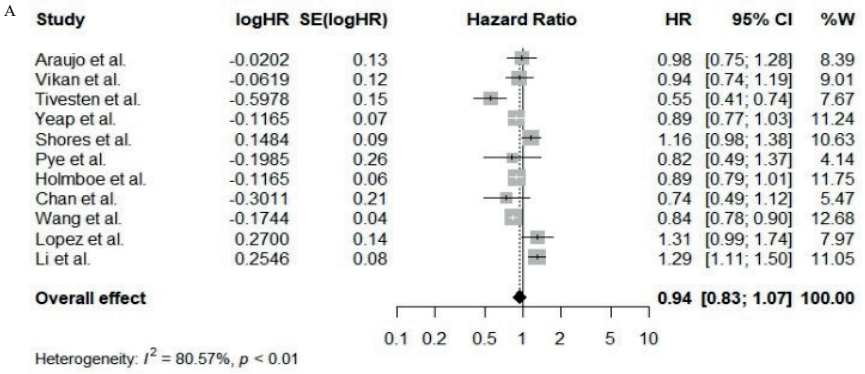
Table S3. Continued

First Author, year (ref)	Bias due to confounding	Bias due to exposure assessment	Bias due to selection of participants	Bias due to post-exposure interventions	Bias due to missing data	Bias due to measurement of the outcome	Bias due to selective reporting of the results	Overall judgement
Zeller et al, 2019 (41)	Some concerns	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Some concerns
Wang et al, 2021 (42)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Wang et al, 2021 (42)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Yeap et al, 2014 (43)	Some concerns	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	Some concerns
Vikan et al, 2009 (44)	Some concerns	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	Some concerns
Trivedi et al, 2001 (45)	High risk	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	High risk
Trivedi et al, 2001 (45)	High risk	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	High risk
Tivesten et al, 2009 (46)	High risk	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	High risk
Srinath et al, 2015 (47)	Some concerns	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	Some concerns
Shores et al, 2014 (48)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Schaffrath et al, 2015 (49)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Pye et al, 2014 (50)	High risk	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	High risk
Sievers et al, 2010 (51)	High risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	High risk
Kalme et al, 2005 (52)	High risk	Low risk	Some concerns	Low risk	Some concerns	Low risk	Low risk	High risk
Khaw et al, 2007 (53)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Haring et al, 2013 (54)	High risk	Low risk	Some concerns	Low risk	Some concerns	Low risk	Low risk	High risk
Hyde et al, 2012 (55)	High risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	High risk
Lin et al, 2011 (56)	High risk	Low risk	Some concerns	Low risk	Some concerns	Low risk	Low risk	High risk

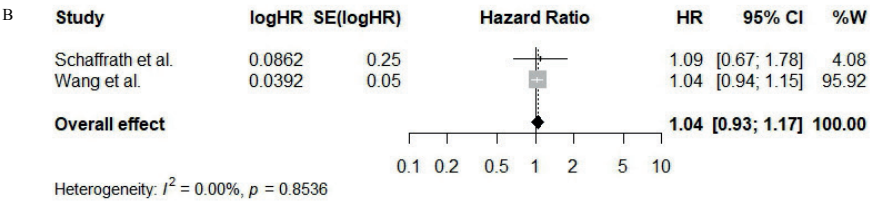
Table S3. Continued

First Author, year (ref)	Bias due to confounding	Bias due to exposure assessment	Bias due to selection of participants	Bias due to post-exposure interventions	Bias due to missing data	Bias due to measurement of the outcome	Bias due to selective reporting of the results	Overall judgement
Benn et al, 2015 (57)	High risk	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	High risk
Enomoto et al, 2008 (58)	High risk	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	High risk
Enomoto et al, 2008 (58)	High risk	Low risk	Some concerns	Low risk	Low risk	Low risk	Low risk	High risk
Guadalupe-Grau et al, 2017 (59)	High risk	Low risk	Some concerns	Low risk	Some concerns	Low risk	Low risk	High risk
Guadalupe-Grau et al, 2017 (59)	High risk	Low risk	Some concerns	Low risk	Some concerns	Low risk	Low risk	High risk
Goodman-Gruen et al, 1995 (60)	High risk	Low risk	Some concerns	Low risk	Some concerns	Low risk	Low risk	High risk
Goodman-Gruen et al, 1995 (60)	High risk	Low risk	Some concerns	Low risk	Some concerns	Low risk	Low risk	High risk
Schederecker et al, 2020 (61)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Schederecker et al, 2020 (61)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Appiah et al, 2022 (66)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Antonio et al, 2022 (63)	High risk	Low risk	Some concerns	Low risk	Some concerns	Low risk	Low risk	High risk
Li et al, 2024 (64)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Mukama et al, 2023 (65)	Some concerns	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Some concerns

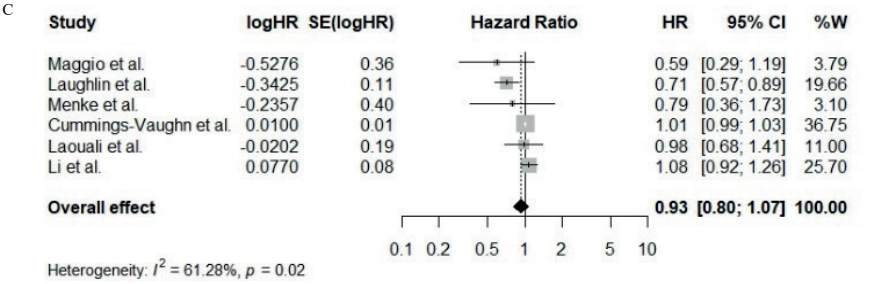
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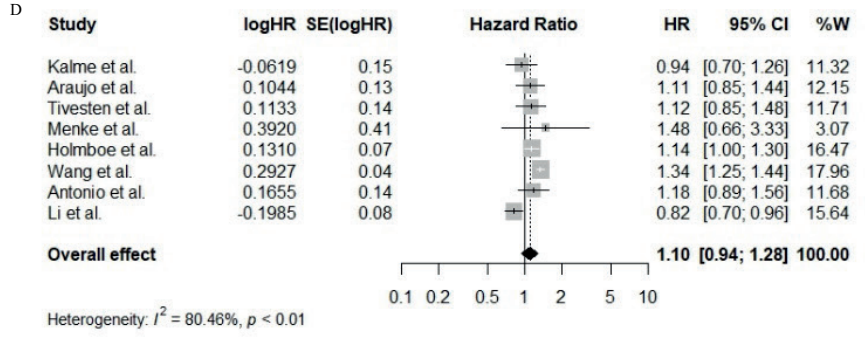
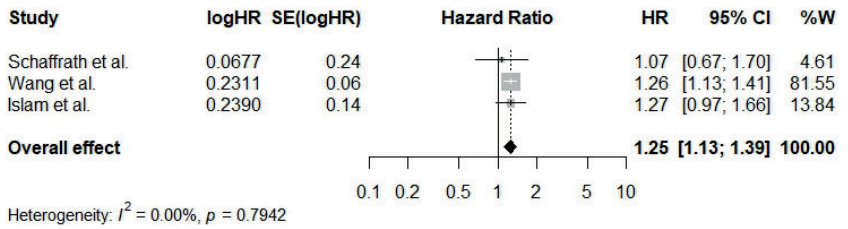
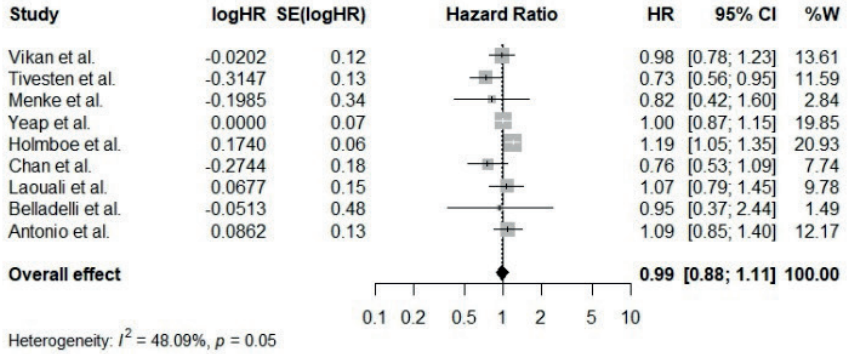


Figure S1.

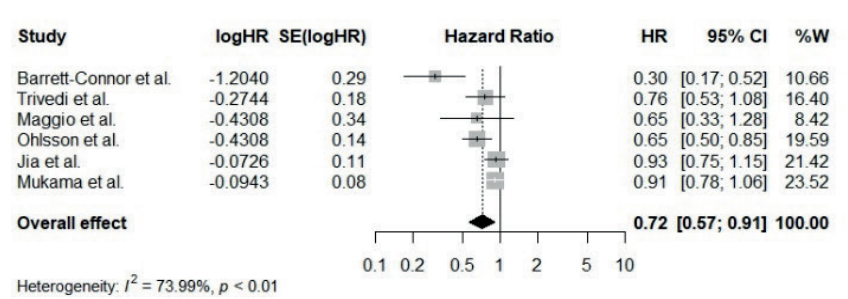
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F



G



H

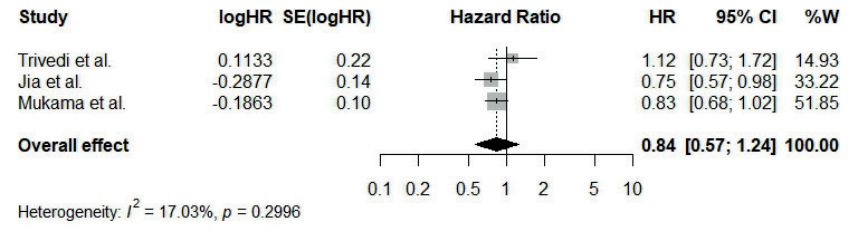
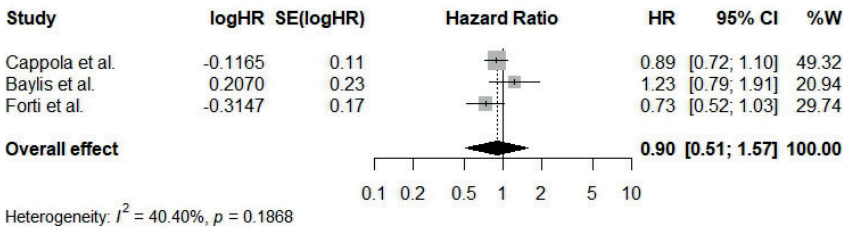


Figure S1.

I



J

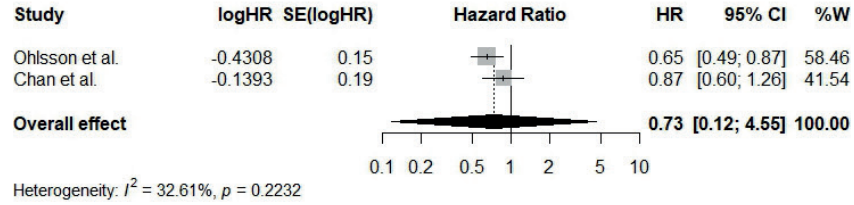
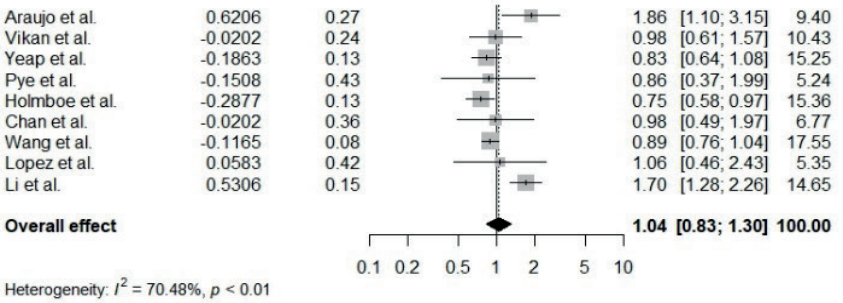
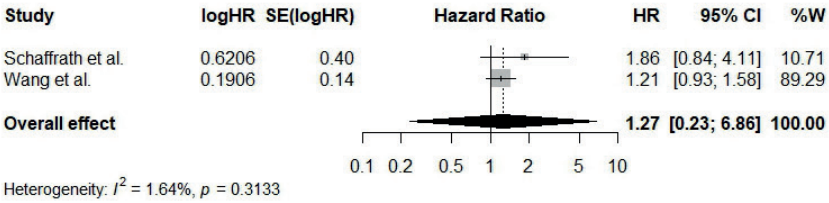


Figure S1. Summary hazard ratios of all-cause mortality for top versus bottom third of **A**) FT-Men **B**) FT-Women; **C**) BT-Men; **D**) SHBG-Men; **E**) SHBG-Women; **F**) E2-Men; **G**) DHEAs-Men; **H**) DHEAs-Women; **I**) DHEAs-Both; **J**) DHEA-Men.

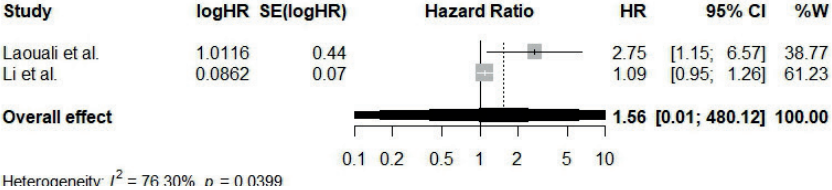
A



B



C



D

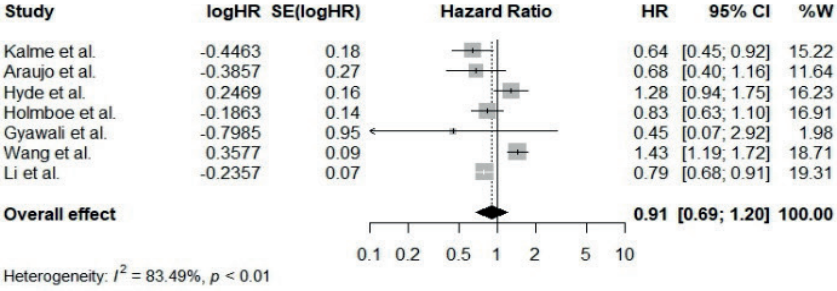


Figure S2.

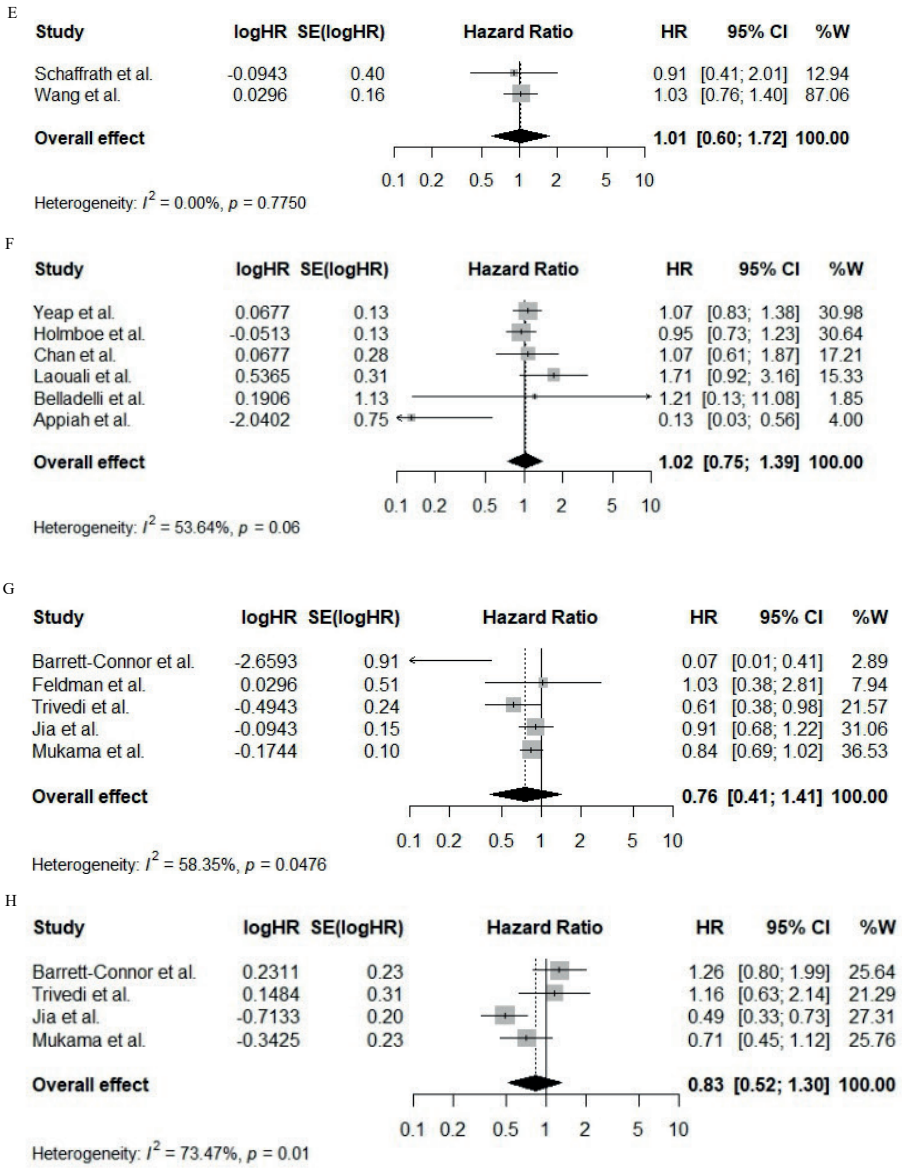
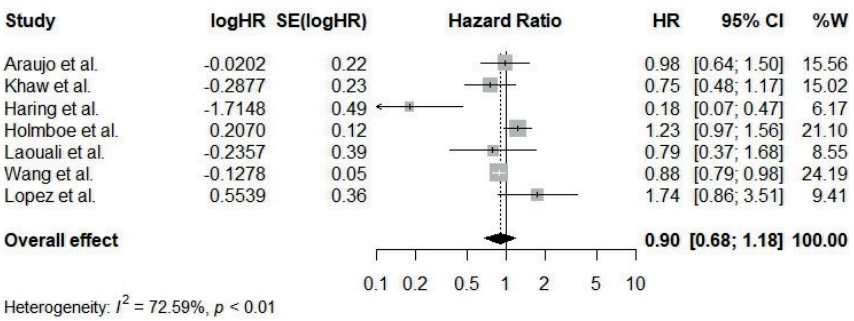
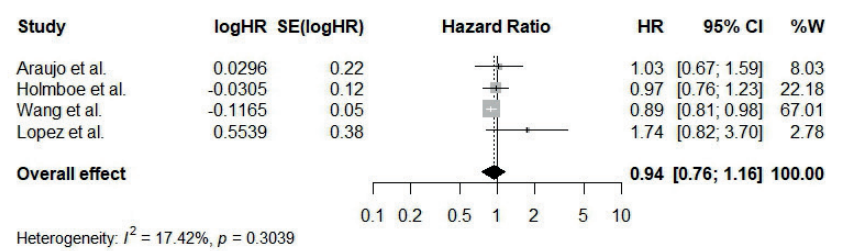


Figure S2. Summary hazard ratios of CVD mortality for top versus bottom third of **A**) FT-Men **B**) FT-Women; **C**) BT-Men; **D**) SHBG-Men; **E**) SHBG-Women; **F**) E2-Men; **G**) DHEAs-Men; **H**) DHEAs-Women.

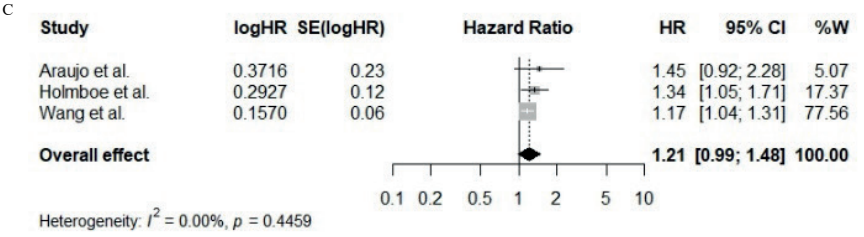
A



B



C



D

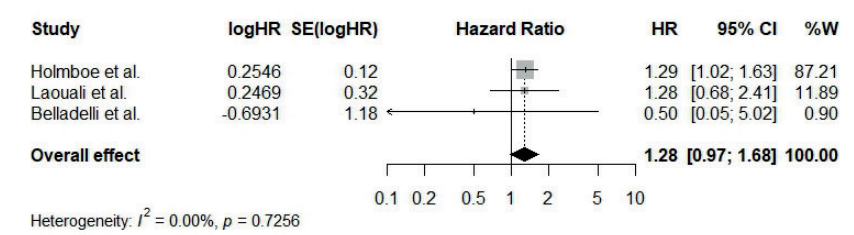


Figure S3. Summary hazard ratios of cancer mortality for top versus bottom third of **A**) TT-Men; **B**) FT-Men; **C**) SHBG-Men; **D**) E2-Men.

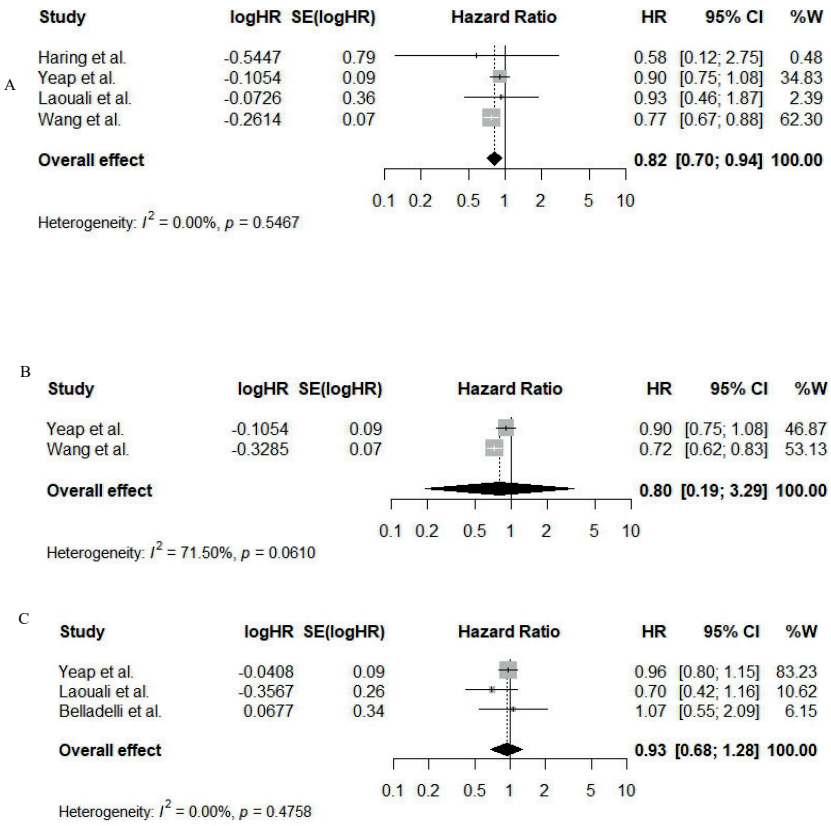


Figure S4. Summary hazard ratios of other causes of mortality for top versus bottom third of **A**) TT-Men; **B**) FT-Men; **C**) E2-Men.

Supplementary information 3

Findings of leave-one-out analyses are presented in **Figure S5-S8**. Briefly, for TT, the association with risk of CVD mortality and death due to the other causes in men; for FT, the association with risk of all-cause, CVD and cancer mortality in men; for BT, the association with the risk of all-cause mortality in men; for SHBG, the association with the risk of all-cause mortality in women; and for DHEAs, the association with the risk of all-cause mortality in the whole population were not robust in leave-one-out analysis. An asymmetric funnel plot was observed for the association between TT and all-cause mortality in men (Egger's p-values: 0.003, **Figure S9-A**), while no indication of publication bias was observed for other analysis. The findings of subgroup analysis for the association between TT and FT and risk of all-cause mortality and association between TT and CVD mortality in

men are summarized in **Table S3**. Subgroup analysis did not identify any factors that substantially contributed to heterogeneity, except whether the included studies adjusted for hypertension or not ($p_{\text{between group}} < 0.05$).

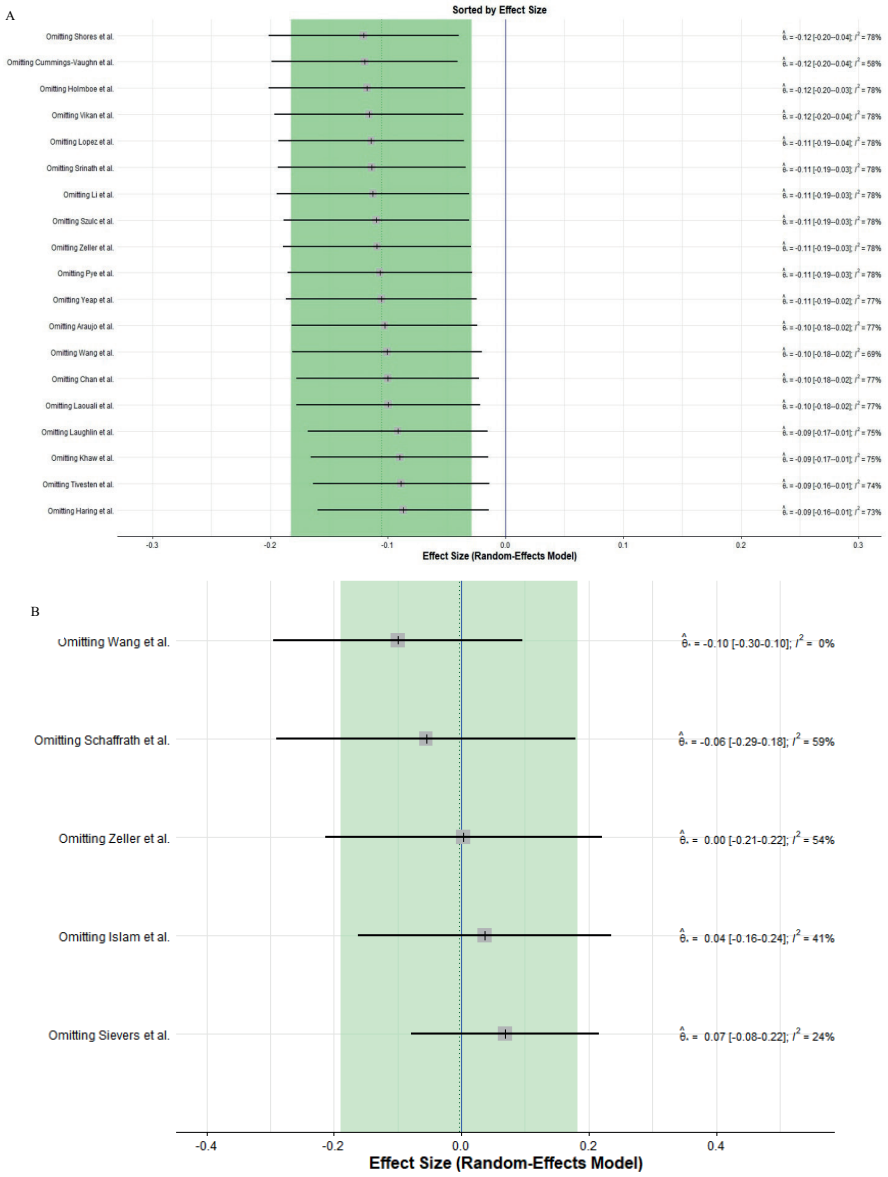


Figure S5.

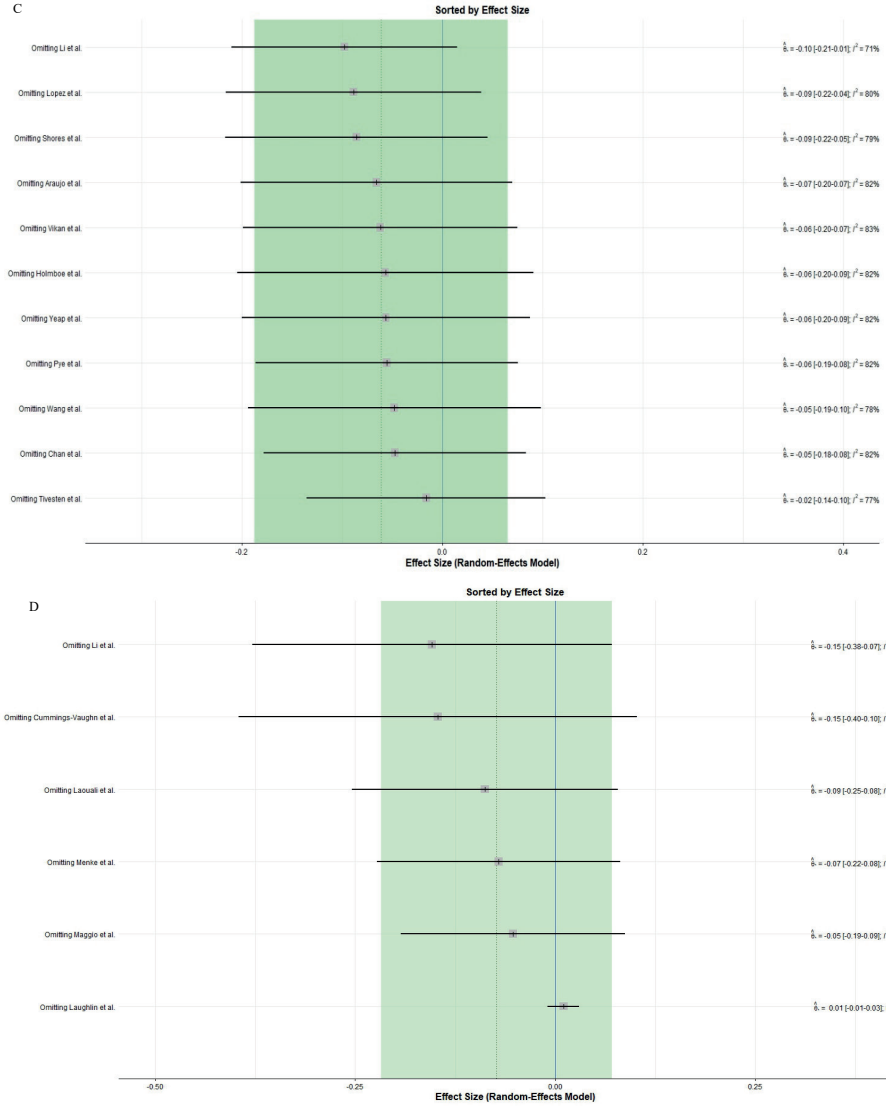

C
Sorted by Effect Size
Omitting Li et al.
Omitting Lopez et al.
Omitting Shores et al.
Omitting Araujo et al.
Omitting Vikan et al.
Omitting Holmboe et al.
Omitting Yeap et al.
Omitting Pye et al.
Omitting Wang et al.
Omitting Chan et al.
Omitting Tivesten et al.
θ̂ = -0.10 [-0.21-0.01]; I² = 71%
θ̂ = -0.09 [-0.22-0.04]; I² = 80%
θ̂ = -0.09 [-0.22-0.05]; I² = 79%
θ̂ = -0.07 [-0.20-0.07]; I² = 82%
θ̂ = -0.06 [-0.20-0.07]; I² = 83%
θ̂ = -0.06 [-0.20-0.09]; I² = 82%
θ̂ = -0.06 [-0.20-0.09]; I² = 82%
θ̂ = -0.06 [-0.19-0.08]; I² = 82%
θ̂ = -0.05 [-0.19-0.10]; I² = 78%
θ̂ = -0.05 [-0.18-0.08]; I² = 82%
θ̂ = -0.02 [-0.14-0.10]; I² = 77%
-0.2
0.0
0.2
0.4
Effect Size (Random-Effects Model)
D
Sorted by Effect Size
Omitting Li et al.
Omitting Cummings-Vaughn et al.
Omitting Laouali et al.
Omitting Menke et al.
Omitting Maggio et al.
Omitting Laughlin et al.
θ̂ = -0.15 [-0.38-0.07]; I² = 67%
θ̂ = -0.15 [-0.40-0.10]; I² = 64%
θ̂ = -0.09 [-0.25-0.08]; I² = 69%
θ̂ = -0.07 [-0.22-0.08]; I² = 68%
θ̂ = -0.05 [-0.19-0.09]; I² = 63%
θ̂ = 0.01 [-0.01-0.03]; I² = 0%
-0.50
-0.25
0.00
0.25
Effect Size (Random-Effects Model)

Figure S5.

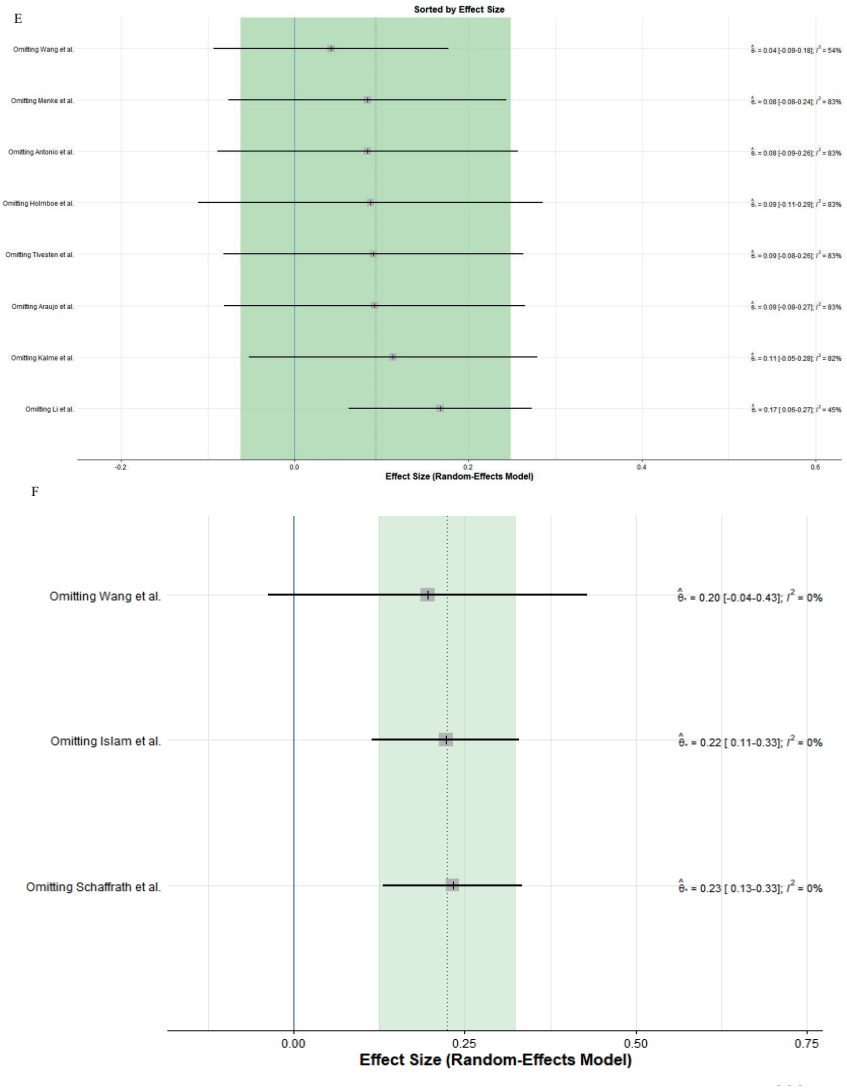

E
Sorted by Effect Size
Omitting Wang et al.
Omitting Menke et al.
Omitting Antonio et al.
Omitting Holmboe et al.
Omitting Thvesten et al.
Omitting Araujo et al.
Omitting Kalmé et al.
Omitting Li et al.
-0.2
0.0
0.2
0.4
0.6
Effect Size (Random-Effects Model)
F
Omitting Wang et al.
Omitting Islam et al.
Omitting Schaffrath et al.
0.00
0.25
0.50
0.75
Effect Size (Random-Effects Model)

Figure S5.

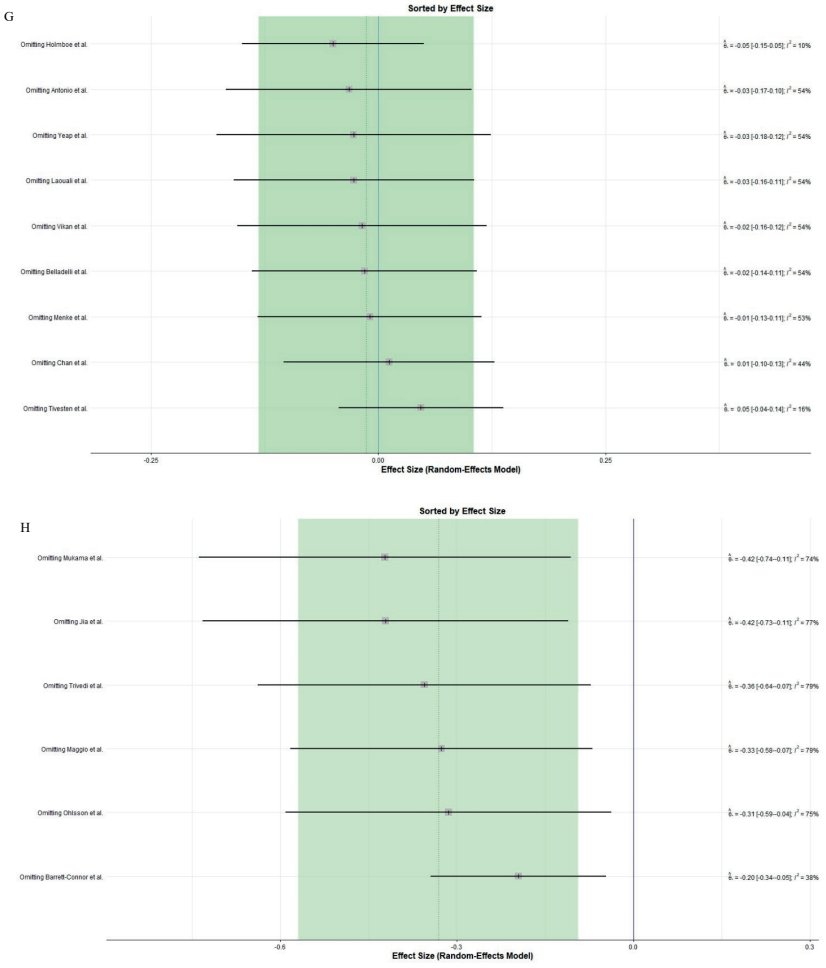

G
Sorted by Effect Size
Omitting Holmboe et al.
Omitting Antonio et al.
Omitting Yeap et al.
Omitting Laouali et al.
Omitting Vikan et al.
Omitting Belladelli et al.
Omitting Menke et al.
Omitting Chan et al.
Omitting Tivesten et al.
-0.25
0.00
0.25
Effect Size (Random-Effects Model)
H
Sorted by Effect Size
Omitting Mukama et al.
Omitting Jia et al.
Omitting Trivedi et al.
Omitting Maggio et al.
Omitting Ohlsson et al.
Omitting Barrett-Connor et al.
-0.6
-0.3
0.0
0.3
Effect Size (Random-Effects Model)

Figure S5.

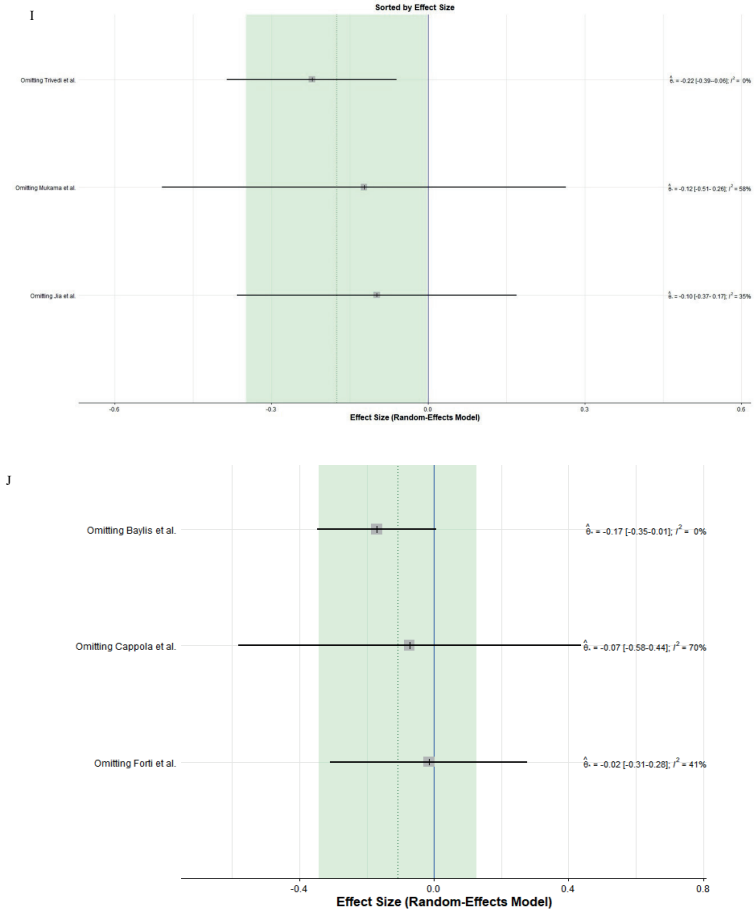


Figure S5. Leave-one-out forest plots of the included studies in the meta-analysis of sex hormones and all-cause mortality. **A**) TT-Men; **B**) TT-Women; **C**) FT-Men; **D**) BT-Men; **E**) SHBG-Men; **F**) SHBG-Women; **G**) E2-Men; **H**) DHEAs-Men; **I**) DHEAs-Women; **J**) DHEAs-Both.

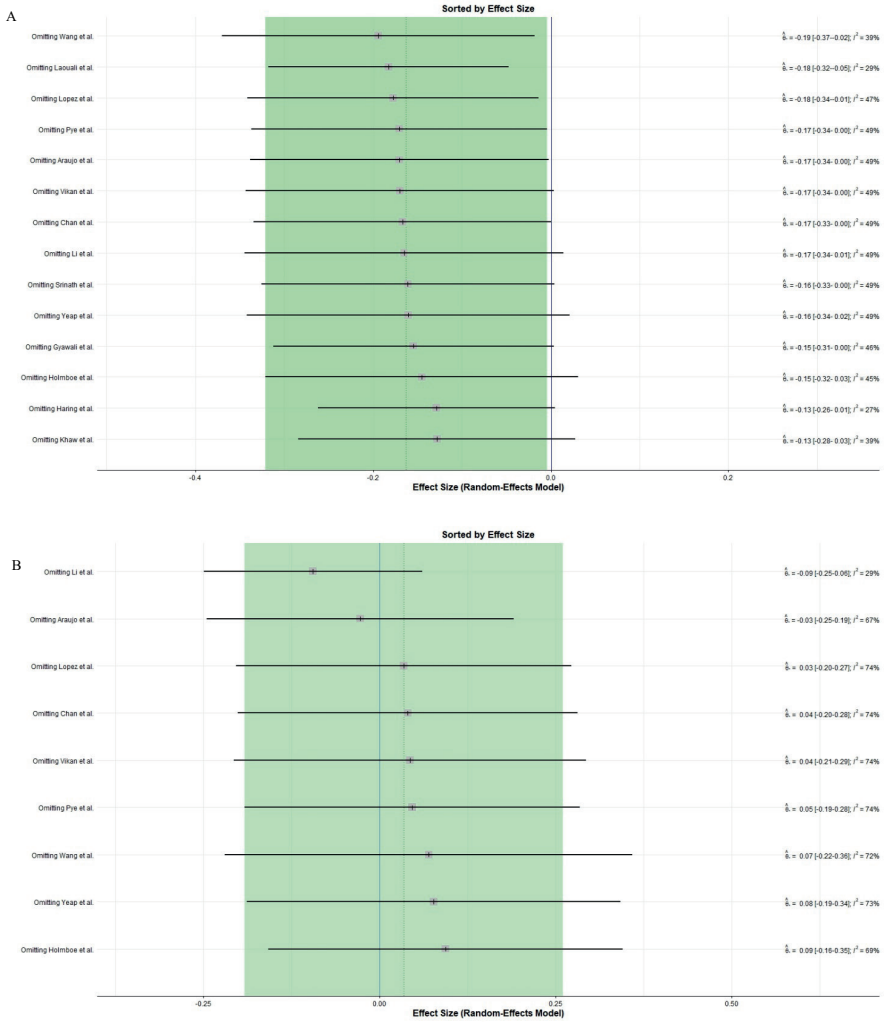

A
Sorted by Effect Size
Omitting Wang et al.
Omitting Laouali et al.
Omitting Lopez et al.
Omitting Pye et al.
Omitting Araujo et al.
Omitting Vikan et al.
Omitting Chan et al.
Omitting Li et al.
Omitting Srinath et al.
Omitting Yeap et al.
Omitting Gyawali et al.
Omitting Holmboe et al.
Omitting Haring et al.
Omitting Khaw et al.
-0.4
-0.2
0.0
0.2
Effect Size (Random-Effects Model)
B
Sorted by Effect Size
Omitting Li et al.
Omitting Araujo et al.
Omitting Lopez et al.
Omitting Chan et al.
Omitting Vikan et al.
Omitting Pye et al.
Omitting Wang et al.
Omitting Yeap et al.
Omitting Holmboe et al.
-0.25
0.00
0.25
0.50
Effect Size (Random-Effects Model)

Figure S6.

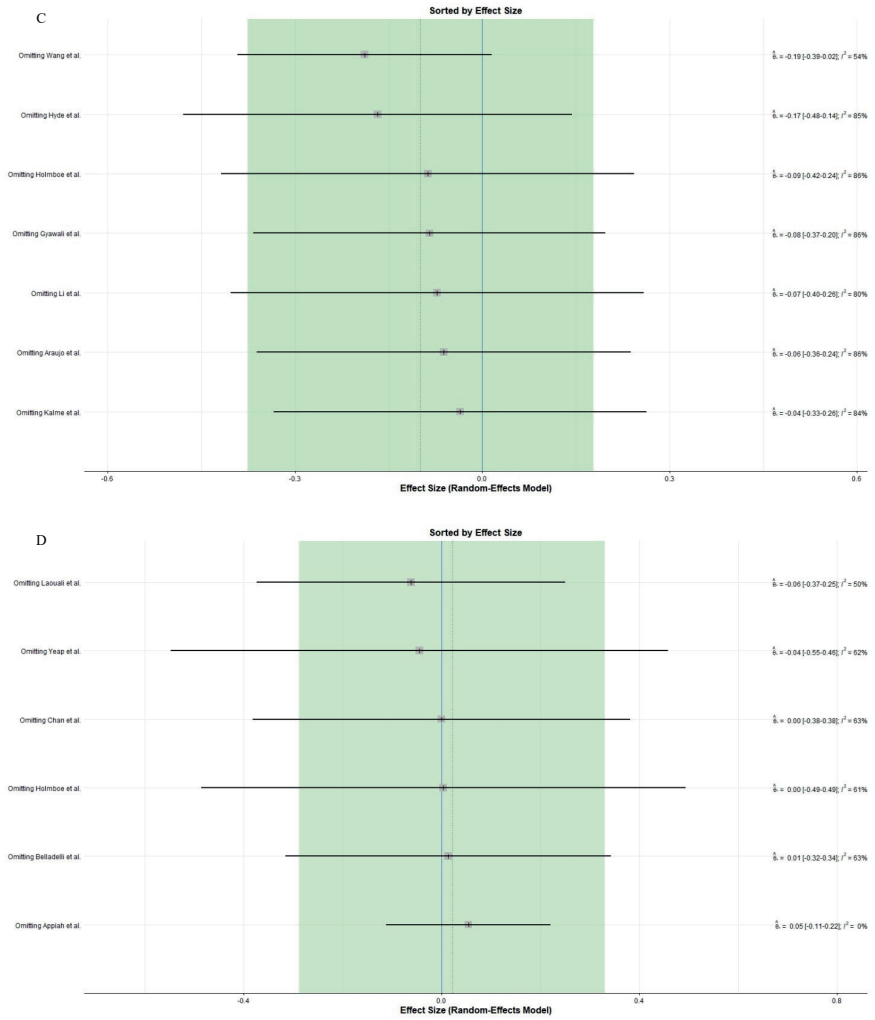

C
Sorted by Effect Size
Omitting Wang et al.
Omitting Hyde et al.
Omitting Holmboe et al.
Omitting Gyawali et al.
Omitting Li et al.
Omitting Araujo et al.
Omitting Kalme et al.
θ̂₀ = -0.19 [-0.39-0.02]; I² = 54%
θ̂₀ = -0.17 [-0.48-0.14]; I² = 85%
θ̂₀ = -0.09 [-0.42-0.24]; I² = 86%
θ̂₀ = -0.08 [-0.37-0.20]; I² = 86%
θ̂₀ = -0.07 [-0.40-0.26]; I² = 80%
θ̂₀ = -0.06 [-0.36-0.24]; I² = 86%
θ̂₀ = -0.04 [-0.33-0.26]; I² = 84%
-0.6
-0.3
0.0
0.3
0.6
Effect Size (Random-Effects Model)
D
Sorted by Effect Size
Omitting Laouali et al.
Omitting Yeap et al.
Omitting Chan et al.
Omitting Holmboe et al.
Omitting Belladelli et al.
Omitting Appiah et al.
θ̂₀ = -0.06 [-0.37-0.25]; I² = 50%
θ̂₀ = -0.04 [-0.55-0.46]; I² = 62%
θ̂₀ = 0.00 [-0.38-0.38]; I² = 63%
θ̂₀ = 0.00 [-0.49-0.49]; I² = 61%
θ̂₀ = 0.01 [-0.32-0.34]; I² = 63%
θ̂₀ = 0.05 [-0.11-0.22]; I² = 0%
-0.4
0.0
0.4
0.8
Effect Size (Random-Effects Model)

Figure S6.

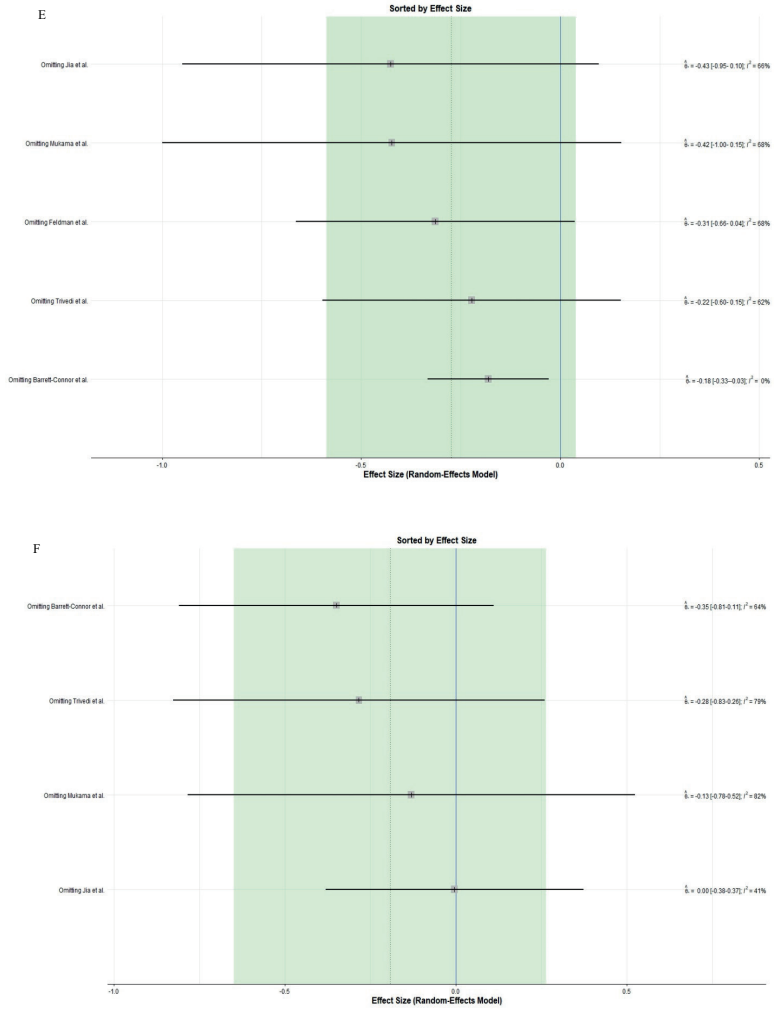


Figure S6. Leave-one-out forest plots of the included studies in the meta-analysis of sex hormones and CVD mortality. **A**) TT-Men; **B**) FT-Men; **C**) SHBG-Men; **D**) E2-Men; **E**) DHEAs-Men; **F**) DHEAs-Women.

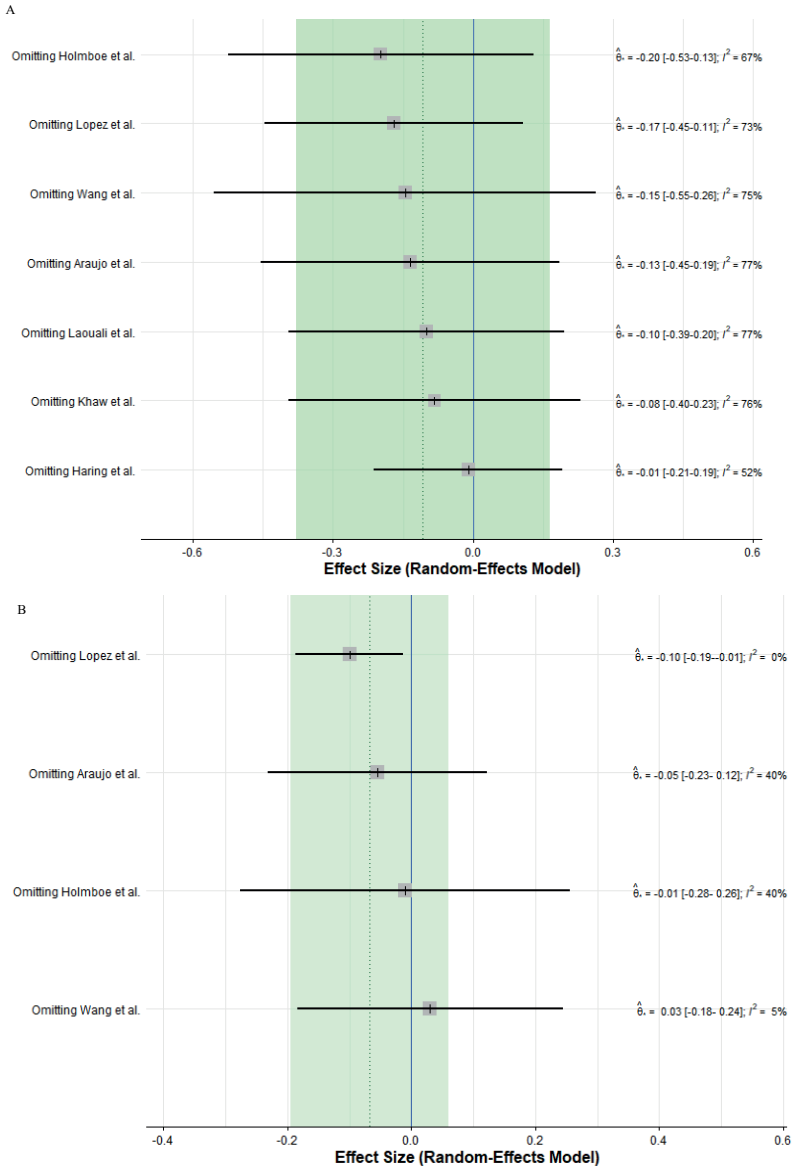

A
Omitting Holmboe et al.
Omitting Lopez et al.
Omitting Wang et al.
Omitting Araujo et al.
Omitting Laouali et al.
Omitting Khaw et al.
Omitting Haring et al.
θ̂. = -0.20 [-0.53-0.13]; I² = 67%
θ̂. = -0.17 [-0.45-0.11]; I² = 73%
θ̂. = -0.15 [-0.55-0.26]; I² = 75%
θ̂. = -0.13 [-0.45-0.19]; I² = 77%
θ̂. = -0.10 [-0.39-0.20]; I² = 77%
θ̂. = -0.08 [-0.40-0.23]; I² = 76%
θ̂. = -0.01 [-0.21-0.19]; I² = 52%
-0.6
-0.3
0.0
0.3
0.6
Effect Size (Random-Effects Model)
B
Omitting Lopez et al.
Omitting Araujo et al.
Omitting Holmboe et al.
Omitting Wang et al.
θ̂. = -0.10 [-0.19--0.01]; I² = 0%
θ̂. = -0.05 [-0.23- 0.12]; I² = 40%
θ̂. = -0.01 [-0.28- 0.26]; I² = 40%
θ̂. = 0.03 [-0.18- 0.24]; I² = 5%
-0.4
-0.2
0.0
0.2
0.4
0.6
Effect Size (Random-Effects Model)

Figure S7.

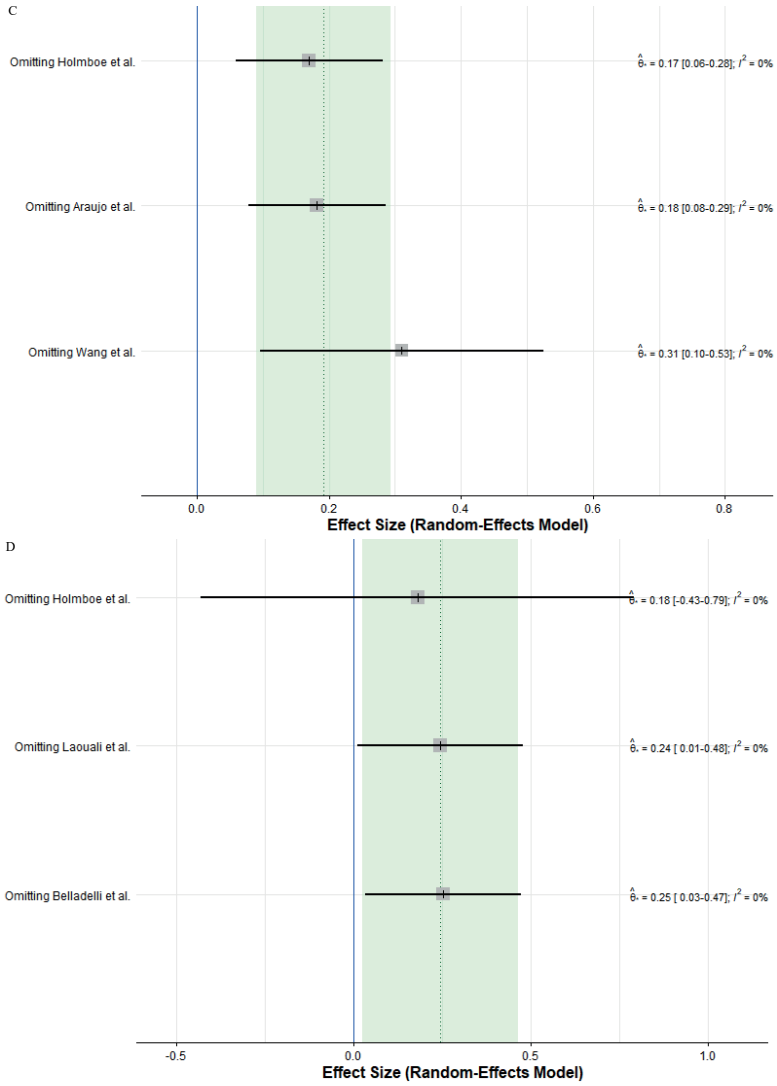


Figure S7. Leave-one-out forest plots of the included studies in the meta-analysis of sex hormones and Cancer mortality. **A**) TT-Men; **B**) FT-Men **C**) SHBG-Men; **D**) E2-Men.

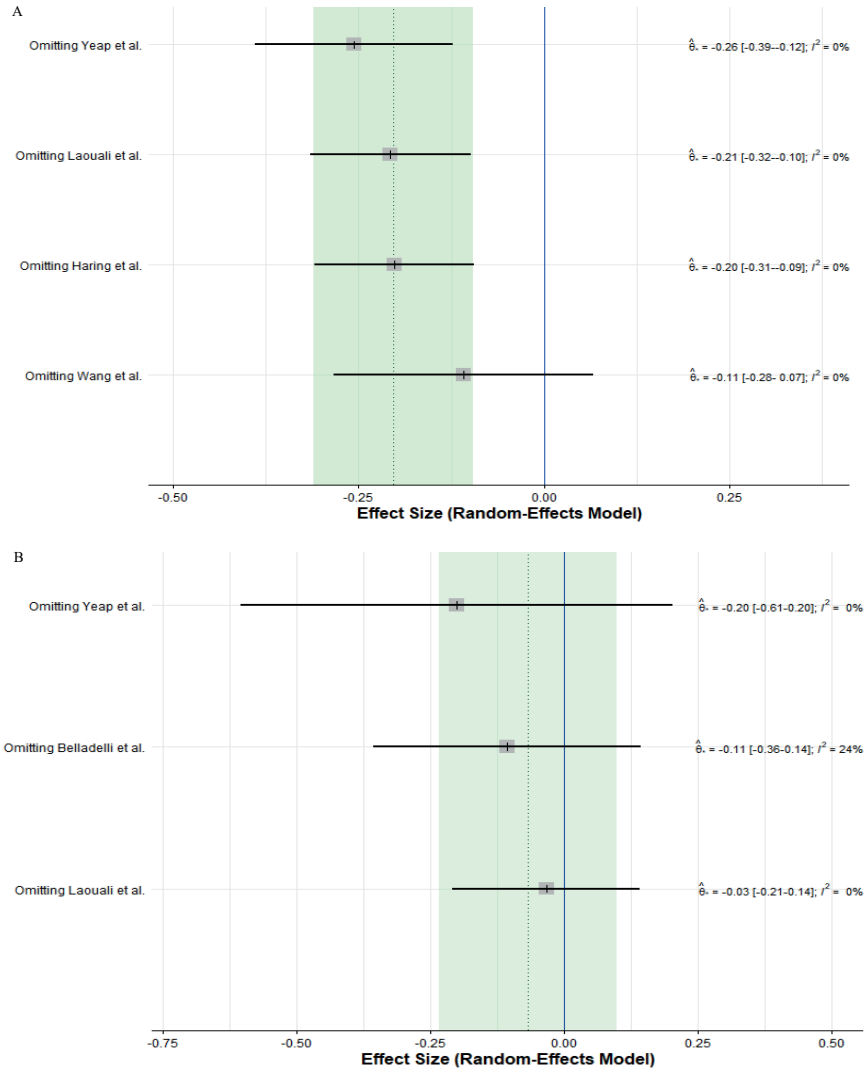


Figure S8. Leave-one-out forest plots of the included studies in the meta-analysis of sex hormones and other causes of mortality. **A**) TT-Men; **B**) E2-Men.

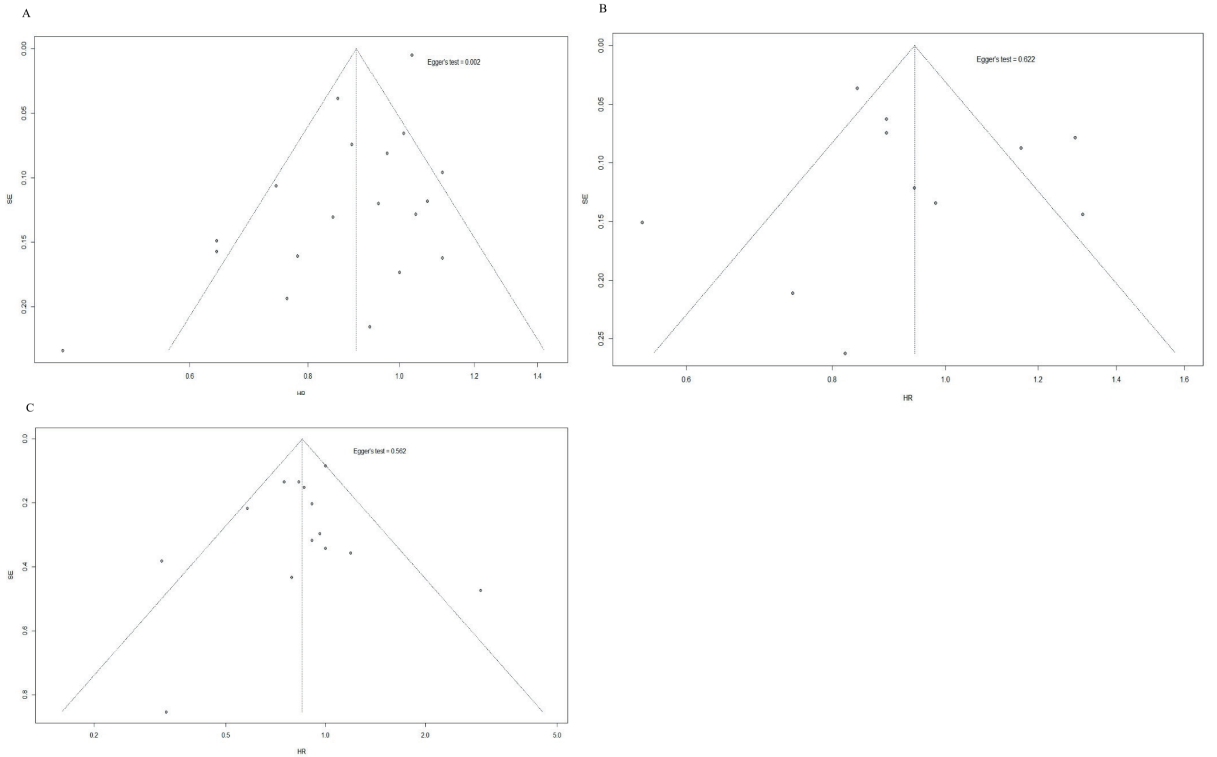


Figure S9. Funnel plots and Egger's test p-values of the included studies in the meta-analysis of sex hormones and all-cause mortality and CVD mortality. **A**) TT-Men; **B**) FT-Men; C) TT-Men (CVD mortality).

Table S4. Summary risk estimates of stratified analyses for association between TT and FT and risk of all-cause and CVD mortality in men.

Subgroups	No. of effect sizes	HR (95%CI)	I^2%	p-value (between group)
All-cause mortality				
TT-Male				
Country				
Europe	10	0.84 (0.75, 0.95)	66.2	0.28
North America	6	0.97 (0.87, 1.09)	60.5	
Australia	2	0.87 (0.76, 0.91)	0.0	
Asia	1	0.97 (0.83, 1.14)	-	
Follow up duration				
<10 years	8	0.90 (0.80, 1.02)	84.6	0.87
≥ 10 years	11	0.89 (0.80, 0.99)	54.2	
Alcohol intake				
No	10	0.95 (0.87, 1.03)	50.9	0.16
Yes	9	0.85 (0.75, 0.97)	72.3	
Physical activity				
No	10	0.95 (0.88, 1.04)	71.7	0.08
Yes	9	0.81 (0.69, 0.95)	72.6	
Blood pressure				
No	13	0.89 (0.81, 0.98)	80.6	0.87
Yes	6	0.91 (0.77, 1.07)	58.4	
Hypertension				
No	12	0.85 (0.77, 0.95)	68.3	0.0006
Yes	7	1.03 (1.01, 1.04)	5.84	
Lipid markers				
No	10	0.88 (0.78, 0.99)	75.7	0.64
Yes	9	0.91 (0.83, 1.001)	48.2	
Hyperlipidemia				
No	15	0.89 (0.81, 0.97)	79.8	0.54
Yes	4	0.93 (0.84, 1.02)	0.10	
Diabetes				
No	11	0.84 (0.74, 0.95)	70.6	0.03
Yes	8	0.98 (0.92, 1.05)	26.7	
FT-Male				
Country				
Europe	5	0.82 (0.73, 0.93)	58.4	0.0001
North America	3	1.14 (0.99, 1.31)	10.7	
Australia	2	0.87 (0.76, 1.00)	0.0	
Asia	1	1.29 (1.12, 1.50)	0.00	

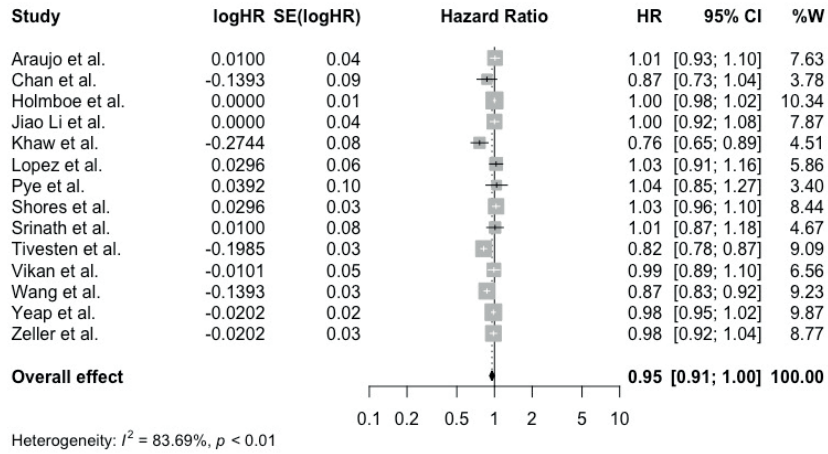
Table S4. Continued

Subgroups	No. of effect sizes	HR (95%CI)	I²%	p-value (between group)
Follow up duration				
<10 years	6	0.91 (0.76, 1.09)	82.8	0.57
≥ 10 years	5	0.98 (0.81, 1.18)	75.5	
Alcohol intake				
No	5	0.88 (0.67, 1.16)	86.3	0.56
Yes	6	0.97 (0.84, 1.12)	75.1	
Physical activity				
No	6	0.93 (0.83, 1.05)	60.1	0.93
Yes	5	0.92 (0.69, 1.22)	88.6	
Blood pressure				
No	7	0.92 (0.78, 1.08)	85.8	0.51
Yes	4	1.00 (0.85, 1.17)	40.2	
Hypertension				
No	7	0.88 (0.78, 0.99)	72.4	0.078
Yes	4	1.10 (0.88, 1.37)	65.2	
Lipid markers				
No	5	0.94 (0.71, 1.26)	88.2	0.92
Yes	6	0.93 (0.82, 1.04)	62.1	
Hyperlipidemia				
No	8	0.87 (0.77, 0.99)	69.6	0.095
Yes	3	1.13 (0.86, 1.50)	85.4	
Diabetes				
No	6	0.91 (0.76, 1.08)	82.8	0.56
Yes	5	0.98 (0.81, 1.18)	73.6	
CVD mortality				
TT-Male				
Country				
Europe	7	0.83 (0.62, 1.10)	71.6	0.89
North America	3	0.98 (0.66, 1.46)	0.0	
Australia	3	0.83 (0.65, 1.05)	0.0	
Asia	1	0.86 (0.64, 1.16)	0.0	
Follow up duration				
<10 years	6	0.86 (0.70, 1.00)	38.3	0.90
≥ 10 years	8	0.85 (0.66, 1.09)	51.8	
Alcohol intake				
No	6	0.85 (0.72, 1.005)	0.0	0.99
Yes	8	0.85 (0.65, 1.12)	67.8	

Table S4. Continued

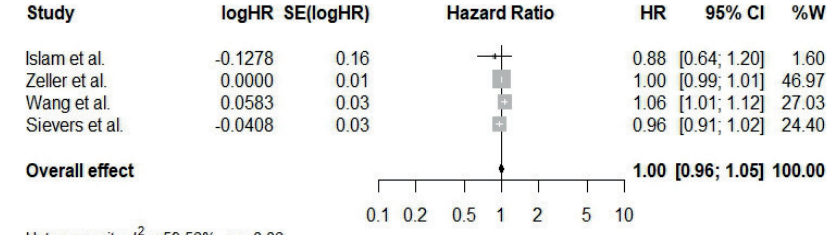
Subgroups	No. of effect sizes	HR (95%CI)	I^2%	p-value (between group)
Physical activity				
No	8	0.95 (0.80, 1.13)	21.0	0.09
Yes	6	0.73 (0.57, 0.94)	45.9	
Blood pressure				
No	10	0.87 (0.70, 1.06)	54.2	0.60
Yes	4	0.80 (0.62, 1.01)	4.5	
Hypertension				
No	8	0.80 (0.64, 0.95)	55.9	0.14
Yes	6	1.01 (0.76, 1.33)	25.4	
Lipid markers				
No	7	0.85 (0.59, 1.21)	63.5	0.77
Yes	7	0.90 (0.79, 1.02)	2.9	
Hyperlipidemia				
No	10	0.80 (0.65, 0.97)	43.8	0.22
Yes	4	1.02 (0.72, 1.44)	58.9	
Diabetes				
No	7	0.76 (0.57, 1.002)	63.8	0.29
Yes	7	0.90 (0.76, 1.08)	12.3	

A



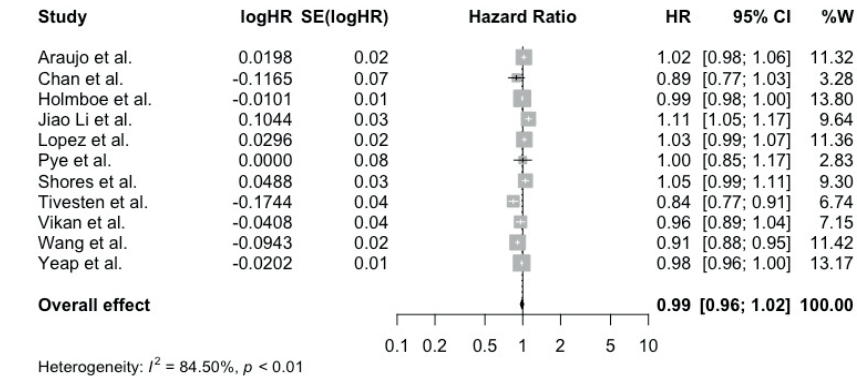
Heterogeneity: I^2 = 83.69%, p < 0.01

B



Heterogeneity: I^2 = 59.56%, p = 0.06

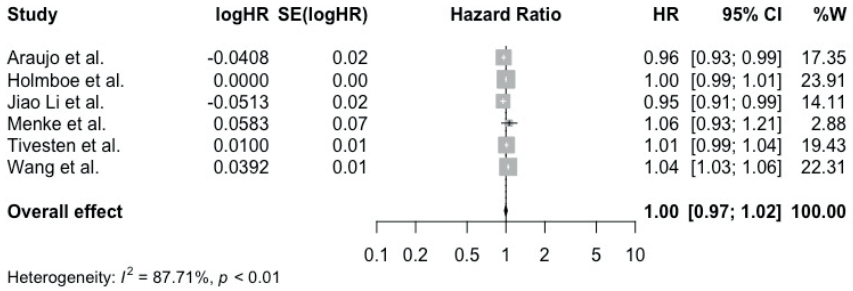
C



Heterogeneity: I^2 = 84.50%, p < 0.01

Figure S10.

D



E

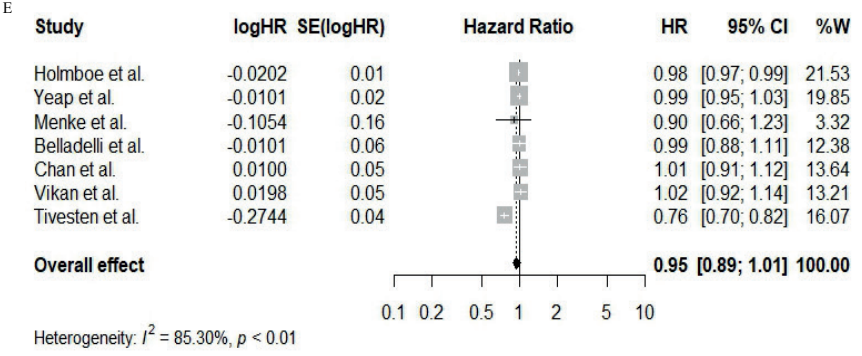
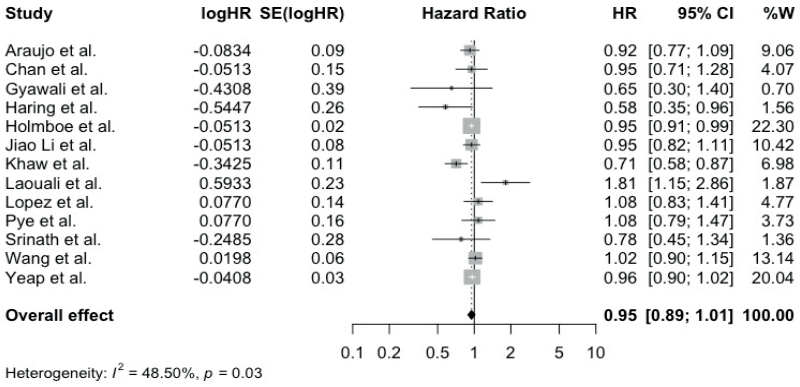
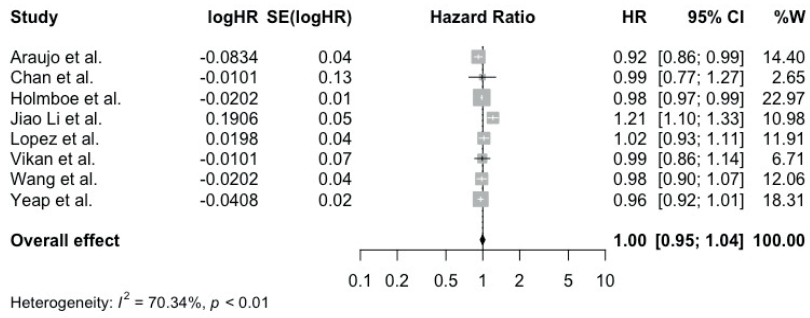


Figure S10. Linear dose response association of sex hormones and all-cause mortality. **A**) TT-Men (per 5nmol/l increment); **B**) TT-Women (per 0.5nmol/l increment); **C**) FT-Men (per 50 pmol/l increment); **D**) SHBG-Men (per 5 nmol/l increment); **E**) E2-Men (per 30 pmol/l).

A



B



C

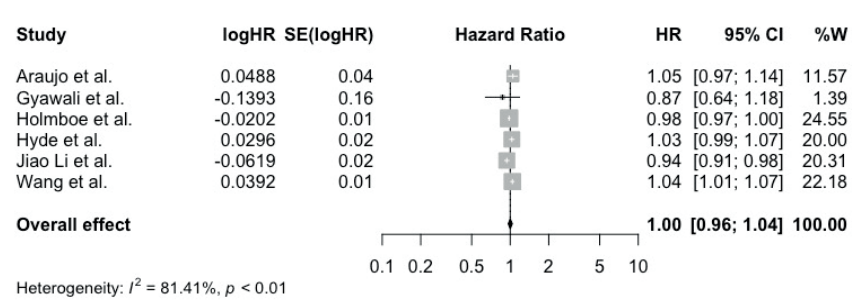


Figure S11.

D

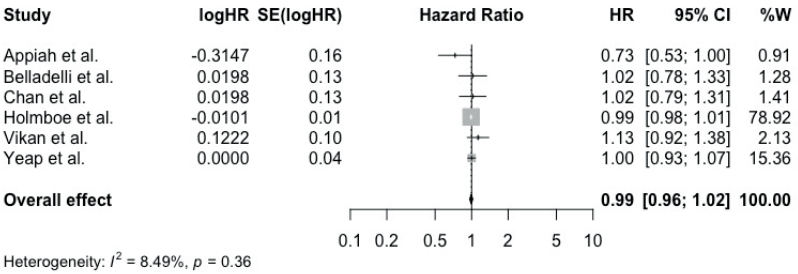


Figure S11. Linear dose response association of sex hormones and CVD mortality. **A**) TT-Men (per 5 nmol/l increment); **B**) FT-Men (per 50 pmol/l increment); **C**) SHBG-Men (per 5 nmol/l increment); **D**) E2-Men (per 30 pmol/l).

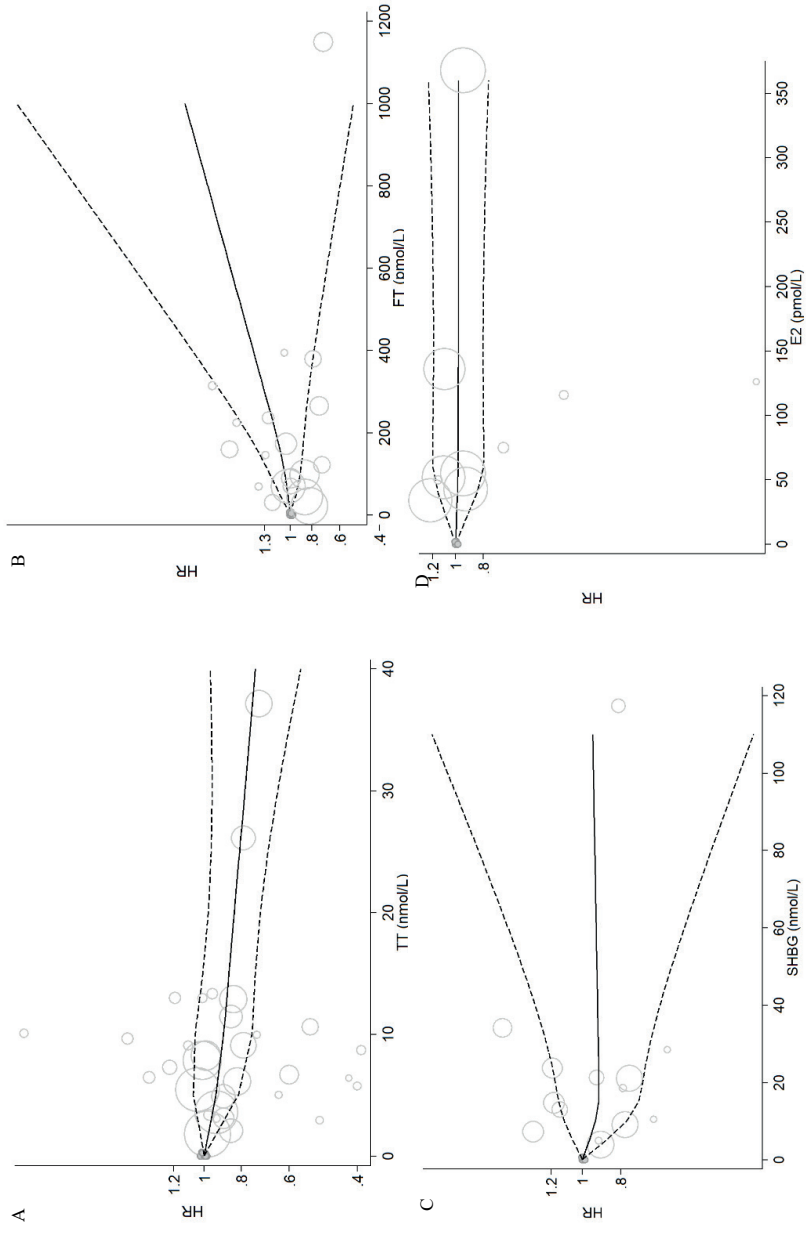


Figure S12. Non-Linear dose response association of sex hormones and CVD mortality. **A**) TT-Men; **B**) FT-Men; **C**) SHBG-Men; **D**) E2-Men.

Table S5. HRs of all-cause and CVD mortality from nonlinear dose-response analyses per hormone.

TT-Men		TT-Women		FT-Men		SHBG-Men		E2-Men	
All-cause mortality									
Dose (nmol/l)	**HR (95%CI)**	**Dose**	**HR (95%CI)**	**Dose**	**HR (95%CI)**	**Dose**	**HR (95%CI)**	**Dose**	**HR (95%CI)**
5	0.88 (0.81, 0.95)	0.1	0.97 (0.93, 1.01)	20	0.96 (0.94, 0.99)	5	0.97 (0.9, 1.05)	20	0.97 (0.85, 1.1)
10	0.84 (0.76, 0.94)	0.5	0.88 (0.72, 1.06)	40	0.93 (0.89, 0.98)	10	0.95 (0.83, 1.1)	40	0.95 (0.79, 1.15)
15	0.87 (0.79, 0.96)	1	0.9 (0.70, 1.15)	80	0.88 (0.81, 0.97)	15	0.96 (0.81, 1.14)	60	0.95 (0.79, 1.13)
20	0.89 (0.82, 0.98)	2	1 (0.69, 1.46)	100	0.87 (0.78, 0.97)	25	1.07 (0.94, 1.22)	80	0.94 (0.81, 1.11)
25	0.92 (0.84, 1.01)	2.5	1.06 (0.68, 1.66)	150	0.87 (0.75, 1.01)	30	1.15 (1.02, 1.29)	100	0.94 (0.82, 1.08)
30	0.95 (0.86, 1.05)	3	1.12 (0.66, 1.90)	300	1.02 (0.69, 1.49)	40	1.32 (1.14, 1.54)	150	0.94 (0.84, 1.04)
35	0.98 (0.87, 1.1)	3.5	1.19 (0.65, 2.18)	400	1.16 (0.65, 2.05)	50	1.52 (1.2, 1.94)	200	0.93 (0.83, 1.04)
40	1.01 (0.88, 1.15)	4	1.26 (0.63, 2.5)	1000	2.47 (0.43, 14.23)	100	3.09 (1.4, 6.78)	300	0.92 (0.74, 1.13)
$p_{nonlinearity}$	0.002		0.047		0.01		0.13		0.719
CVD mortality									
5	0.93 (0.82, 1.07)	-	-	20	1.01 (0.98, 1.04)	5	0.96 (0.87, 1.06)	20	0.99 (0.92, 1.07)
10	0.89 (0.75, 1.06)	-	-	80	1.04 (0.92, 1.18)	10	0.93 (0.77, 1.11)	60	0.98 (0.8, 1.2)
15	0.86 (0.74, 1.01)	-	-	100	1.06 (0.90, 1.23)	15	0.91 (0.72, 1.15)	90	0.98 (0.8, 1.2)
20	0.83 (0.71, 0.98)	-	-	170	1.12 (0.87, 1.44)	30	0.91 (0.68, 1.23)	100	0.98 (0.8, 1.2)
25	0.81 (0.68, 0.96)	-	-	200	1.16 (0.87, 1.55)	40	0.92 (0.64, 1.32)	150	0.98 (0.8, 1.19)
30	0.78 (0.64, 0.95)	-	-	300	1.3 (0.83, 2.05)	50	0.92 (0.60, 1.42)	200	0.98 (0.8, 1.2)
35	0.76 (0.60, 0.96)	-	-	350	1.38 (0.81, 2.36)	80	0.93 (0.47, 1.83)	250	0.98 (0.79, 1.2)
40	0.74 (0.56, 0.96)	-	-	1000	2.95 (0.52, 16.63)	100	0.94 (0.40, 2.19)	300	0.98 (0.78, 1.22)
$p_{nonlinearity}$	0.80	-	-		0.25		0.50		0.91

Table S6. GRADE evidence for the association of sex hormones and risk of all-cause and cause-specific mortality in men.

Certainty assessment							№ of patients		Effect		Certainty	Importance
№ of studies	**Study design**	**Risk of bias**	**Inconsistency**	**Indirectness**	**Imprecision**	**Other considerations**	**Participants**	**Cases (event rate)**	**Relative (95% CI)**	**Absolute (95% CI)**		
TT-All cause mortality												
19	observational studies	serious[a]	serious[b]	not serious	not serious	publication bias strongly suspected dose response gradient[c]	196054	15222 (7.8%)	**HR 0.90** (0.83 to 0.97)	**7 fewer per 1,000** (from 13 fewer to 2 fewer)	⨁⨁◯◯ Low	CRITICAL
TT-CVD mortality												
14	observational studies	serious[d]	not serious	not serious	serious[e]	none	188730	3546 (1.9%)	**HR 0.85** (0.73 to 1.00)	**3 fewer per 1,000** (from 5 fewer to 0 fewer)	⨁⨁◯◯ Low	CRITICAL
TT-Cancer mortality												
7	observational studies	serious[f]	serious[g]	not serious	serious[h]	none	171956	4172 (2.4%)	**HR 0.90** (0.68 to 1.18)	**2 fewer per 1,000** (from 8 fewer to 4 more)	⨁◯◯◯ Very low	CRITICAL
TT-Other causes mortality												
4	observational studies	not serious	not serious	not serious	not serious	none	160944	1944 (1.2%)	**HR 0.82** (0.73 to 0.91)	**2 fewer per 1,000** (from 3 fewer to 1 fewer)	⨁⨁⨁⨁ High	CRITICAL
BT-All cause mortality												
6	observational studies	serious[i]	not serious[j]	not serious	not serious	none	6766	1820 (26.9%)	**HR 0.93** (0.80 to 1.07)	**16 fewer per 1,000** (from 47 fewer to 16 more)	⨁⨁⨁◯ Moderate	CRITICAL
BT-CVD mortality												
2	observational studies	not serious	serious[k]	not serious	serious[ka]	none	4286	342 (8%)	**HR 1.56** (0.64 to 3.78)	**42 more per 1,000** (from 28 fewer to 190 more)	⨁⨁◯◯ Low	CRITICAL
FT-All cause mortality												
11	observational studies	serious[l]	serious[m]	not serious	not serious	dose response gradient	184609	12293 (6.7%)	**HR 0.94** (0.83 to 1.07)	**4 fewer per 1,000** (from 11 fewer to 4 more)	⨁⨁⨁◯ Moderate	CRITICAL
FT-CVD mortality												
9	observational studies	serious[n]	Serious[na]	not serious	Serious[nb]	none	180959	2842 (1.6%)	**HR 1.04** (0.83 to 1.30)	**1 more per 1,000** (from 3 fewer to 5 more)	⨁◯◯◯ Very low	CRITICAL

Table S6. Continued

Certainty assessment							№ of patients		Effect		Certainty	Importance
№ of studies	**Study design**	**Risk of bias**	**Inconsistency**	**Indirectness**	**Imprecision**	**Other considerations**	**Participants**	**Cases (event rate)**	**Relative (95% CI)**	**Absolute (95% CI)**		
FT-Cancer mortality												
4	observational studies	serious[o]	not serious	not serious	not serious	none	167350	3750 (2.2%)	**HR 0.94** (0.82 to 1.06)	**1 fewer per 1,000** (from 4 fewer to 1 more)	⨁⨁⨁◯ Moderate	CRITICAL
FT-Other causes mortality												
2	observational studies	not serious	serious[p]	not serious	not serious	none	158652	1849 (1.2%)	**HR 0.80** (0.64 to 0.99)	**2 fewer per 1,000** (from 4 fewer to 0 fewer)	⨁⨁⨁◯ Moderate	CRITICAL
SHBG-All cause mortality												
8	observational studies	serious[q]	not serious[r]	not serious	serious[ra]	none	169993	9015 (5.3%)	**HR 1.10** (0.94 to 1.28)	**5 more per 1,000** (from 3 more to 14 more)	⨁⨁◯◯ Low	CRITICAL
SHBG-CVD mortality												
7	observational studies	serious[s]	serious[t]	not serious	serious[u]	none	171383	2246 (1.3%)	**HR 0.91** (0.69 to 1.20)	**1 fewer per 1,000** (from 4 fewer to 3 more)	⨁◯◯◯ Very low	CRITICAL
SHBG-Cancer mortality												
3	observational studies	serious[v]	not serious	not serious	not serious	none	161971	3594 (2.2%)	**HR 1.21** (1.09 to 1.34)	**5 more per 1,000** (from 2 more to 7 more)	⨁⨁⨁◯ Moderate	CRITICAL
DHEA-All cause mortality												
2	observational studies	serious[w]	not serious	not serious	not serious	none	4448	647 (14.5%)	**HR 0.73** (0.55 to 0.97)	**37 fewer per 1,000** (from 63 fewer to 4 fewer)	⨁⨁⨁◯ Moderate	CRITICAL
DHEAs-All cause mortality												
6	observational studies	very serious[x]	serious[y]	not serious	not serious	none	9482	2412 (25.2%)	**HR 0.72** (0.57 to 0.91)	**64 fewer per 1,000** (from 100 fewer to 20 fewer)	⨁◯◯◯ Very low	CRITICAL
DHEAs-CVD mortality												
5	observational studies	serious[z]	not serious[aa]	not serious	serious[ab]	none	7595	884 (11.6%)	**HR 0.76** (0.56 to 1.04)	**27 fewer per 1,000** (from 49 fewer to 4 more)	⨁⨁◯◯ Low	CRITICAL

Table S6. Continued

Certainty assessment							№ of patients		Effect		Certainty	Importance
№ of studies	**Study design**	**Risk of bias**	**Inconsistency**	**Indirectness**	**Imprecision**	**Other considerations**	**Participants**	**Cases (event rate)**	**Relative (95% CI)**	**Absolute (95% CI)**		
E2- All cause mortality												
9	observational studies	serious[ac]	not serious	not serious	not serious	none	19507	4444 (22.8%)	**HR 0.99** (0.88 to 1.11)	**2 fewer per 1,000** (from 24 fewer to 22 more)	⨁⨁⨁◯ Moderate	CRITICAL
E2-CVD mortality												
6	observational studies	serious[ae]	not serious[ag]	not serious	serious[ah]	none	13352	968 (7.2%)	**HR 1.02** (0.75to 1.39)	**1 more per 1,000** (from 18 fewer to 27 more)	⨁⨁◯◯ Low	CRITICAL
E2-Cancer mortality												
3	observational studies	very serious[af]	not serious	not serious	not serious	none	6904	600 (8.7%)	**HR 1.28** (1.03 to 1.59)	**23 more per 1,000** (from 2 more to 48 more)	⨁⨁◯◯ Low	CRITICAL
E2-Other causes mortality												
3	observational studies	very serious[af]	not serious	not serious	not serious	none	5271	470 (8.9%)	HR 0.93 (0.79 to 1.10)	6 fewer per 1,000 (from 18 fewer to 8 more)	⨁⨁◯◯ Low	CRITICAL

Abbreviations: TT, total testosterone; FT, Free testosterone; BT, bioavailable testosterone; SHBG, sex-hormone-binding globulin; DHEA, dehydroepiandrosterone; DHEAs, dehydroepiandrosterone-sulfate; E2, estradiol.

CI: confidence interval; HR: hazard Ratio

Explanations:

a. Downgraded since 21.05% of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out

b. Downgraded by one level due to inconsistency since I2= 76.45%, (P<0.01), that was unexplained by sensitivity analyses and subgroup analysis

c. Downgraded by one level due to strong publication bias, Eggers test=0.003

d. Downgraded since 14.28% of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out Risk of bias

e. Downgraded by one level due to imprecision: effect estimate overlaps null effect and 95% CI includes important benefit/harm (SRR <0.75 and/or >1.25)

f. Downgraded since 14.28 % of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out

g. Downgraded by one level due to inconsistency since I2= 72.59%, (P<0.001), that was unexplained by sensitivity analyses and subgroup analysis

h. Downgraded by one level due to imprecision: effect estimate overlaps null effect and 95% CI includes important benefit/harm (SRR <0.75 and/or >1.25)

i. Downgraded since 33.33% of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out

j. Downgraded by one level due to inconsistency since I2= 67.09%, (P<0.001), that was unexplained by sensitivity analyses and subgroup analysis

k. Downgraded by one level due to inconsistency since I2= 76.30%, (P=0.04)

Table S6. Continued

ka. Downgraded by one level due to imprecision: effect estimate overlaps null effect and 95% CI includes important benefit/harm (SRR <0.75 and/or >1.25)
l. Downgraded since 27.27 % of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out
m. Downgraded by one level due to inconsistency since I2= 80.57 %, (P<0.01), that was unexplained by sensitivity analyses and subgroup analysis
n. Downgraded since 33.33% of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out
na. No downgrade for inconsistency as the presence of inter-study heterogeneity (I2=70.48%, p< 0.01) was explained by the removal of Li et al. (I2=29%, p>0.05)
nb. Downgraded by one level due to imprecision: effect estimate overlaps null effect and 95% CI includes important benefit/harm (SRR <0.75 and/or >1.25)
o. Downgraded since 25% of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out
p. Downgraded by one level due to inconsistency since I2= 71.50 %, (P<0.06), that was unexplained by sensitivity analyses and subgroup analysis
q. Downgraded since 50% of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out
r. No downgrade for inconsistency as the presence of inter-study heterogeneity (I2=80.46%, p< 0.01) was explained by the removal of Li et al. (I2=45%, p>0.05)
ra. Downgraded by one level due to imprecision: effect estimate overlaps null effect and 95% CI includes important benefit/harm (SRR <0.75 and/or >1.25)
s. Downgraded since 42.85% of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out
t. Downgraded by one level due to inconsistency since I2= 83.49 %, (P<0.01), that was unexplained by sensitivity analyses and subgroup analysis
u. Downgraded by one level due to imprecision: effect estimate overlaps null effect and 95% CI includes important benefit/harm (SRR <0.75 and/or >1.25)
v. Downgraded since 33% of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out
w. Downgraded since 50% of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out
x. Downgraded since 66% of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out
y. Downgraded by one level due to inconsistency since I2= 73.9 %, (P<0.01), that was unexplained by sensitivity analyses and subgroup analysis
z. Downgraded since 40% of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out
aa. No downgrade for inconsistency as the presence of inter-study heterogeneity (I2=58.35%, p= 0.05) was explained by the removal of Barrett-Connor et al. (I2=0%, p>0.05)
ab. Downgraded by one level due to imprecision: effect estimate overlaps null effect and 95% CI includes important benefit/harm (SRR <0.75 and/or >1.25)
ac. Downgraded since 55.55% of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out
ae. Downgraded since 50% of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out
ag. No downgrade for inconsistency as the presence of inter-study heterogeneity (I2=53.64%, p=0.06) was explained by the removal of Appiah et al. (I2=0%, p>0.05)
ah. Downgraded by one level due to imprecision: effect estimate overlaps null effect and 95% CI includes important benefit/harm (SRR <0.75 and/or >1.25)
af. Downgraded by two levels as all of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out

Table S7. GRADE evidence table for the association of sex hormones and risk of all-cause and cause-specific mortality in women.

Certainty assessment							№ of patients		Effect		Certainty	Importance
№ of studies	**Study design**	**Risk of bias**	**Inconsistency**	**Indirectness**	**Imprecision**	**Other considerations**	**Participants**	**Cases (event rate)**	**Relative (95% CI)**	**Absolute (95% CI)**		
TT-All-cause mortality												
5	observational studies	serious[a]	not serious	not serious	not serious	dose response gradient	107362	3009 (2.8%)	**HR 1.00** (0.83 to 1.20)	**0 fewer per 1,000** (from 5 fewer to 6 more)	⨁⨁⨁⨁ High	CRITICAL
TT-CVD mortality												
2	observational studies	not serious	not serious	not serious	serious[b]	none	94952	346 (0.4%)	**HR 1.23** (0.98 to 1.54)	**1 more per 1,000** (from 0 fewer to 2 more)	⨁⨁⨁◯ Moderate	CRITICAL
FT-All cause mortality												
2	observational studies	not serious	not serious	not serious	not serious	none	94839	2435 (2.6%)	**HR 1.04** (0.94 to 1.15)	**1 more per 1,000** (from 2 fewer to 4 more)	⨁⨁⨁⨁ High	CRITICAL
FT-CVD mortality												
2	observational studies	not serious	not serious	not serious	serious[b]	none	94839	346 (0.4%)	**HR 1.27** (0.98 to 1.64)	**1 more per 1,000** (from 0 fewer to 2 more)	⨁⨁⨁◯ Moderate	CRITICAL
SHBG-All cause mortality												
3	observational studies	not serious	not serious	not serious	not serious	none	100819	2635 (2.6%)	**HR 1.25** (1.13 to 1.38)	**6 more per 1,000** (from 3 more to 10 more)	⨁⨁⨁⨁ High	CRITICAL
SHBG-CVD mortality												
2	observational studies	not serious	not serious	not serious	serious[b]	none	95284	346 (0.4%)	**HR 1.01** (0.76 to 1.35)	**0 fewer per 1,000** (from 1 fewer to 1 more)	⨁⨁⨁◯ Moderate	CRITICAL
DHEAs-All cause mortality												
3	observational studies	serious[c]	not serious	not serious	not serious[d]	none	6013	1690 (28.1%)	**HR 0.84** (0.71 to 1.00)	**39 fewer per 1,000** (from 72 fewer to 0 more)	⨁⨁⨁◯ Moderate	CRITICAL

Table S7. Continued

Certainty assessment							№ of patients		Effect		Certainty	Importance
№ of studies	**Study design**	**Risk of bias**	**Inconsistency**	**Indirectness**	**Imprecision**	**Other considerations**	**Participants**	**Cases (event rate)**	**Relative (95% CI)**	**Absolute (95% CI)**		
DHEAs-CVD mortality												
4	observational studies	serious[e]	not serious[f]	not serious	serious[d]	none	6955	931 (13.4%)	**HR 0.83** (0.52to 1.30)	**21 fewer per 1,000** (from 62 fewer to 37 more)	⨁⨁◯◯ Low	CRITICAL

Abbreviations: TT, total testosterone; FT, Free testosterone; BT, bioavailable testosterone; SHBG, sex-hormone-binding globulin; DHEA, dehydroepiandrosterone; DHEAs, dehydroepiandrosterone-sulfate; E2, estradiol.

CI: confidence interval; HR: hazard Ratio

Explanations:

a. Downgraded since 20 % of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out

b. Downgraded by one level due to imprecision: small number of cases (n<400) and effect estimate overlaps null effect and 95% CI includes important benefit/harm (SRR <0.75 and/or >1.25)

c. Downgraded since 33.33% of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out

d. Not downgraded: effect estimate overlaps null effect but 95% CI does not include important benefit/harm (SRR <0.75 and/or >1.25)

e. Downgraded since50% of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out

f. No downgrade for inconsistency as the presence of inter-study heterogeneity (I2=73.74%, p< 0.01) was explained by the removal of Jia et al (I2=41%, p>0.05)

g. Downgraded by one level due to imprecision: small number of cases (n<400), effect estimate overlaps null effect and 95% CI includes important benefit/harm (SRR <0.75 and/or >1.25) and number of events were unclear for two studies

Table S8. GRADE evidence table for the association of DHEAs and all-cause mortality in men and women.

Certainty assessment							Nº of patients		Effect		Certainty	Importance
Nº of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	Participants	Cases (event rate)	Relative (95% CI)	Absolute (95% CI)		
DHEAs-All cause mortality												
3	observational studies	serious[b]	not serious	not serious	serious[a,c]	none	2124	248 (11.7%)	**HR 0.90** (0.71 to 1.14)	**11 fewer per 1,000** (from 32 fewer to 15 more)	⨁◯◯◯ Very low	CRITICAL

Abbreviations: DHEAs, dehydroepiandrosterone-sulfate; CI: confidence interval; HR: hazard Ratio

Explanations:

a. Downgraded by one level due to imprecision: small number of cases (n<400) and effect estimate overlaps null effect and 95% CI includes important benefit/harm (SRR <0.75 and/or >1.25)

b. Downgraded since 66 % of the evidence from studies rated as serious risk of bias and residual confounding cannot be ruled out

c. Downgraded by one level due to imprecision: small number of cases (n<400), effect estimate overlaps null effect and 95% CI includes important benefit/harm (SRR <0.75 and/or >1.25) and number of events were unclear for two studies

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Chapter 7

Endogenous sex steroid hormones, sex hormone binding globulin and risk of all-cause and cardiovascular mortality in patients with established cardiovascular diseases: a systematic review and meta-analysis of prospective studies

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Abstract

Background and Aims: Endogenous sex steroid hormones are involved in numerous regulatory mechanisms of the cardiovascular system, and their imbalances are frequently observed in patients with established cardiovascular disease (CVD). However, their prognostic significance for mortality and cardiovascular mortality remains unclear. This study aims to systematically synthesize existing research and quantify the association between endogenous sex steroid hormones, sex hormone binding globulin (SHBG), and the risk of all-cause and cardiovascular mortality in individuals with established CVD.

Methods: Six bibliographic databases were systematically searched for relevant articles based on population-based cohort studies. Data from the included studies were extracted, and the risk of bias was assessed using the Risk of Bias In Non-randomised Studies of Exposures (ROBINS-E) tool. Pooled hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated using a random-effects model, comparing the highest versus lowest levels of sex hormones/SHBG.

Results: Twelve studies with a total of 5,981 patients with established CVD were included in the systematic review. The results of meta-analysis found no significant association between endogenous total testosterone levels and risk of either all-cause ((HR (95%CI): 0.78 (0.56 to 1.09), n=5 studies) or CVD (HR (95%CI): 1.30 (0.28 to 6.01), n=3) mortality in men. Most studies were classified as having a high risk of bias, mainly due to potential confounding and missing data. The findings on other sex hormones and studies in women were insufficient to perform a meta-analysis and are presented narratively.

Conclusion: Our meta-analysis found no evidence for an association between endogenous sex hormones and mortality outcomes in patients with CVD. Future studies are needed to establish whether measuring sex hormones in patients with CVD could have any impact on clinical management.

Keywords: Sex steroid hormones, mortality, death, CVD, SHBG.

Introduction

Endogenous sex steroid hormones and sex hormone-binding globulin (SHBG) play essential roles in several physiological processes and have been implicated in the development of chronic conditions such as obesity, insulin resistance, type 2 diabetes, and cardiovascular disease (1-13). Additionally, these hormones and SHBG have been associated with mortality risk in the general population (13, 14), with sex-specific patterns observed in previous meta-analyses (14). However, evidence on the relationship between endogenous sex steroid hormones, SHBG, and mortality risk in individuals with cardiovascular disease (CVD) remains limited.

This gap is particularly important given that hormonal imbalances have been reported in patients with CVD (15, 16), and previous studies examining the association between these hormones and mortality in this population have produced inconsistent findings. Some studies in men suggest that lower testosterone levels are associated with an increased risk of mortality in patients with coronary heart disease (17) and heart failure (18). However, no significant associations have been observed in male patients with acute coronary syndromes (19) or in men with systolic chronic heart failure (20). While one study found that higher estradiol levels are associated with all-cause mortality in men referred to angiography (21), another study showed that both high and low estradiol levels are associated with risk of mortality in men with systolic chronic heart failure (22).

Furthermore, evidence on the association between endogenous sex steroid hormones and mortality risk in women with cardiovascular disease is scarce and remains inconclusive. Some studies have reported positive associations; for example, between estradiol and short-term mortality in postmenopausal women with acute stroke (23), and between higher SHBG levels and all-cause mortality in women at high cardiovascular risk (24). However, other studies found no significant associations for SHBG or dehydroepiandrosterone sulfate (DHEAs) in women with acute stroke (25). Given the limited and inconsistent evidence, this systematic review and meta-analysis aims to synthesize existing studies and quantify the association between endogenous sex steroid hormones, SHBG, and mortality risk in individuals with established CVD.

Methods

This systematic review was designed and carried out in accordance with two recent guidelines for systematic reviews, and the findings are reported following the PRISMA recommendations (27-29). The study protocol was registered in the PROSPERO database (registration number: CRD42022329605).

Data Sources and Search Strategy

To identify potentially relevant studies, we combined keywords related to sex hormones and mortality. We conducted searches in Embase.com, MEDLINE ALL (Ovid), Web of Science Core Collection, Cochrane Central, and PubMed (publisher), with the search updated until February 26, 2025. Additionally, the first 200 hits from Google Scholar were included. Additionally, we applied filters to exclude conference abstracts, case reports, studies involving non-adult populations, and animal studies. The search strategy was developed by an expert research librarian. The search results were imported into EndNote and de-duplicated using the method outlined by Bramer et al. (26). The specific details of the search strategy and keywords are presented in **Table S1**. To further enhance our search, we manually reviewed the references of the included studies and relevant published reviews.

Eligibility Criteria

We included original studies that met the following criteria: (i) they had an observational prospective design; (ii) they were conducted in a patients with established cardiovascular disease; (iii) they measured at least one of the following exposures: total testosterone, bioavailable testosterone, free testosterone, dehydroepiandrosterone (DHEA), DHEAs, and estradiol, or SHBG; (iv) they reported associations with all-cause or cause-specific mortality, including CVD, cancer, and other causes; and (v) they provided adjusted effect estimates (relative risk (RR), hazard ratio (HR), or odds ratio (OR) with 95% confidence intervals (95% CI).

We excluded retrospective studies, conference abstracts, ecological studies, case reports, case series, letters to the editor, conference proceedings, narrative reviews, systematic reviews, and meta-analyses. Studies conducted on animals, children, or adolescents were also excluded. In addition, we excluded studies focusing on other chronic diseases, studies conducted in the general population, and studies that did not clearly specify that participants were diagnosed with cardiovascular disease.

Study Selection and Data Extraction

All titles and abstracts were screened in duplicate by two independent teams of researchers (MA, HRD, and SB) based on the eligibility criteria. Subsequently, the selected full texts were reviewed again in duplicate by MA and HRD. Data from the included studies were extracted using a pre-designed form created by MA, HRD and SB. The extracted information included the first author's name, study design, study title, established type of CVD, publication year, location, number of participants, sex distribution of the population, age, follow-up duration, levels of sex hormones, methods of sex hormone assessment, number of deaths, causes of mortality, adjustments, and hazard/risk/odds ratios.

Risk of Bias Assessment

Risk of bias assessments were conducted by HRD, MA, and SB, with any discrepancies resolved through consensus. The risk of bias for cohort studies was evaluated using the latest version of the ROBINS-E tool (Risk Of Bias In Non-randomized Studies - of Exposures) (26). This tool provides a structured approach to assessing the risk of bias in observational epidemiological studies and shares many characteristics with the risk of bias (ROB) 2 tool for randomized clinical trials and the ROBINS-I tool for non-randomized intervention studies. Indeed, it provides further development of ROBINS-I. The tool includes 7 domains and considers potential biases resulting from confounding, assessment of exposure, selection of participants, post-exposure interventions, missing data, measurement of the outcome, and selective reporting of the results (26).

Statistical analysis

We used the random-effects model by Hartung-Knapp method for small number of studies (e.g., ≤5), which considers both between-study and within-study variations (heterogeneity), to calculate the summary HR and 95% CI of all-cause and cause-specific mortality for the highest and lowest categories of sex hormones. In the analysis, we used the most extensively adjusted HRs reported. For studies that only reported risk estimates on a continuous scale, if possible, we converted the HRs per-unit increment to the highest and lowest level of exposure using the average difference between the midpoints of the upper and lower categories of that relevant study in the analysis (27, 28). Heterogeneity between studies was assessed using the I^2 (29). Sensitivity analysis was performed to explore if the results were robust using leave-one-out analysis, excluding each study at a time from analysis. We performed the statistical analyses of our meta-analysis in R (Version 4.0.5) using meta, dmetar, and dosresmeta packages. $P < 0.05$ was considered significant.

Results

Eligible studies

Out of 13,135 references screened, twelve studies were included in the systematic review. Among included studies, eight studies examined the association between total testosterone levels and the risk of death in populations with established CVD (19, 20, 22, 30-34), with only six studies in men providing sufficient data to be included in the meta-analysis of total testosterone with the risk of all-cause and CVD mortality. One study was excluded due to insufficient data for estimate conversion (31), and another was conducted among women (30). The rest of the studies (n=6) were included only in the narrative analysis (23, 30, 31, 35-37).

A detailed outline of the study selection process is presented in **Figure 1**.

Study characteristics and assessment of risk of bias

The included studies were published between 2009 and 2023 and comprised a total of 5,981 patients with established CVD, among whom 709 deaths were due to all causes and 151 were attributed specifically to CVD (**Table 1**). We found no studies reporting on death due to other causes or cancer as the outcomes.

Out of these, nine studies were conducted in European countries (19, 22, 23, 30, 33-37), two in Asian countries (20, 31), and one in the United States (32). Nine studies focused on men (19, 20, 22, 31-35, 37), two on women (23, 30), and one included both men and women in a combined analysis (36). Six studies were conducted among patients with (systolic) heart failure (20, 22, 30, 31, 33, 37), two with acute coronary syndrome (19, 34), one with acute myocardial infarction (35), one with acute stroke (35), one with atherosclerotic CVD (32), and one ischemic heart disease (36). Additionally, most had a mean participant age over 50 years (20, 22, 23, 30, 31, 33-37) with median follow-up of 34 months.

Among the six studies included only in the narrative analysis (23, 30, 31, 35-37), the majority were rated as having some concerns, primarily due to potential confounding bias based on the ROBINS-E. The other six studies were included in the meta-analysis (19, 20, 22, 32-34). The majority of these studies were classified as having a high risk of bias, primarily due to potential confounding bias and missing data (**Table S2**).

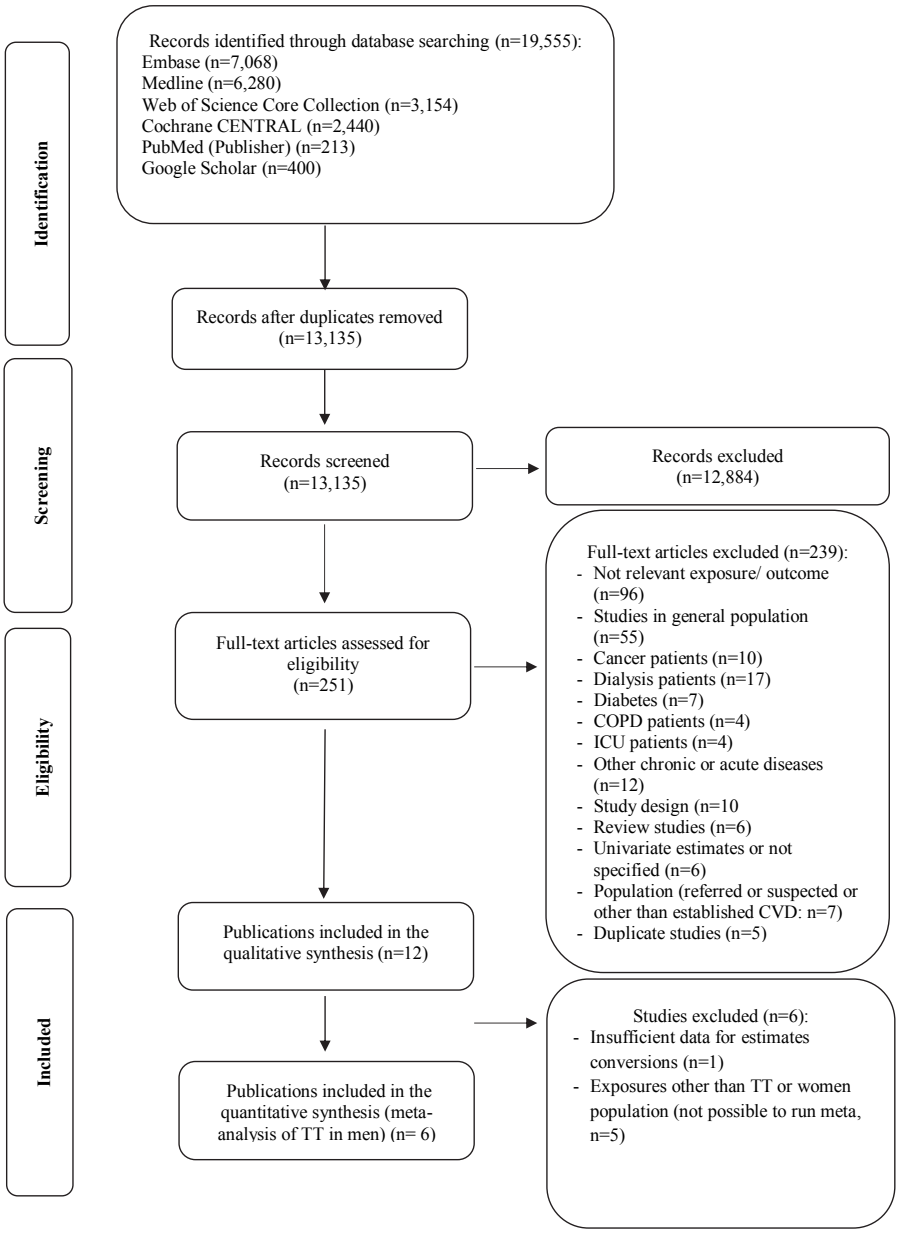


Figure 1. Study selection procedure.

Table 1. Characteristics of the included studies in the systematic review.

Study				Participants				Exposures				
Author, year	**Country**	**Design**	**Follow-up**	**Sex**	**Health status**	**Age**	**No. of Participants**	**Sex hormones**	**Measurement methods**	**All-cause**	**CVD**	**Adjustments**
Marra et al., 2023 (30)	Italy	Prospective cohort	3 y	F	HF	66.2±10.3	94	TT	Immunoassay	18	-	Age, NYHA class, LVEF, impaired eGFR, NT-proBNP, haemoglobin levels, aetiology, HF duration, and peak VO2.
Raparelli et al., 2022 (36)	Italy	Prospective cohort	23.7 m	B	Ischemic heart disease	69.8±11.5	434	Ratio TT/E2	Chemiluminescent microparticle immunoassay	41	-	Sex, ACS presentation, obstructive type of CAD.
Gencer et al., 2021 (19)	Switzerland	Prospective cohort	1 y	M	ACS	Min: 18	1054	TT	ECL	42	35	Global Registry of Acute Coronary Events risk score (composite of age, heart rate, SBP, creatinine, cardiac arrest at admission, ST segment deviation, abnormal troponin enzyme and Killip classification), preexisting DM and inflammation (hs-CRP).
Boden et al., 2020 (32)*	USA	Prospective cohort	3 y	M	Atherosclerotic CVD	40	2118	TT	Liquid chromatography coupled with tandem mass spectrometry.	109	55	Age, BMI, history of angina, DM, and HTN.
Yoshihisa et al., 2018 (31)	Japan	Prospective cohort	3.5 y	M	HF	65.9	618	TT	ECL	180	-	Age, BMI, NYHA class III or IV, LVEF, ischemic etiology, HTN, DM, dyslipidemia, CKD, anemia, atrial fibrillation.
Pesonen et al., 2016 (34)**	Sweden	Prospective cohort	5.6 y	M	ACS	62.3±9.4	264	TT	Radioimmunoassay	-	45	Age, current smoking, cholesterol, CRP, weight.
Pappa et al., 2012 (23)	Greece	Prospective cohort	30 d	F	Acute stroke	73.6±12.9	302	E2	Spectria E2-sensitive radioimmunoassay	25	-	Arterial hypertension, National institutes of health stroke scale
Wu et al., 2011 (20)	China	Prospective cohort	3.4 y	M	Systolic CHF	68.5±5.4	175	TT, FT, SHBG	TT: ECL; FT: Vermeulen equation; SHBG: ELISA	54	-	Age, NYHA class, LVEF, SBP, comorbidities, medications, NT-proBNP, e-GFR, plasma albumin, hemoglobin.
Guder et al., 2010 (33)	Germany	Prospective cohort	859 d	M	HF	64±13	191	TT, FT, DHEAs, SHBG	TT, DHEAs, SHBG: Immunoassays; FT: Vermeulen equation.	53	-	Age, NYHA class, renal function, atrial fibrillation, SBP, CRP, N-terminal pro-brain natriuretic peptide (NT-proBNP), intake of diuretics, cortisol.
Militaru et al., 2010 (35)	Romania	Prospective cohort	30 d	M	Acute MI	61.7±12.9	126	FT	Chemoluminiscence assay	16	-	Age, BMI, LDL and HDL cholesterol, TG, cigarette moking, DM, history of HTN, LVEF, hs-CRP and NT-proBNP.
Pascual-Figal et al., 2009 (37)	Spain	Prospective cohort	3 y	M	HF	53.1±10.6	104	SHBG	Radioimmune test	-	16	Age, BMI, spironolactone, loop diuretics.

Table 1. Continued

Study				Participants				Exposures				
Author, year	**Country**	**Design**	**Follow-up**	**Sex**	**Health status**	**Age**	**No. of Participants**	**Sex hormones**	**Measurement methods**	**All-cause**	**CVD**	**Adjustments**
Jankowska et al., 2009 (22)	Poland	Prospective cohort	3 y	M	Systolic CHF	58±12	501	E2, TT, DHEAs	E2: ECL; TT, DHEAs: Immunoassays.	171	-	Age, BMI, NYHA class, HF etiology, LVEF, plasma NT-proBNP, serum sodium, hemoglobin, estimated GFR, plasma total cholesterol, serum uric acid, therapy with loop diuretic, therapy with spironolactone, SBP, history of HTN, DM, and smoking

y: Years, d: Days; m: Months; M: male; F: Female; B: Both; TT: Total testosterone; FT: Free testosterone; DHEAs: Dehydroepiandrosterone sulfate; SHBG: Sex hormone-binding globulin; E2: Estradiol; CVD: Cardiovascular diseases; ACS: Acute coronary syndrome; BMI: Body mass index; HF: Heart failure; CHF: Chronic heart failure; MI: Myocardial infarction; DM: Diabetes mellitus; HTN: Hypertension; CRP: C-reactive protein; SBP: Systolic blood pressure; TG: triglycerides; NT-proBNP: N-terminal pro-B-type natriuretic peptide; NYHA: New York Heart Association; eGFR: estimated glomerular filtration rate; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; LVEF: Left ventricular ejection fraction; CAD: Coronary artery disease; ECL: Electrochemiluminescence. *This study was originally designed as a trial; however, the analyses included here are post hoc analyses of men with available baseline plasma testosterone concentrations. This study was originally designed as a case-control study, but the analyses presented here focus on 264 patients (cases) who were followed for a median of 5.6 years.

Findings of the narrative analysis

In a cohort of 302 women with acute stroke, estradiol emerged as an independent determinant of 1-month mortality (OR: 2.28; 95% CI: 1.27–4.07) (23). In 94 women with heart failure, testosterone deficiency (defined as serum total testosterone <25 ng/d) was strongly associated with all-cause mortality (HR: 8.33; 95% CI: 5.36–15.11) (30).

In 126 men with acute myocardial infarction, low levels of free testosterone at admission were independently associated with an increased risk of short-term all-cause mortality (OR: 0.56; 95% CI: 0.40–0.83) (35). Similarly, in a cohort of 618 men with heart failure, higher levels of total testosterone were associated with a lower risk of all-cause mortality (HR: 0.92; 95% CI: 0.86–0.99) (31). Additionally, in 104 men with heart failure, SHBG was associated with a higher risk of CVD mortality (HR: 1.05; 95% CI: 1.02–1.08) (37). In 501 men with systolic chronic heart failure, higher estradiol concentrations were associated with increased mortality risk (HR: 2.40; 95% CI: 1.35–4.28), whereas higher DHEAs levels were associated with a lower risk of death (HR: 0.63; 95% CI: 0.47–0.84) (22). By contrast, other studies did not observe statistically significant associations. For instance, in 191 men with heart failure, higher levels of free testosterone (HR: 0.92; 95% CI: 0.84–1.07), DHEAs (HR: 0.97; 95% CI: 0.91–1.03), and SHBG (HR: 1.06; 95% CI: 0.92–1.23) were not significantly related to all-cause mortality after (33). Similarly, in 175 men with systolic heart failure, neither SHBG (HR: 1.08; 95% CI: 0.88–1.26) nor free testosterone (HR: 0.92; 95% CI: 0.82–1.06) were associated with risk of all-cause mortality (20). Finally, among 434 men and women with ischemic heart disease, a lower total testosterone to estradiol ratio was associated with a higher risk of all-cause mortality (HR: 3.49; 95% CI: 1.24–9.80). These results are reported for the combined population of men and women (36).

Findings of the meta-analysis

Five studies, including a total of 4,039 male participants and 429 all-cause mortality events, were included in the meta-analysis of total testosterone levels with the risk of all-cause mortality in men. The comparison between the highest versus lowest levels of total testosterone showed no significant association with risk of all-cause mortality (HR: 0.78; 95% CI: 0.56–1.09; I^2 = 30.83%, **Figure 2**). Sensitivity analysis using the leave-one-out method revealed that excluding the study by Boden et al. resulted in a statistically significant association, with no between study heterogeneity (HR: 0.68; 95% CI: 0.53–0.86; I^2 = 0.0%, **Figure S1**).

Three studies, including a total of 3,436 male participants and 135 cardiovascular-related deaths, were included in the meta-analysis examining the association

between total testosterone levels and the risk of CVD mortality in men. The analysis found no significant association between total testosterone levels and the risk of CVD mortality (HR: 1.30; 95% CI: 0.28–6.01; I^2 = 38.20%, **Figure 3**). These findings were not sensitive to the exclusion of any individual study (**Figure S2**).

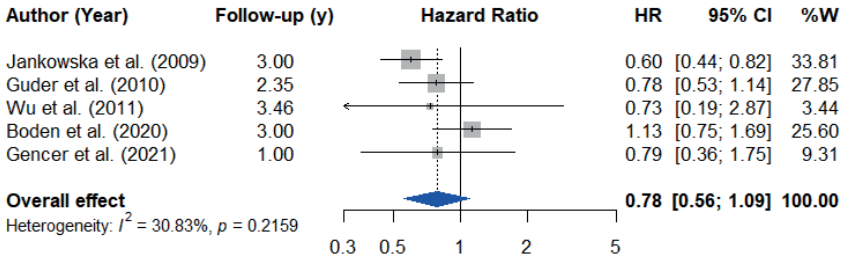


Figure 2. Pooled hazard ratio for total testosterone and all-cause mortality in men with stablished CVD. y: Year

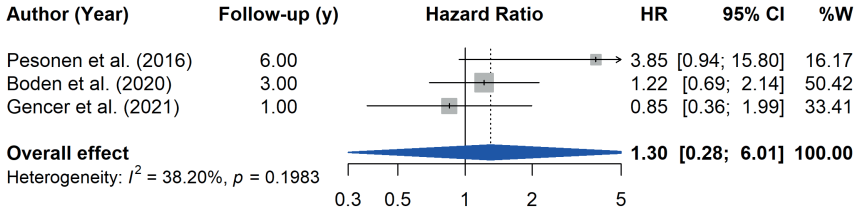


Figure 3. Pooled hazard ratio for total testosterone and CVD mortality in men with stablished CVD. y: Year

Discussion

To the best of our knowledge, this is the first systematic review and meta-analysis to examine the association between endogenous sex hormones and SHBG, and the risk of all-cause and CVD mortality in patients with established CVD. No significant association was observed between higher endogenous total testosterone levels and the risk of either all-cause or CVD mortality in men. These findings should be interpreted with caution, as the male patient participants included in the studies varied widely in their underlying conditions and characteristics. Evidence regarding other endogenous sex hormones, SHBG, and studies involving women remains limited and inconclusive.

In the general population, an individual participant data (IPD) meta-analysis of nine prospective cohort studies with at least five years of follow-up found a non-linear association between total testosterone levels and all-cause or CVD mortality. Specifically, all-cause mortality risk increased in men with total testosterone levels below 7.4 nmol/L, while CVD mortality risk rose at levels below 5.3 nmol/L (13). Findings from another systematic review and meta-analysis of prospective cohort studies in the general male population revealed a U-shaped association between endogenous total testosterone levels and the risk of all-cause mortality, whereas no significant association was observed with CVD mortality (14). In our study we were limited by the number of included articles to perform non-linear analysis, which could have provided more insights into the relationship between testosterone and all-cause mortality. Among patients with CVD, a randomized, double-blind, placebo-controlled trial involving 5,246 men with preexisting CVD or at high risk for CVD demonstrated that testosterone replacement therapy (TRT) was noninferior to placebo in relation to the incidence of major adverse cardiac events. Additionally, the occurrence of secondary endpoint events, including any component of the composite of cardiovascular death, nonfatal myocardial infarction, nonfatal stroke, or coronary revascularization was similar between the two groups (38). The recent meta-analysis of 30 randomized controlled trials in hypogonadal men found that TRT did not elevate the risk of cardiovascular events or mortality. These trials included participants with common cardiovascular risk factors, such as diabetes and hypertension (39). Furthermore, an IPD meta-analysis including 3,431 men with hypogonadism found no evidence that TRT increased short- to medium-term cardiovascular risk. Notably, the included randomized trials were not limited to men free of cardiovascular disease at baseline and encompassed participants with pre-existing conditions such as myocardial infarction, coronary heart disease, atherosclerosis, heart failure, and diabetes, among other cardiovascular comorbidities (40).

Several explanations may account for the discrepancies between findings in the general population and patients with established CVD. Studies in the general population typically include healthier individuals with greater hormonal variability, whereas individuals with CVD often have advanced disease states, where hormonal effects may be attenuated by competing risks. Additionally, severe or acute illness can disrupt the hormonal system which may lead to temporary hypogonadism in men (41-44). Further, differences in CVD subtypes across studies may influence hormone-mortality relationship and limited number of studies and/or sample sizes in CVD-specific studies reduce the power to detect associations. While most studies adjust for metabolic factors (for example blood pressure, diabetes, body mass

index), residual confounding might be of concern for studies in CVD patients as they often neglect lifestyle confounders such as physical activity and dietary intakes. Also, existing studies in patients with CVD have been constrained by shorter follow-up durations compared to those in the general population, with none providing longitudinal data beyond 10 years.

Several limitations should be acknowledged. This analysis synthesized findings from multiple cohort studies that varied in hormonal assessment methods, sample sizes, follow-up durations, and statistical adjustments. In particular, the inconsistency in hormone measurement techniques, especially for total testosterone in women and estradiol in postmenopausal women and men, is a notable concern. Methods such as immunoassays are known to be less reliable at low hormone concentrations due to method-specific biases and limited sensitivity. Additionally, we were unable to conduct meta-analyses for women or for endogenous sex steroid hormones other than total testosterone due to the limited number of eligible studies. Additionally, due to the limited number of studies, we were unable to perform any subgroup analyses to explore potential sources of heterogeneity or differences between studies. This limitation should be considered when interpreting the pooled results.

Our paper underscores a critical gap in the literature concerning women with CVD. A meta-analysis of observational cohort studies in the general population identified a J-shaped association between total testosterone levels and all-cause mortality in women, and found that higher SHBG levels were linked to increased mortality risk (14). However, the relevance of these findings to women with established CVD (23, 30) remains unclear. In particular, there is a complete lack of data on the association between SHBG and mortality in this population, despite SHBG's potential role in the risk of chronic diseases independent of sex hormone levels (9-12). Furthermore, considering known sex hormone variations across ethnic groups, there is a need for studies in more diverse populations to better understand ethnic and sex-based differences in hormonal influences on CVD outcomes. Moreover, further research should prioritize the development of clinically relevant reference ranges for hormone levels in patients with CVD and investigate whether hormone-guided therapy could benefit specific high-risk subgroups.

The findings of this review suggest that in individuals with established CVD, the effects of endogenous sex hormones on mortality risk may be less pronounced than in the general population, potentially overshadowed by the underlying disease process. Consequently, the associations observed between sex hormones and mortality risk in general population may not directly translate to patients

with established cardiovascular diseases. Clinicians should be cautious when considering these findings for secondary prevention, as the evidence in this specific context is still limited. Furthermore, the notable lack of data on women with CVD presents a significant gap in our understanding of clinical practice. Given the current evidence, there is insufficient justification to incorporate sex hormone levels into routine risk assessment or management strategies for women with CVD. This is particularly relevant considering the cardioprotective effects of estrogen in women (45, 46). More research is needed to determine whether sex hormone levels might offer valuable insights for clinical care in these populations.

Conclusion

This systematic review and meta-analysis provides suggestive evidence for a link between total testosterone and all-cause mortality in men, while limited evidence exists for other sex hormones and in women. IPD meta-analysis should be considered for future research to understand whether sex hormones could be used for clinical insights in patients with CVD.

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Supplementary materials: Endogenous sex steroid hormones, sex hormone binding globulin and risk of all-cause and cardiovascular mortality in patients with established cardiovascular diseases: a systematic review and meta-analysis of prospective studies

Table S1. Search strategy

Database	Search strategy
Embase.com	('sex hormone binding globulin'/de OR 'androgen'/de OR 'androgen blood level'/exp OR 'estradiol'/de OR 'estradiol blood level'/de OR 'testosterone'/de OR 'testosterone blood level'/de OR 'dehydroepiandrosterone'/de OR 'dehydroepiandrosterone sulfate'/de OR (androgen* OR 'sex hormone*' OR 'sex steroid*' OR testosterone* OR 'sexual hormone*' OR shbg OR dehydroepiandrosterone* OR androstenolone* OR dhea OR dheas OR 'dehydroepiandrosterone sulfate' OR 'dhea s' OR estradiol* OR oestradiol* OR prasterone*):ab,ti) AND ('mortality'/exp OR 'death'/exp OR 'mortality risk'/de OR 'mortality risk score'/de OR 'cardiovascular disease'/de OR 'coronary artery disease'/de OR 'heart muscle ischemia'/de OR 'heart infarction'/de OR 'Coronary Disease'/de OR 'cerebrovascular accident'/de OR 'heart failure'/de OR (mortalit* OR death* OR fatal* OR 'cardiovascular disease*' OR 'coronary disease*' OR 'coronary artery disease*' OR 'heart disease*' OR 'coronary heart disease*' OR 'ischemic heart disease*' OR 'ischaemic heart disease*' OR 'myocardial ischemia*' OR 'myocardial ischaemia*' OR 'myocardial infarct*' OR 'heart infarct*' OR stroke* OR 'sudden cardiac death' OR 'heart failure*' OR 'cardiovascular event*' OR 'cardiac event*' OR MACE OR MACEs OR survival* OR CVD OR CHD):ab,ti) AND ('cohort analysis'/de OR 'prospective study'/de OR 'follow up'/de OR 'longitudinal study'/de OR 'hazard ratio'/de OR (((hazard* OR odds OR risk* OR inciden*) NEXT/1 (ratio* OR proportion*)) OR cohort* OR prospectiv* OR longitudinal OR follow-up* OR followup* OR followed-up OR population-based OR case-cohort* OR nested case-control* OR 'relative risk*' OR inciden*):ab,ti) NOT ([animals]/lim NOT [humans]/lim) NOT (animal*:ti OR mice:ti OR rat:ti OR rats:ti OR in-vivo:ti OR in-vitro:ti OR tissue:ti OR tissues:ti OR cell*:ti) NOT (([newborn]/lim OR [infant]/lim OR [child]/lim OR [adolescent]/lim) NOT [adult]/lim) NOT ('pregnancy'/exp OR pregnan*:ti OR fetus:ti OR fetal:ti OR foetus:ti OR foetal:ti OR neonatal*:ti OR perinatal*:ti OR maternal*:ti OR infant*:ti OR child*:ti OR newborn*:ti OR pediatric*:ti OR paediatric*:ti OR juvenile*:ti) NOT ([Conference Abstract]/lim OR [Letter]/lim OR [Note]/lim OR [Editorial]/lim OR [Review]/lim) NOT ('systematic review'/exp OR 'meta analysis'/exp OR 'case report'/de OR 'case study'/de OR 'case report*':ti OR 'case stud*':ti OR 'case series':ti OR review*:ti OR 'meta-analysis':ti)

Table S1. Continued

Database	Search strategy
Medline (Ovid)	(Sex Hormone-Binding Globulin/ OR Androgens/ OR Estradiol/ OR Testosterone/ OR Dehydroepiandrosterone/ OR Dehydroepiandrosterone Sulfate/ OR (androgen* OR sex hormone* OR sex steroid* OR testosterone* OR sexual hormone* OR shbg* OR dehydroepiandrosterone* OR androstenolone* OR dhea OR dheas OR dehydroepiandrosterone sulfate OR dhea-s OR estradiol* OR oestradiol* OR prasterone*).ab,ti.) AND (exp Mortality/ OR exp Death/ OR Cardiovascular Diseases/ OR Coronary Disease/ OR Coronary Artery Disease/ OR Myocardial Ischemia/ OR Myocardial Infarction/ OR exp Stroke/ OR Heart Failure/ OR (mortalit* OR death* OR fatal* OR cardiovascular disease* OR coronary disease* OR coronary artery disease* OR heart disease* OR coronary heart disease* OR ischemic heart disease* OR ischaemic heart disease* OR myocardial ischemia* OR myocardial ischaemia* OR myocardial infarct* OR heart infarct* OR stroke* OR sudden cardiac death* OR heart failure* OR cardiovascular event* OR cardiac event* OR MACE OR MACEs OR survival* OR CVD OR CVDs OR CHD).ab,ti.) AND (Cohort Studies/ OR Prospective Studies/ OR Follow-Up Studies/ OR Longitudinal Studies/ OR (((hazard* OR odds OR risk* OR inciden*) adj1 (ratio* OR proportion*)) OR cohort* OR prospectiv* OR longitudinal OR follow-up* OR followup* OR followed-up OR population-based OR case-cohort* OR nested case-control* OR relative risk* OR (inciden* AND risk*)).ab,ti.) NOT (exp animals/ NOT humans/) NOT (animal*.ti. OR mice.ti. OR rat.ti. OR rats.ti. OR in-vivo.ti. OR in-vitro.ti. OR tissue.ti. OR tissues.ti. OR cell*.ti.) NOT ((exp infant/ OR exp child/ OR adolescent/) NOT (exp adult/)) NOT (exp Pregnancy/ OR pregnan*.ti. OR fetus.ti. OR fetal.ti. OR foetus.ti. OR foetal.ti. OR neonatal*.ti. OR perinatal*.ti. OR maternal*.ti. OR infant*.ti. OR child*.ti. OR newborn*.ti. OR pediatric*.ti. OR paediatric*.ti. OR juvenile*.ti.) NOT (letter* OR news OR comment* OR editorial* OR congres*).pt. NOT ("review"/ OR systematic review/ OR meta-analysis/ OR case reports/ OR case report*.ti. OR case series.ti. OR case stud*.ti. OR review*.ti. OR meta-analysis.ti.) NOT (Hormone Replacement Therapy/ OR replacement.ti OR (hormone adj2 treatment*).ti OR (hormone adj2 therap*).ti)
Cochrane Central Register of Controlled Trials (CENTRAL)	((androgen* OR sex NEXT hormone* OR sex NEXT steroid* OR testosterone* OR sexual NEXT hormone* OR shbg OR dehydroepiandrosterone* OR androstenolone* OR dhea OR dheas OR "dehydroepiandrosterone sulfate" OR "dhea-s" OR estradiol* OR oestradiol* OR prasterone*):ab,ti) AND ((mortalit* OR death* OR fatal* OR cardiovascular NEXT disease* OR coronary NEXT/2 disease* OR heart NEXT disease* OR ischemic NEXT heart NEXT disease* OR ischaemic NEXT heart NEXT disease* OR myocardial NEXT ischemia* OR myocardial NEXT ischaemia* OR myocardial NEXT infarct* OR heart NEXT infarct* OR stroke* OR "sudden cardiac death" OR heart NEXT failure* OR cardiovascular NEXT event* OR cardiac NEXT event* OR MACE OR MACEs OR survival* OR CVD OR CHD):ab,ti) AND ((((hazard* OR odds OR risk* OR inciden*) NEXT/1 (ratio* OR proportion*)) OR cohort* OR prospectiv* OR longitudinal OR follow NEXT up* OR followup* OR followed NEXT up OR population NEXT based OR case NEXT cohort* OR nested NEXT case NEXT control* OR relative NEXT risk* OR inciden*):ab,ti) NOT (replacement:ti OR hormone NEXT/2 treatment*:ti OR hormone NEXT/2 therap*:ti) NOT (pregnan*:ti OR fetus:ti OR fetal:ti OR foetus:ti OR foetal:ti OR neonatal*:ti OR perinatal*:ti OR maternal*:ti OR infant*:ti OR child*:ti OR newborn*:ti OR pediatric*:ti OR paediatric*:ti OR juvenile*:ti)

Table S1. Continued

Database	Search strategy
Web of Science Core Collection	Focus on precision: search terms for sex hormones and CV-mortality, all-cause mortality only in *Title* and *Keyword Plus* TI=((androgen* OR "sex hormone*" OR "sex steroid*" OR testosterone* OR "sexual hormone*" OR shbg OR dehydroepiandrosterone* OR androstenolone* OR dhea OR dheas OR "dehydroepiandrosterone sulfate" OR "dhea-s" OR estradiol* OR oestradiol* OR prasterone*) AND (mortalit* OR death* OR fatal* OR "cardiovascular disease*" OR coronary NEAR/2 disease* OR "heart disease*" OR "ischemic heart disease*" OR "ischaemic heart disease*" OR "myocardial ischemia*" OR "myocardial ischaemia*" OR "myocardial infarct*" OR "heart infarct*" OR stroke* OR "sudden cardiac death*" OR "heart failure*" OR "cardiovascular event*" OR "cardiac event*" OR MACE OR MACEs OR survival* OR CVD OR CHD)) OR KP=((androgen* OR "sex hormone*" OR "sex steroid*" OR testosterone* OR "sexual hormone*" OR shbg OR dehydroepiandrosterone* OR androstenolone* OR dhea OR dheas OR "dehydroepiandrosterone sulfate" OR "dhea-s" OR estradiol* OR oestradiol* OR prasterone*) AND (mortalit* OR death* OR fatal* OR "cardiovascular disease*" OR coronary NEAR/2 disease* OR "heart disease*" OR "ischemic heart disease*" OR "ischaemic heart disease*" OR "myocardial ischemia*" OR "myocardial ischaemia*" OR "myocardial infarct*" OR "heart infarct*" OR stroke* OR "sudden cardiac death*" OR "heart failure*" OR "cardiovascular event*" OR "cardiac event*" OR MACE OR MACEs OR survival* OR CVD OR CHD)) AND TS=(((hazard* OR odds OR risk* OR inciden*) NEAR/1 (ratio* OR proportion*)) OR cohort* OR prospectiv* OR longitudinal OR "follow up*" OR followup* OR "followed up" OR "population based" OR "case cohort*" OR "nested case control*" OR "relative risk*" OR inciden*) NOT TS=((animal* OR plant* OR rat OR rats OR mouse OR mice OR murine OR pigs OR primate* OR nonhuman*) NOT (human* OR patient*)) Exclude Document Types: Review Articles
PubMed (publisher)	(Sex Hormone-Binding Globulin[mh] OR Androgens[mh] OR Estradiol[mh] OR Testosterone[mh] OR Dehydroepiandrosterone[mh] OR Dehydroepiandrosterone Sulfate[mh] OR (androgen*[tiab] OR sex hormone*[tiab] OR sex steroid*[tiab] OR testosterone*[tiab] OR sexual hormone*[tiab] OR shbg*[tiab] OR dehydroepiandrosterone*[tiab] OR androstenolone*[tiab] OR dhea[tiab] OR dheas[tiab] OR dehydroepiandrosterone sulfate[tiab] OR dhea-s[tiab] OR estradiol*[tiab] OR oestradiol*[tiab] OR prasterone*[tiab])) AND (Mortality[mh] OR Death[mh] OR Cardiovascular Diseases[mh] OR Coronary Disease[mh] OR Coronary Artery Disease[mh] OR Myocardial Ischemia[mh] OR Myocardial Infarction[mh] OR Stroke[mh] OR Heart Failure[mh] OR (mortalit*[tiab] OR death*[tiab] OR fatal*[tiab] OR cardiovascular disease*[tiab] OR coronary disease*[tiab] OR coronary artery disease*[tiab] OR heart disease*[tiab] OR coronary heart disease*[tiab] OR ischemic heart disease*[tiab] OR ischaemic heart disease*[tiab] OR myocardial ischemia*[tiab] OR myocardial ischaemia*[tiab] OR myocardial infarct*[tiab] OR heart infarct*[tiab] OR stroke*[tiab] OR sudden cardiac death*[tiab] OR heart failure*[tiab] OR cardiovascular event*[tiab] OR cardiac event*[tiab] OR MACE[tiab] OR MACEs[tiab] OR survival*[tiab] OR CVD[tiab] OR CVDs[tiab] OR CHD[tiab])) AND (Cohort Studies[mh] OR Prospective Studies[mh] OR Follow-Up Studies[mh] OR Longitudinal Studies[mh] OR (((hazard*[tiab] OR odds[tiab] OR risk*[tiab] OR inciden*[tiab]) AND (ratio*[tiab] OR proportion*[tiab])) OR cohort*[tiab] OR prospectiv*[tiab] OR longitudinal[tiab] OR follow-up*[tiab] OR followup*[tiab] OR followed-up[tiab] OR population-based[tiab] OR case-cohort*[tiab] OR nested case-control*[tiab] OR relative risk*[tiab] OR (inciden*[tiab] AND risk*[tiab]))) NOT (animals[mh] NOT humans[mh]) NOT (letter[pt] OR news[pt] OR comment[pt] OR editorial[pt] OR congress[pt]) AND publisher[sb]

Table S1. Continued

Database	Search strategy
Google Scholar	"sex|sexual hormone|hormones|steroids"|androgen|testosterone|estradiol|oestradiol|dehydroepiandrosterone|dhea|dheas mortality|death|fatal|cardiovascular|cardiac|coronary|ischemic|heart|myocardial|stroke|survival cohort|prospective|"follow-up"|followup|longitudinal

Table S2. ROBINS-E judgement for each domain and overall.

First Author, year (ref)	Bias due to confounding	Bias due to exposure assessment	Bias due to selection of participants	Bias due to post-exposure interventions	Bias due to missing data	Bias due to measurement of the outcome	Bias due to selective reporting of the results	Overall judgement
Militaru et al., 2010 (1)	Some concerns	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Some concerns
Pappa et al., 2012 (2)	Some concerns	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Some concerns
Pascual-Figal et al., 2009 (3)	Some concerns	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Some concerns
Marra et al., 2023 (4)	High risk	Some concerns	Low risk	Low risk	Low risk	Low risk	Low risk	High risk
Raparelli et al., 2022 (5)	Some concerns	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	Some concerns
Yoshihisa et al., 2018 (6)	High risk	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	High risk
Boden et al., 2020 (7)	Some concerns	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	Some concerns
Gencer et al., 2021 (8)	High risk	Low risk	Low risk	Low risk	Some concerns	Low risk	Low risk	High risk
Guder et al., 2010 (9)	High risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	High risk
Jankowska et al., 2009 (10)	Some concerns	Low risk	Low risk	Low risk	High risk	Low risk	Low risk	High risk
Pesonen et al., 2016 (11)	High risk	Low risk	Some concerns	Low risk	Some concerns	Low risk	Low risk	High risk
Wu et al., 2011 (12)	High risk	Low risk	Some concerns	Low risk	Some concerns	Low risk	Some concerns	High risk

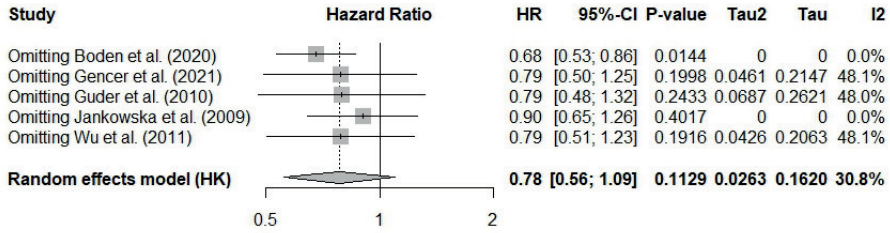


Figure S1. Leave-one-out analysis evaluating the association between total testosterone levels and all-cause mortality in men with established CVD.

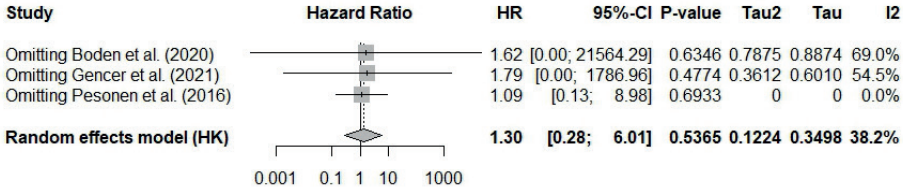


Figure S2. Leave-one-out analysis evaluating the association between total testosterone levels and CVD mortality in men with established CVD.

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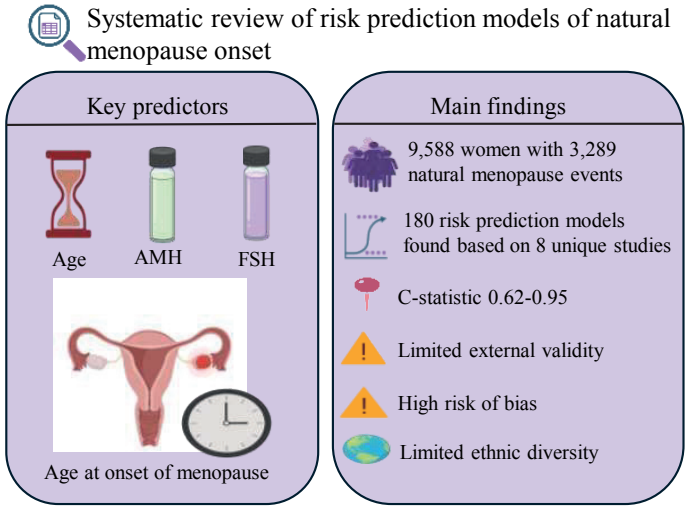


Chapter 8

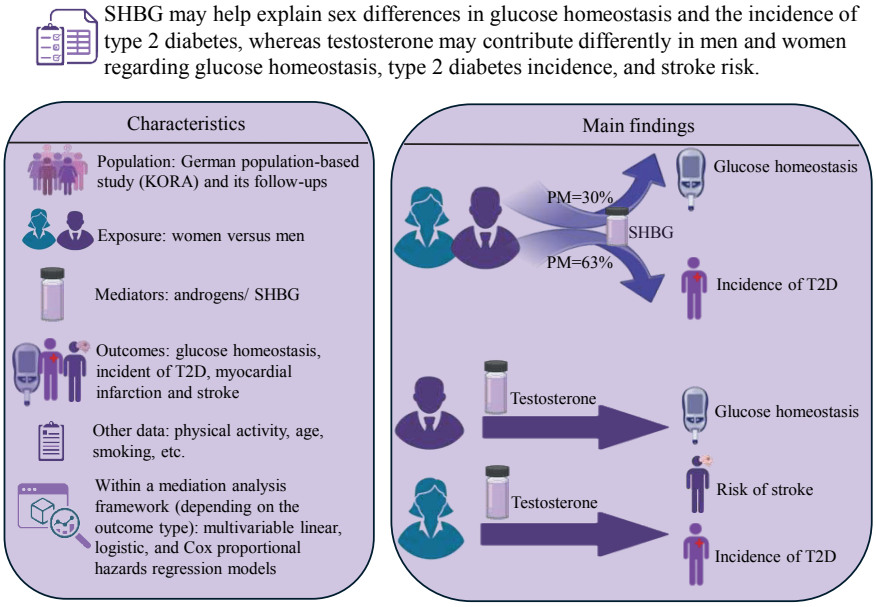
General discussion

Overview

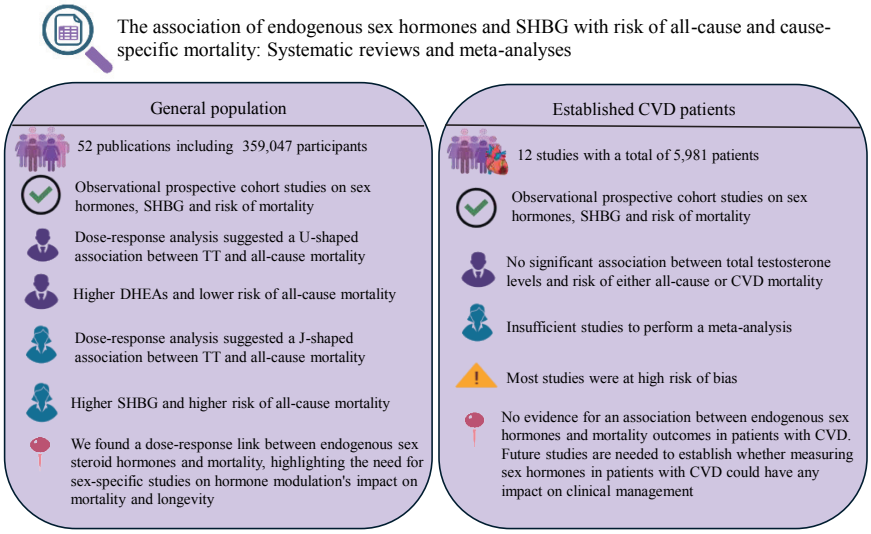
This thesis investigated the role of endogenous sex hormones and sex hormone–binding globulin (SHBG) across the life course, from the menopausal transition to mortality, in relation to cardiometabolic health. The first study chapter (Chapter 2) focused specifically on women and evaluated existing risk prediction models for the onset of menopause, a key reproductive transition and critical phase in women's lives that is increasingly recognized as a risk factor for subsequent cardiovascular and other chronic diseases. While sex hormones were not the main focus of this chapter, they are closely related to the overall theme of this thesis, as hormonal changes drive the menopausal transition. Hormonal markers, including follicle-stimulating hormone (FSH) and anti-Müllerian hormone (AMH), were among the most commonly studied predictors of menopause timing in the risk prediction models (Graphical Abstract 1). The subsequent chapters included both women and men. Using data from the KORA study, we examined whether endogenous sex hormones and SHBG contribute to explaining sex differences in glucose homeostasis, type 2 diabetes (T2D), and cardiovascular disease (Graphical Abstract 2). Finally, systematic reviews and meta-analyses of prospective cohort studies were conducted to evaluate the associations of circulating sex hormones and SHBG with all-cause and cause-specific mortality in the general population as well as among patients with established CVD (Graphical Abstract 3). Overall, the thesis approaches the topic from complementary perspectives: first by addressing menopause as a female-specific, hormone-driven transition increasingly recognized as an independent cardiometabolic risk factor; subsequently by examining whether sex differences in cardiometabolic diseases may be partly explained by differences in endogenous sex hormone and SHBG concentrations between women and men; and finally by investigating the associations of circulating sex hormones and SHBG with mortality as the ultimate clinical endpoint.



Graphical Abstract 1.



Graphical Abstract 2.



Graphical Abstract 3.

Main findings and implications

In Chapter 2, we systematically reviewed existing risk prediction models for the onset of natural menopause (ONM). Overall, these models demonstrated limited predictive performance and poor generalizability. Many were developed in selected populations and lacked rigorous external validation. This review highlighted substantial heterogeneity and important methodological limitations across studies. Common shortcomings included insufficient external validation, inconsistent incorporation of hormonal and clinical predictors, and limited transparency in model development and reporting. By systematically evaluating existing approaches, we identified key gaps in the literature and outlined directions for improving future model development. These findings underscore the need for more robust and standardized modelling strategies that integrate hormonal, clinical, and lifestyle factors to enhance predictive accuracy and applicability across diverse populations.

Accurate prediction of ONM holds significant clinical and public health relevance. Early identification of women at risk of premature or early menopause could facilitate timely interventions to mitigate downstream health risks, including CVD, metabolic dysfunction, and overall mortality (1). This is of particular importance because accumulating evidence indicates that women who experience premature or early menopause, typically defined as onset before the age of 45, are at substantially higher risk for CVD and all-cause mortality (2). Given that menopause

marks a physiological transition characterized by increased abdominal adiposity, adverse lipid shifts, and heightened insulin resistance, improved prediction models could play an important role in both preventive care and personalized risk stratification (3). Ultimately, advancing the accuracy and generalizability of ONM prediction models may help optimize women's reproductive and cardiometabolic health across the life course.

In Chapters 3 and 4, we examined whether SHBG and androgens mediate the association between sex and glucose homeostasis, as well as incident T2D, using data from the KORA study. SHBG partially mediated the association between sex and fasting glucose (PM = 30%) and more strongly mediated sex differences in T2D incidence (PM = 63%), suggesting that SHBG may help explain the observed sex differences in glucose control and the incidence of T2D.

In contrast, total testosterone (TT) demonstrated an inconsistent mediatory role. While higher TT levels appeared beneficial for glucose metabolism or the incidence of T2D among men, the opposite pattern was observed among women, highlighting the complex, context-dependent effects of androgens on metabolic health. This sexual dimorphism underscores that hormonal influences on glycemic control are not uniform across sexes but instead reflect distinct physiological mechanisms and regulatory pathways. Previous studies, particularly those using Mendelian Randomization (MR) approaches, have provided evidence for a potential causal role of SHBG and androgens in T2D risk (4-6). However, these studies typically examined the causal hormone–disease associations within each sex separately and did not explicitly evaluate the mediating role of these hormones in explaining sex differences in glycemic outcomes and incidence of T2D. By applying formal mediation analysis, our study adds a crucial layer of understanding: it quantifies how much of the sex disparity in glucose homeostasis and incidence of T2D can be statistically explained by variations in SHBG and androgen levels. This approach therefore bridges the gap between mechanistic and epidemiologic perspectives, providing empirical support for the hormonal pathways underlying sex-specific metabolic risk.

Our findings further highlight SHBG as a potential biomarker capturing part of the sex-related variation in glucose regulation and incidence of T2D. If replicated in other cohorts, SHBG could contribute to sex-specific risk stratification and earlier identification of individuals at higher risk of T2D. Whether SHBG also represents a potential therapeutic target would require evidence from interventional studies.

Our findings are consistent with current clinical understanding that testosterone-related interventions are inherently sex-specific. In men, testosterone replacement therapy (TRT) is clinically indicated for hypogonadism and has been shown to improve body composition, insulin sensitivity, and glycemic control (7, 8). However, TRT is prescribed only under strict clinical criteria and is not currently used as a preventive strategy for T2D. In contrast, in women, elevated endogenous testosterone levels, as observed in conditions such as polycystic ovary syndrome (PCOS), are associated with insulin resistance, dyslipidemia, and increased T2D risk (9, 10). Therefore, therapeutic lowering of androgen levels, rather than replacement, may be beneficial in hyperandrogenic states. These opposing effects underscore the necessity of sex-tailored hormonal approaches in both research and clinical settings. Collectively, our findings emphasize that interventions involving testosterone must consider sex-specific hormonal physiology to avoid unintended metabolic consequences and to optimize therapeutic outcomes.

In Chapter 5, we expanded the investigation to cardiovascular outcomes, specifically myocardial infarction (MI) and stroke, within the same KORA cohort. We found an inconsistent mediatory role for TT and dihydrotestosterone (DHT) in the association between sex and risk of stroke, suggesting that androgen-related pathways may contribute differentially to cerebrovascular risk across sexes. No mediating role was observed for other androgens or SHBG in relation to stroke, and neither androgens nor SHBG mediated the risk of MI.

Previous evidence has established sex differences in the incidence of cardiovascular events, specifically MI and stroke, as well as in their related risk factors (11, 12). However, the biological underpinnings of these differences remain only partially understood. While androgen levels and SHBG have been linked to cardiometabolic risk in prior epidemiological and MR studies, most have focused on direct associations within each sex rather than assessing their role as mediators of sex-based differences in cardiovascular outcomes (13-15).

By formally evaluating mediation pathways, our analysis adds an important mechanistic perspective to the literature. The inconsistent mediatory role of TT and DHT for stroke risk, but not MI, in men and women suggests that androgen-related vascular effects may differ by outcome type and possibly by vascular territory or hormonal context. This pattern is consistent with the hypothesis that alternative pathways, particularly lipid metabolism, inflammation, and endothelial dysfunction may play more dominant roles in coronary events (16-19).

Our findings have some implications for clinical and preventive cardiovascular research. First, they suggest that androgen-related pathways may not uniformly account for sex differences across all cardiovascular outcomes. While TT and DHT might contribute to cerebrovascular risk differences between men and women, their role in coronary disease appears to be less pronounced. This underscores the need for outcome-specific and sex-aware cardiovascular risk assessment, rather than assuming shared hormonal mechanisms across vascular beds.

In Chapter 6, we conducted a large systematic review and meta-analysis examining the associations of endogenous sex hormones and SHBG with the risk of all-cause and cause-specific mortality in the general population. The dose-response analysis revealed a significant U-shaped association between TT and all-cause mortality in men, and a J-shaped association in women. Higher SHBG concentrations were significantly associated with a greater risk of all-cause mortality in women, whereas no association was observed in men. Additionally, higher DHEA-S levels were linked to a lower risk of all-cause mortality in men, with no corresponding association in women.

While the relationship between endogenous sex hormones, SHBG, and mortality risk has been extensively studied in men, evidence among women remains limited and inconsistent. To address this gap, we performed a comprehensive systematic review and meta-analysis that, for the first time, assessed the certainty of evidence using the GRADE framework. This approach allowed us to provide a structured evaluation of study quality and confidence in the observed associations, highlighting critical gaps in the existing literature for both men and women.

Our dose-response analyses further extended current knowledge by identifying non-linear associations between endogenous testosterone levels and all-cause mortality risk. These findings are consistent with a recent individual-participant-data (IPD) meta-analysis reporting similar non-linear patterns among men (20), reinforcing the notion that both low and high testosterone levels may be detrimental to longevity. Together, these results emphasize the importance of considering hormonal balance and optimal physiological ranges, rather than absolute concentrations, in understanding endocrine contributions to mortality risk.

In the final Chapter, we extended our systematic review and meta-analysis to investigate the associations of endogenous sex hormones and SHBG with the risk of all-cause and cause-specific mortality among patients with established CVD,

an analysis that, to our knowledge, had not been conducted previously. We found no significant association between endogenous TT concentrations and the risk of either all-cause or CVD-specific mortality in men. The majority of studies included in this analysis were rated as having a high risk of bias, primarily due to inadequate adjustment for confounders, small sample sizes, and missing data. Evidence on other sex hormones and data from studies in women were too limited to permit meta-analysis.

These findings suggest that the relationships observed between endogenous sex hormones, SHBG, and mortality in the general population may not directly translate to populations with established CVD. The presence of underlying cardiovascular pathology, pharmacological treatments, and disease-related metabolic alterations could attenuate or obscure hormonal associations that are evident in healthier individuals. Moreover, reverse causation, whereby advanced disease influences hormone levels, cannot be ruled out in this clinical context.

Our study highlights the importance of context-specific evaluation when interpreting the prognostic value of sex hormones. While low or high hormone concentrations may predict mortality risk in community-based cohorts, such markers may lose discriminatory power once CVD has developed. This underscores the need for longitudinal studies that account for disease severity, treatment status, and dynamic hormonal changes over time.

Methodological considerations

Beyond the chapter-specific limitations discussed earlier, there are three main methodological issues to consider for this thesis as a whole. Two of these relate to the original observational studies (Chapters 3–5), and one relates to the systematic reviews and meta-analyses (Chapters 2, 6, and 7).

Low number of incident cases and limited statistical power

An important methodological limitation of this thesis is the relatively low number of incident cases for outcomes including T2D, stroke, and MI in the studies described in Chapters 3–5. The limited event counts reduced statistical power, widened confidence intervals, and increased uncertainty around the effect estimates. The limited number of events also restricted our ability to perform stratified analyses, for example, by obesity and menopausal status, as further subdivision of the dataset would have resulted in unstable estimates. To partially mitigate the impact of event sparsity, particularly in the mediation analyses, we applied non-parametric bootstrapping to obtain more robust confidence intervals that do not rely on normality assumptions. Although bootstrapping improves the certainty of the indirect-effect estimates, the

findings should still be interpreted with caution given the limited number of outcome events. This is especially important for sensitivity analysis models with several confounders, where low event numbers increase the risk of overfitting and reduce the certainty of the effect estimates. For these reasons, main models were prioritized, and the results of more complex models should be interpreted with caution. Finally, because all analyses were conducted within a single regional cohort (KORA), the generalizability of the findings to other populations may be limited.

Potential sources of bias

Selection bias

In the studies presented in Chapters 3–5, a general methodological concern is the presence of missing data on mediators (sex hormones and SHBG) and outcomes (glucose homeostasis, incident T2D, stroke, and MI). Missingness may have arisen due to loss to follow-up or incomplete data collection. Exclusion of participants with missing mediator or outcome data may have introduced selection bias if the probability of missingness was related to both the exposure and the outcomes, potentially affecting the validity of the observed associations. However, we did not observe meaningful differences in key baseline characteristics between participants included in the analyses and those excluded, suggesting that the impact of selection bias may be limited.

Information bias

While information bias is more commonly discussed in relation to exposures and outcomes, in the present studies it is more likely to have affected the measurement of confounding factors. In Chapters 3–5, key confounders, including smoking status, alcohol consumption, physical activity, and medication use, were collected through standardized interviews conducted by trained medical staff. The use of standardized protocols and trained personnel likely improved the consistency and overall quality of data collection. However, these variables were primarily self-reported and are therefore inherently subjective. As a result, they may be prone to various forms of information bias, including misclassification bias, recall bias, response bias, and interviewer bias. Participants may not accurately recall past behaviours or exposures, particularly for variables such as long-term lifestyle habits or medication use. In addition, socially undesirable behaviours, such as smoking or excessive alcohol consumption, may be underreported, while healthier behaviours may be overreported, leading to systematic misclassification. Interviewer-related factors, such as differences in questioning or interpretation, may also introduce variability despite standardized procedures.

Such misclassification of confounders can result in incomplete adjustment in multivariable models, thereby contributing to residual confounding. Depending on whether the misclassification is differential or non-differential with respect to the exposure or outcome, this could bias the observed associations in either direction or attenuate true effects. Therefore, although efforts were made to account for major confounders, the potential for measurement error in these self-reported variables should be considered when interpreting the findings.

Confounding

Confounding arises from variables that are associated with both the exposure and the outcome but are not part of the causal pathway between them (21). In all analyses presented in Chapters 3–5, we adjusted for potential confounders identified based on prior literature and the proposed directed acyclic graphs (DAGs).

We attempted to control for confounding by using multivariable models that adjusted for a wide range of potential confounders, including demographic, clinical, and lifestyle factors. However, given the observational nature of the included studies, residual and unmeasured confounding cannot be entirely ruled out. Residual confounding may arise from imperfect measurement of covariates, misclassification, or incomplete adjustment, while unmeasured confounding may occur when relevant variables are not captured or included in the analyses. Such biases could lead to either overestimation or underestimation of the true associations, depending on the direction and magnitude of the relationships between the confounders, exposures, and outcomes.

In Chapter 6, which presents a systematic review and meta-analysis of observational cohort studies on sex hormones, SHBG, and mortality risk in the general population, medication use represents an important potential source of unmeasured or residual confounding. Many of the included studies did not report or adequately adjust for medication use, despite the fact that various medications (e.g., hormone therapy, lipid-lowering agents, antidiabetic drugs, and antihypertensive agents) may influence both circulating hormone levels and mortality risk. In addition, some studies did not account for comorbidities. Unmeasured or insufficiently adjusted comorbid conditions may have contributed to residual confounding, as diseases such as CVD, T2D, chronic kidney disease, liver disease, cancer, and other metabolic or inflammatory disorders can influence both hormone concentrations and mortality risk. The omission of these factors may therefore have biased the observed associations.

Similarly, in Chapter 7, focusing on patients with established cardiovascular disease, several additional factors may contribute to unmeasured or residual confounding. In this clinical population, medication use is particularly relevant, as treatments such as statins, antihypertensives, and hormone-modulating therapies may affect both hormone levels and survival outcomes. In addition, lifestyle factors, including smoking status, alcohol consumption, and physical activity, may not have been consistently measured or fully adjusted for across studies. These factors are strongly related to both hormonal profiles and mortality risk, and incomplete control for them may have influenced the observed associations.

Heterogeneity and publication bias

Systematic reviews and meta-analyses are widely regarded as among the highest forms of evidence across clinical and epidemiologic research, including etiological, diagnostic, prognostic, and therapeutic questions because they synthesize all relevant studies using rigorous, transparent methods (22). Inevitably, a systematic review brings together studies that differ in population characteristics, study design, and definitions of exposures and outcomes. This variation, referred to as heterogeneity, is an important aspect to consider, assess, and report when summarizing evidence (23).

In Chapter 2, we identified studies that developed or evaluated risk prediction models for the ONM. The limitations of the existing evidence are discussed in detail in the corresponding chapter. A major limitation was the lack of sufficient external validation for individual risk prediction models. Because of this, it was not possible to reliably combine or compare their predictive performance across different settings and populations. Consequently, conducting a formal meta-analysis was not feasible (24).

In Chapters 6 and 7, we observed varying degrees of heterogeneity across studies, with I^2 estimates ranging from 0% to 83%. In our systematic review and meta-analysis on endogenous sex hormones, SHBG, and mortality risk in the general population (Chapter 6), we explored potential sources of heterogeneity based on study characteristics such as country, duration of follow-up, as well as categories defined by whether studies adjusted for key metabolic factors (e.g., hypertension, diabetes, physical activity, lipid markers, and blood pressure). We also conducted leave-one-out analyses. These approaches helped explain some of the substantial heterogeneity observed in several models. In contrast, in Chapter 7, the number of available studies for each analysis among patients with established CVD was

generally small, limiting our ability to investigate sources of heterogeneity through subgroup or sensitivity analyses.

Another threat to the validity of a meta-analysis is publication bias, which may arise from the underreporting of null or negative findings (25). If the body of studies retrieved for review is biased, the pooled estimates may be distorted. In the meta-analyses presented in this thesis, traditional approaches, including visual inspection of funnel plots and Egger's test, generally suggested a minimal impact of publication bias in most analyses. However, these methods have inherent limitations, particularly their reliance on subjective visual assessment and reduced reliability when the number of available studies is small, as was often the case in our evaluations (typically fewer than ten studies). Therefore, despite undertaking a comprehensive and systematic literature search, the possibility of publication bias due to selective non-publication of negative or non-significant results cannot be entirely excluded.

Future directions

The findings from this thesis collectively underscore the complex and context-dependent roles of endogenous sex hormones and SHBG in shaping cardiometabolic outcomes and mortality in both men and women. Building on these insights, several key avenues for future research can be identified.

First, the prediction of reproductive aging and menopause timing remains an important but underdeveloped area. Future research should aim to develop and externally validate robust, generalizable models for predicting the ONM. Such efforts may combine hormonal, genetic, metabolic, and lifestyle predictors using modern statistical and machine learning methods. Longitudinal, multiethnic cohort studies are needed to ensure calibration and transportability across populations. Translating these models into practice will require testing whether more accurate prediction of menopause timing can help guide early prevention of cardiometabolic and bone-related diseases. Linking menopause prediction to downstream health trajectories such as metabolic and cardiovascular risks may further bridge reproductive and preventive medicine and enhance women's health management across the life span.

The observed mediatory role of SHBG in explaining sex differences in glucose homeostasis and incidence of T2D underscores the need to elucidate its causal and biological mechanisms. Future studies should leverage MR studies integrated with multi-omics approaches, particularly proteomics and metabolomics to dissect how

SHBG modulates hepatic lipid metabolism, insulin sensitivity, and inflammatory pathways. In parallel, sex-stratified longitudinal cohorts with repeated hormonal and multi-omics measurements are essential to clarify the sequence of events, specifically whether changes in SHBG and androgens occur before metabolic deterioration or arise as a consequence of it.

Although recent evidence suggests that SHBG may function as an active metabolic regulator influencing lipid and glucose metabolism (26, 27), complementary studies in human cell models and animal systems are needed to validate these findings and elucidate the underlying mechanisms beyond its traditional carrier role. These mechanistic investigations will bridge observational and genetic findings, providing a foundation for sex-specific therapeutic targets.

Our mediation findings mirror earlier evidence showing that TT influences glucose homeostasis and incidence of T2D in a sex-specific manner but are established here within a different statistical framework that enables a direct comparison between men and women. Resolving these discrepancies demands sex-stratified genetic instruments and analyses in future MR studies. Critically, current genome-wide association studies (GWAS) of sex hormones are limited by their predominant use of mixed-sex cohorts, which mask sex-specific signals and yield limited power in female subgroups (28). Recent genetic studies have confirmed these limitations, showing that the overlap between variants associated with sex hormone traits in men and women is minimal, except for SHBG, and that even shared loci often exert divergent effects across sexes (5). This low cross-sex replication rate not only undermines the reliability of genetic instruments for causal inference but also perpetuates a male-biased understanding of hormone-metabolism interactions, as evidenced by underpowered female strata in landmark studies (5). To address this, expanding well-powered, sex-specific GWAS with targeted recruitment to achieve adequate statistical power in women will be indispensable for accurately mapping the genetic architecture of these pathways and enabling robust, unbiased MR analyses.

The inconsistent mediatory role of TT and DHT in stroke risk between men and women, together with the absence of mediation observed for MI in this thesis, suggests that hormone–vascular associations may be outcome-specific. Future research should therefore focus on identifying vascular-bed-specific mechanisms by exploring how androgens and SHBG influence endothelial function, coagulation, and inflammation across different arterial territories. Studies that include imaging biomarkers and detailed vascular assessments such as measures of subclinical

atherosclerosis, arterial stiffness, and microvascular function could provide insight into sex-specific vascular profiles.

In this context, mediation analysis will be useful to better understand the biological pathways involved and to determine whether hormonal effects on cardiovascular outcomes act through intermediate factors such as lipid metabolism, blood pressure regulation, or inflammation. Combining data on lipid profiles, inflammatory markers, and hormone levels will also be important to better understand shared risk pathways that may differ between men and women. These approaches could help improve personalized and sex-specific cardiovascular risk assessment and prevention strategies that consider hormonal context.

If confirmed by future studies, this outcome-specific and sex-divergent pattern challenges the notion of uniform androgen action and instead supports a vascular-bed– and disease-specific framework for hormone–cardiovascular associations. Future research might therefore move beyond a "one-size-fits-all" model of cardiovascular disease and focus on hormone effects that may differ across specific vascular beds. Using imaging techniques and multi-marker mediation analyses could help clarify why testosterone shows different associations across cardiovascular subtypes.

The non-linear and sex-specific associations observed between endogenous TT and risk of all-cause mortality emphasize the need to consider hormonal balance, rather than isolated hormone thresholds, in understanding endocrine contributions to longevity. Future research should incorporate time-varying analyses and repeated hormone measurements to capture how dynamic changes in androgens and SHBG relate to long-term mortality trajectories.

Establishing sex- and age-specific reference ranges that reflect optimal hormonal balance for survival, rather than single cutoffs, would enhance both the interpretability and clinical applicability of findings. Given the variability in measurement methods across existing studies, future research should employ hormone assays that meet the highest analytical standards, ideally liquid chromatography–tandem mass spectrometry (LC–MS/MS), to minimize measurement error and information bias, particularly for hormones present at low concentrations, such as testosterone in postmenopausal women, which may fall below the limit of detection when measured using less sensitive assays. Standardization of hormone assessment and analytical approaches across cohorts will be critical for improving reproducibility and comparability of results.

Most of the methodological limitations and research gaps identified for studies in the general population also apply to investigations in patients with established CVD. Future work should therefore prioritize well-designed longitudinal studies with repeated hormone assessments to capture dynamic hormonal changes in relation to disease progression and treatment. Standardization of hormone assays (e.g., LC–MS/MS), analytical approaches, and adjustment for disease- and therapy-related confounders remain essential to improve comparability and validity.

Future research could also benefit from IPD meta-analyses to more comprehensively investigate the associations of endogenous sex hormones and SHBG with a range of chronic diseases in both general-population and patient-based studies. Such approaches would enable harmonized exposure definitions, consistent adjustment for confounders, and more detailed sex-specific and non-linear analyses, thereby improving the robustness and comparability of findings across studies.

Finally, evidence on the role of endogenous sex hormones in cardiovascular disease among women remains limited. Future research should prioritize well-powered, sex-specific studies, particularly in women across different menopausal stages, to better characterize hormonal associations with cardiovascular outcomes. Such studies should incorporate repeated hormone measurements and carefully account for hormonal transitions, including menopause and hormone therapy use, to improve understanding of sex-specific cardiovascular risk profiles.

Conclusion

Taken together, the findings of this thesis demonstrate that endogenous sex hormones and SHBG contribute to cardiometabolic health in complex, outcome-specific, and often sex-divergent ways. These results support current prevention guidelines, including those of the European Society of Cardiology, which emphasize the importance of incorporating sex into cardiovascular risk assessment and clinical management (29). Such recommendations reflect the growing recognition that traditional risk models often overlook sex-specific biological mechanisms and may not fully capture differences in disease presentation, progression, and outcomes. By integrating evidence synthesis with large-scale cohort analyses, this thesis provides both epidemiologic and mechanistic insights that advance our understanding of hormonal influences on cardiometabolic health across the life course. Collectively, this work highlights the need to move beyond one-size-fits-all approaches toward more precise, sex-aware, and outcome-specific prevention strategies that explicitly account for hormonal context in cardiometabolic health.

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Appendices

Summary
Samenvatting
PhD Portfolio
About Author
Acknowledgments
List of publications

Summary

Sex differences in cardiometabolic diseases are well documented, yet the biological mechanisms underlying these differences remain incompletely understood. Endogenous sex hormones and sex hormone–binding globulin (SHBG) have been proposed as important contributors, given their central role in metabolic regulation and cardiovascular physiology. This thesis aimed to investigate the role of endogenous sex hormones and SHBG across the life course, from the menopausal transition to mortality, in relation to cardiometabolic health.

Chapter 2 of this thesis presented a systematic review of risk prediction models for natural menopause. Menopause represents a major reproductive transition in women and is increasingly recognized as an independent risk factor for cardiometabolic diseases later in life. By evaluating existing prediction models for the onset of menopause, this work highlighted both the potential and the current limitations of these approaches in identifying women who may experience earlier or later menopause. Improving such predictive tools could enhance our understanding of individual risk assessment and its implications for long-term cardiometabolic prevention strategies.

Chapters 3 to 5 examined whether endogenous sex hormones and SHBG contribute to explaining sex differences in cardiometabolic risk factors and diseases. Using population-based data from the KORA study, we investigated the associations between sex, circulating hormone concentrations, and key cardiometabolic outcomes, including glucose homeostasis, type 2 diabetes, and cardiovascular disease. A mediation analysis framework was applied to explore the complex relationships between sex, sex hormones, cardiometabolic intermediates, and clinical outcomes. This approach allowed us to assess the extent to which differences in endogenous hormone levels and SHBG may explain observed sex differences in cardiometabolic health. Our findings suggest that the role of androgens and SHBG may be context-dependent and varies across vascular beds and disease types, indicating that their effects cannot be generalized across different forms of cardiovascular disease.

In Chapters 6 and 7, we synthesized existing epidemiological evidence on the associations between circulating sex hormones, SHBG, and mortality. Through systematic reviews and meta-analyses of prospective cohort studies, we evaluated the relationships between hormone levels and both all-cause and cause-specific mortality in the general population as well as among patients with established

cardiovascular disease, an area that had not previously been comprehensively synthesized. These findings contribute to the growing body of evidence suggesting that circulating sex hormones and SHBG may serve as markers of overall health status and long-term disease risk.

Finally, Chapter 8 integrates the findings of this thesis and discusses their broader implications for research and clinical practice. It also outlines important methodological considerations and highlights directions for future research to address remaining knowledge gaps regarding the role of sex hormones in cardiometabolic health across the life course.

Samenvatting

Sekseverschillen in cardiometabole ziekten zijn goed gedocumenteerd, maar de biologische mechanismen die aan deze verschillen ten grondslag liggen, worden nog niet volledig begrepen. Endogene geslachtshormonen en sex hormone–binding globulin (SHBG) worden beschouwd als belangrijke factoren die mogelijk bijdragen aan deze verschillen, gezien hun centrale rol in metabole regulatie en cardiovasculaire fysiologie. Het doel van dit proefschrift was daarom om de rol van endogene geslachtshormonen en SHBG gedurende de levensloop in relatie tot cardiometabole gezondheid te onderzoeken, van de menopauzale overgang tot sterfte.

Hoofdstuk 2 van dit proefschrift presenteert een systematische review van risico-voorspellingsmodellen voor het optreden van de natuurlijke menopauze. De menopauze markeert het einde van het reproductieve leven van vrouwen en wordt steeds vaker erkend als een onafhankelijke risicofactor voor cardiometabole ziekten op latere leeftijd. Door bestaande voorspellingsmodellen voor het optreden van de menopauze te evalueren, bracht dit werk zowel het potentieel als de huidige beperkingen van deze modellen bij het identificeren van vrouwen die een eerdere of latere menopauze kunnen ervaren aan het licht. Verbetering van dergelijke voorspellingsinstrumenten kan bijdragen aan een beter begrip van reproductieve veroudering en de gevolgen daarvan voor cardiometabole gezondheid op de lange termijn.

In Hoofdstukken 3 tot en met 5 werd onderzocht of endogene geslachtshormonen en SHBG bijdragen aan het verklaren van sekseverschillen in cardiometabole risicofactoren en ziekten. Met behulp van gegevens uit de populatiegebaseerde KORA-studie onderzochten we de verbanden tussen sekse, circulerende hormoonconcentraties en belangrijke cardiometabole uitkomsten, waaronder glucosestofwisseling, type 2 diabetes en cardiovasculaire ziekten. Daarnaast werd een mediatieanalyse toegepast om de complexe relaties tussen sekse, geslachtshormonen, cardiometabole intermediaire factoren en klinische uitkomsten te onderzoeken. Deze benadering maakte het mogelijk om te beoordelen in hoeverre verschillen in endogene hormoonspiegels en SHBG de waargenomen sekseverschillen in cardiometabole gezondheid kunnen verklaren. Onze bevindingen suggereren dat de rol van androgenen en SHBG contextafhankelijk kan zijn en kan variëren tussen verschillende vaatbedden en ziektebeelden, wat aangeeft dat hun effecten niet kunnen worden gegeneraliseerd naar alle vormen van cardiovasculaire ziekten.

In Hoofdstukken 6 en 7 werd bestaand epidemiologisch bewijs over de verbanden tussen circulerende geslachtshormonen, SHBG en mortaliteit samengebracht. Door middel van systematische reviews en meta-analyses van prospectieve cohortstudies evalueerden we de relaties tussen hormoonspiegels en zowel totale mortaliteit als oorzaak-specifieke mortaliteit, zowel in de algemene bevolking als bij patiënten met reeds bestaande cardiovasculaire ziekten, een onderwerp dat nog niet eerder uitgebreid systematisch was samengevat. Deze bevindingen dragen bij aan de groeiende hoeveelheid bewijs dat circulerende geslachtshormonen en SHBG mogelijk kunnen fungeren als indicatoren van de algehele gezondheidstoestand en het risico op ziekte op de lange termijn.

Tot slot brengt Hoofdstuk 8 de bevindingen van dit proefschrift samen en bespreekt het de bredere implicaties voor onderzoek en klinische praktijk. Daarnaast worden belangrijke methodologische overwegingen besproken en worden richtingen voor toekomstig onderzoek voorgesteld om bestaande kennislacunes met betrekking tot de rol van geslachtshormonen in cardiometabole gezondheid gedurende de levensloop verder te verkennen.

PhD Portfolio

Name of PhD student: Hamidreza Raeisidehkordi
UMCU department: Julius Center – Department of Global Public Health and Bioethics
PhD period: January 2023 – March 2026
Promotors: Prof. dr. O.H. Franco Duran, Prof. dr. Y.T. van der Schouw
Co-promotors: Dr. T. Muka, Dr. A. Chatelan

MSc Epidemiology (Track: Clinical Epidemiology)		
Courses	**Year**	**ECTs**
Introduction to Epidemiology	2024	1.5
Introduction to Statistics	2024	1.5
Study Design in Etiologic Research	2024	3.0
Presenting Your Research Confidently	2024	0.5
Classical Methods in Data Analysis	2024	6.0
Modern Methods in Data Analysis	2024	4.5
Writing Research Proposals	2024	1.5
Mixed Models	2025	1.5
Systematic Review and Meta-Analysis of Prognosis Studies	2025	1.5
Clinical Epidemiology	2025	1.5
Clinical Trials and Drug Risk Assessment	2025	1.5
Research Ethics an Introduction	2025	1.5
Systematic Reviews in Intervention Research	2025	1.5
Survival Analysis	2025	1.5
Advanced Topics in Causal Research	2025	1.5
Prognostic Research	2025	1.5
Advanced Diagnostic Research	2025	1.5
Missing data	2025	1.5
Machine Learning and Applications in Medicine	2025	1.5
Research project	2024-2025	56
Conferences and scientific meetings		
WEON – poster presentation	2024	1
ESPEN- poster presentation	2025	1
Julius Symposium- poster presentation	2025	1
Teaching activities		
START block	2024	-
Other		
Research Internship-Amsterdam UMC	March–August 2023	-
Microbiome Data Analysis (Erasmus Summer Programme, Erasmus MC)	2023	1.5
Peer review of articles for scientific journals	2023-2026	-

About Author



Hamidreza Raeisi-Dehkordi was born on 7 June 1992 in Shahrekord, Iran. He obtained his Bachelor's degree in Nutrition Sciences from Shiraz University of Medical Sciences, Iran, in 2016. He subsequently pursued a Master's degree in Public Health Nutrition at Shahid Sadoughi University of Medical Sciences in Yazd, Iran, which he completed in 2019.

Following his master's degree, he contributed to securing funding through the Swiss National Science Foundation (SNSF) SPIRIT project and joined the Institute of Social and Preventive Medicine (ISPM) at the University of Bern, Switzerland, in December 2021 as a Scientific Researcher. After one year in Bern, he moved to the Netherlands to begin his PhD at the Julius Center for Health Sciences and Primary Care, UMC Utrecht, under the supervision of Professors Oscar H. Franco and Yvonne T. van der Schouw as promotors, and Dr. Taulant Muka and Dr. Angeline Chatelan as co-promotors.

Alongside his PhD, he completed the Postgraduate Programme in Epidemiology at Utrecht University between 2023 and 2025, earning a Master of Science (MSc) in Epidemiology. His research focused on nutritional, lifestyle, and cardiovascular epidemiology, with particular emphasis on sex hormones, sex differences, and cardiometabolic health.

Following the completion of his PhD, he joined Tilburg University as a postdoctoral researcher, where he continues his research in nutritional and lifestyle epidemiology, with a particular focus on cardiometabolic health.

Acknowledgment

There it is! I still find it hard to believe that I have finally reached this milestone. Looking back, my PhD journey was anything but straightforward. It was filled with uncertainty, challenges, and moments when I questioned whether I would ever make it to this point.

My journey truly began in December 2021, when I arrived at the Institute of Social and Preventive Medicine (ISPM) at the University of Bern. From the very beginning, there was uncertainty surrounding the funding of my PhD, and that uncertainty remained with me until the very last stages of the project. There were times when I was not at my best, mentally or emotionally, but I always tried to give everything I had. Looking back now, I realize that I could never have walked this path alone. This thesis is not only the result of my own efforts, but also of the unwavering support, encouragement, and kindness of my family, friends, colleagues, collaborators, and mentors.

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of research matters far more than simply producing more work, and that research is most valuable when it is meaningful and applicable beyond academia. I am especially grateful that you trusted me to work with you at Epistudia. This was a very different and valuable experience, as it showed me how academic knowledge can be used beyond universities and research institutes. It encouraged me to accept new challenges, take risks, and explore opportunities outside my comfort zone. Thank you also for supporting me even when I made mistakes. I learned from you that, although we always do our best to provide accurate and reliable evidence, mistakes can sometimes happen. What matters most is that we remain transparent, take responsibility, learn from them, and continue improving. Your support during difficult moments helped me approach challenges with greater confidence and maturity. I am deeply grateful for all your guidance and encouragement throughout this journey. You were always available whenever I needed to discuss an idea, seek advice, or clarify an important point. Your constant encouragement to "*keep it up and shine*" gave me confidence and motivation at many important moments. Beyond the work itself, I am genuinely grateful for your trust, encouragement, and friendship along the way.

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