Sex Hormones and Cardiometabolic Risk

Marija Glišić

ACKNOWLEDGMENTS

The work presented in this thesis was conducted at the Cardiovascular Group of the Department of Epidemiology, and at Division of Vascular Medicine and Pharmacology at Department of Internal Medicine, Erasmus MC, Rotterdam, the Netherlands.

The research presented in this thesis was partially supported by The Erasmus Mundus–Western Balkans (ERAWEB) scholarship. The majority of studies described in this thesis involved the Rotterdam Study, which is supported by the Erasmus MC and the Erasmus University Rotterdam, the Netherlands Organization for Scientific Research (NOW), the Netherlands Organization for Health Research and Development (ZonMw), the Dutch Heart Foundation, the Research Institute for Diseases in Elderly (RIDE), the Ministry of Education, Culture, and Science, the Ministry of Health Welfare and Sports, the European Commission, and the municipality of Rotterdam. The contribution of the inhabitants, general practitioners and pharmacists of the Ommoord district to the Rotterdam Study is gratefully acknowledged.

Publication of this thesis was kindly supported by the Department of Epidemiology of Erasmus Medical Center and by Erasmus University Rotterdam.

ISBN: 978-94-6361-171-8

Layout and printed by: Optima Grafische Communicatie, Rotterdam, the Netherlands (www.ogc.nl)

Cover Photo by 24-design (www.24-design.com)

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Geslachtshormonen en cardiometabool risico

Thesis

to obtain the degree of Doctor from the

Erasmus University Rotterdam

by command of the rector magnificus

Prof. Dr Rutger Engels

and in accordance with the decision of the Doctorate Committee.

The public defence shall be held on
Wednesday, October 31st, 9:30 am.

by

Marija Glišić

Born in Loznica, Serbia

Erasmus University Rotterdam

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	Irma Karabegović

To my sister, parents and granny

And to Stevan

Manuscripts that form the basis of this thesis

Chapter 2

Glisic M, Rojas LZ*, Asllanaj E*, Vargas KG, Kavousi M, Ikram MA, Fauser BCJM, Laven JSE, Muka T, Franco OH. Sex steroids, sex hormone-binding globulin and levels of N-terminal pro-brain natriuretic peptide in postmenopausal women. Int J Cardiol. 2018 Jun 15;261:189-195. doi: 10.1016/j.ijcard.2018.03.008.

Rojas LZ*; Rueda-Ochoa OL*; Asllanaj E*; Portilla E; Gonzáles-Jaramillo V; Nano J, Ikram MA, Burgess S, Franco OH; **Glisic M***; Muka T*. Mendelian randomization provides evidence for a causal role of dehydroepiandrosterone sulfate in decreased NT-proBNP levels in a Caucasian population (under review).

Glisic M, Mujaj B, Rueda-Ochoa OL, Asllanaj E, Laven JSE, Kavousi M, Ikram MK, Vernooij MW, Ikram MA, Franco OH, Bos D, Muka T. Associations of Endogenous Estradiol and Testosterone Levels With Plaque Composition and Risk of Stroke in Subjects With Carotid Atherosclerosis. Circ Res. 2018 Jan 5;122(1):97-105. doi: 10.1161/CIRCRESAHA.117.311681

O'Reilly MW*, **Glisic M***, Kumarendran B, Subramanian A, Manolopoulos KN, Tahrani AA, Keerthy D, Muka T, Toulis KA, Hanif W, G. Thomas N, Franco OH, Arlt W, Nirantharakumar K. Serum testosterone and sex-specific risk of incident type 2 diabetes: a longitudinal UK primary care database study (Submitted to journal).

Glisic M, Kastrati N*, Meun C*, Asllanaj E, Sedaghat S, Ikram MA, Laven J.S.E., Nirantharankumar K, Franco O.H., Muka T. Prognostic value of dehydroepiandrosterone in type 2 diabetes: The Rotterdam Study (under review).

Chapter 3

Glisic M, Shahzad S, Tsoli S, Chadni M, Asllanaj E, Rojas LZ, Brown E, Chowdhury R, Muka T, Franco OH. Association between progestin-only contraceptive use and cardiometabolic outcomes: A systematic review and meta-analysis. Eur J Prev Cardiol. 2018 Jul;25(10):1042-1052. doi: 10.1177/2047487318774847

Oliver-Williams C*, **Glisic M***, Shahzad S, Brown E, Pellegrino Baena C, Chadni M, Chowdhury R, Franco OH*, Muka T *. The route of administration, timing, duration and dose of postmenopausal hormone therapy and cardiovascular outcomes in women: a systematic review (under review)

Chapter 4

Glisic M, Kastrati N*, Gonzalez-Jaramillo V*, Bramer WM, Ahmadizar F, Chowdhury R, Danser AHJ, PhD, Roks AJM, Voortman T, Franco OH, Muka T. Associations between phytoestrogens, glucose homeostasis and risk of diabetes in women: a systematic review and meta-analysis. **Accepted for publication in Advances in Nutrition.**

Glisic M, Kastrati N[*], Musa J[*], Milic J, Asllanaj E, Portilla Fernandez E, Nano J, Ochoa Rosales C, Amiri M , Kraja B, Bano A, Bramer WM, Roks AJM, Danser AHJ, Franco OH , Muka T. Phytoestrogen supplementation and body composition in postmenopausal women: A systematic review and meta-analysis of randomized controlled trials. Maturitas 115 (2018) 74-83.

*denotes equal contribution

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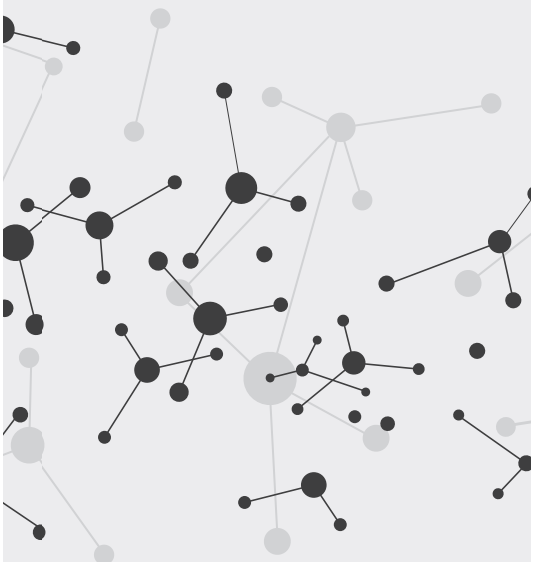
Chapter 1

General Introduction



Sex Disparities in Cardiometabolic Outcomes

Cardiometabolic diseases include cardiovascular diseases (CVD), type 2 diabetes (T2D) and their associated risk factors including metabolic syndrome (increased blood pressure, high blood sugar/cholesterol/triglyceride levels) and obesity[1]. Cardiovascular diseases are a leading cause of death in the world accounting for more than one-third of deaths annually[2]. Prevalence and incidence of T2D, a major risk factor for CVD, is increasing rapidly, with more than 340 million people living with diabetes worldwide[3]. During the past decades, there have been some truly significant advances in management and treatment of cardiometabolic diseases, but yet they remain the leading cause of death and disability worldwide and a major public health concern[4].

Sex differences are described in nearly all human diseases and their prevalence, severity and prognosis[5,6,7]. CVD has been taken as a classical example of sexual dimorphism in human diseases[8]. Women are considered to be protected from CVD before menopause; the prevalence of CVD is far less in premenopausal women compared to age-matched men, however, this sex advantage for women gradually disappears with increased age, and is associated with reduced estrogen levels after menopause[9,10]. Also, T2D which often manifests during mid-life and thus coincides with the timing of the menopausal transition in women is associated with higher risk of CVD and stroke in women compared to men[11]. Indeed, menopausal transition is associated with a worsening of cardiometabolic risk profile including adverse changes in blood lipids, blood pressure and body composition. Ovarian hormone insufficiency at the time of the menopause has been suggested as an important determinant of the decline in cardiometabolic health in aging women[9]. Certainly, after menopause, estradiol levels decrease by 80% as compared to premenopausal levels and the ratio of estrogens and androgens significantly changes, while the decline in testosterone in aging men is not as dramatic, as it amounts to 30 to 40%[8]. Therefore, aging related sex hormone-changes have been suggested to be an important player in modifying cardiometabolic risk over the life span, particularly in women (**Figure 1**)[12,13].

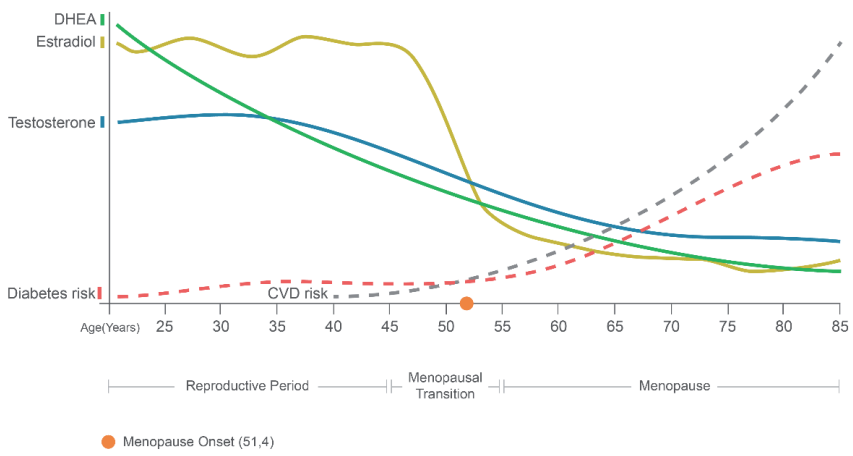


Figure 1. Age-related variations in serum sex hormones and cardiometabolic risk in women.

Endogenous Sex Hormones and Cardiovascular risk in Men and Women

Studies of circulating cardiovascular biomarkers, such as the natriuretic peptides (NPs), may provide a biologic basis to better understand the sex-related differences in cardiovascular risk. NPs such as brain natriuretic peptide (BNP) and its inactive precursor amino-terminal-B-type-natriuretic peptide (NT-proBNP) have a diagnostic and prognostic role in heart failure (HF) and a predictive value in CHD[14]. BNP exerts hormonal and autocrine/paracrine protective cardiovascular effects[15]. Sex is suggested to be one of the most important determinants of circulating NP levels[15]. Women have been reported to have higher NP levels during their life span as compared to men[16]. Also, in women, NP levels change by menopause status, with women after menopause having lower levels of NPs[17]. Although the exact mechanisms underlying these observed sex-differences have not been described yet, emerging evidence suggests that sex hormones play an important role in the regulation of NPs[15]. Estrogens can have a stimulating effect on the NP system[18,19], while, androgens may exert an inhibitory effect[16]. Also, dehydroepiandrosterone (DHEA) and its sulphate conjugate (DHEAs), hormones that give rise to both estrogens and androgens, are inversely associated with plasma levels of NPs independently of age and other clinical variables in subjects with HF[20]. Therefore, the lower NP levels during the whole lifespan in men and in aging women could potentially explain the lack of cardiovascular protection in men and

postmenopausal women as compared to premenopausal women[17]. However, previous studies looking at these associations have been primarily focused on premenopausal women, and a gap exists on whether endogenous sex hormones affect NT-proBNP levels in postmenopausal women, and the role of age. Also, due to the observational nature of previous studies on this topic, it is not known whether the described associations are causal or explained by confounding and reverse causation.

Besides affecting NPs levels, estradiol may play cardioprotective role in premenopausal women via multiple mechanisms: affecting (i) body fat distribution, (ii) glucose-insulin homeostasis, (iii) vasodilatation and (iv) plasma lipoprotein levels[21]. However, the protective effect of estradiol may be diminished with aging, and animal studies show that this could be due to the decrease in estradiol levels, but also due to a decrease in estradiol receptor responsiveness[22]. The so called "timing hypothesis" has been proposed after observational data showed that risk of stroke roughly doubles during the 10 years after menopause[23] and that a certain stroke risk exists with menopausal hormone therapy (HT) use[24]. For example, ischemic stroke risk is higher in men as compared to women, except in the oldest age groups (>85 years of age) in which women tend to have higher or similar stroke incidence[25]. Although, younger women have lower age-specific ischemic stroke mortality than men, above age of 65 women have poorer prognosis, resulting in stroke being the third leading cause of death in women, and the fifth in men[25]. The prevalence of carotid atherosclerosis and soft/vulnerable atherosclerotic plaques, which are important risk factors of stroke, is higher in younger men as compared to women, however, male predominance in atherosclerosis declines after the age of 50 years, with the plaque prevalence being similar in elderly men and women[26]. The age trend in females and the significant increase in prevalent atherosclerotic changes around the age of 50 years (which overlaps with average age of menopause onset) supports the timing hypothesis. The "timing hypothesis" theorizes that estradiol has harmful vascular effects in elderly women in contrast to neutral or beneficial effects in younger women[27]. Nevertheless it is suggested that in elderly women the switch from protective to harmful estradiol effect may be due to changes in estrogen receptor signalling[28] or a consequence of age-related hyper-inflammatory state[29] the exact mechanisms are not fully understood. Investigating whether estradiol and the other endogenous hormones are associated with composition of plaque, a

determinant factor in stroke pathology, could provide some insights into sex-differences in stroke.

Endogenous Sex Hormones and Type 2 Diabetes

Age-related testosterone deficiency in men has been associated with several deleterious health effects[21] in particular with obesity-related chronic diseases, including T2D[30-33]. In contrast, in young women with polycystic ovarian syndrome (PCOS), which is a marker of hyperandrogenism, there is an increase in prevalence of several metabolic factors (dyslipidaemia, insulin resistance, hypertension, obesity) which could lead to increased risk of T2D, but also of heart disease and stroke as compared to women without PCOS[34]. In line with this, a recent meta-analysis indicated that higher testosterone level can significantly decrease the risk of T2D in men[35]. In contrast, in women (without PCOS), studies reported either no association[36] or an increased T2D risk with higher serum testosterone levels[37,38]. Low circulating sex hormone-binding globulin (SHBG) has been consistently identified as a risk factor for T2DM in both sexes in a number of smaller studies and meta-analyses[39,40]. Furthermore, DHEA and DHEAs display the most pronounced decrease with age of all sex hormones [20], and evidence from animal and human experimental studies indicate a vital role of DHEA(s) in T2D[41]. Recent data from large prospective population-based cohort studies show that the serum level of DHEA is inversely associated with risk of T2D independently of intermediate risk factors for T2D[42,43]. Also, randomized controlled trials have reported that DHEA replacement can reduce abdominal fat and improve insulin sensitivity, and other data show a prognostic value of DHEA in T2D[44]. Although substantial evidence suggests sex hormones (estrogen, testosterone, and DHEAs) as important factors in modifying diabetes risk, the association is more complex than might be anticipated, and at least appears to be dependent on sex and on aging, which not all previous studies have taken into account[45]. Also, the association between sex hormones and T2D, and their role in T2D prognosis, can be confounded by changes in body composition that are observed in disorders of androgen excess and deficiency and in menopausal transition (e.g. postmenopausal women being 3-fold more likely to develop obesity and premenopausal women)[46,47], which needs to be taken into account when studying the link between sex hormones and T2D in both sexes.

Exogenous Sex Hormones and Cardiovascular Risk in Women

Ovarian insufficiency, a sharp decline in endogenous estradiol and an increase in androgen-estrogen ratio during the menopausal transition could be closely related to women's health and quality of life after menopause[48]. Menopause is considered the end of a woman's reproductive life. It is defined as the permanent cessation of menstrual period as a consequence of gradual loss of ovarian follicular activity[49]. Also, up to 50-80% of women during menopause will experience menopausal symptoms such as hot flushed and night sweats which humper quality of life in women but also could lead to increased risk of T2D and CVD[50-53]. HT is the most effective treatment for menopausal symptoms[51], however it has been associated with some undesirable health consequences on cardiovascular health and breast cancer risk[54]. The current clinical guidelines suggest positive risk-benefit ratio for HT in younger healthy women (aged 50-60 years) however, the adverse effects in women after the age of 60 and CVD risk with different HT regimes used, route of HT administration and duration of HT use remains controversial[55]. Further evidence on how these factors affect CVD risk related to HT use in women could help to guide better clinical management of CVD risk in women using HT. During the menopausal transition, oral contraceptives are not only used to prevent pregnancies but also to tackle menopausal symptoms[56]. Although endogenous estradiol, which is altered by oral contraceptives, seems to be beneficial in preventing CVD in premenopausal women, contraceptive use has been associated with increased CVD risk, with highest risk observed with combined oral contraceptives (COCs)[54]. In particular, increased risk of venous thromboembolism and lipid abnormalities[57], MI[58] and stroke[59] have been reported with COC. Indeed, Cochrane meta-analysis reported 1.6-fold increased stroke and MI risks in women using COCs, with the highest risk for pills with > 50 microgram of estrogen. COC pills with lower estrogen content were suggested to be safer in comparison with COC with higher estrogen content. Indeed, the COC pill containing levonorgestrel and low dose estrogen (30 µg of estrogen), as compared to higher dosages of estrogen, was the safest oral form of COC in regard of thrombotic risk [60]. This suggests that the adverse CVD effects of COC are mainly attributed to the estrogen content of these contraceptives. In line with this, the progestin-only contraceptives (POCs) appeared to be safer in regard of CVD risk[61]. However, due to the low incidence of CVDs during the reproductive period, little evidence exists on how POCs affect the various cardiometabolic outcomes among women of reproductive age[54]. Therefore, an updated and comprehensive

quantitative review of the existing literature would be a good approach to study this topic. A review may overcome the problem of low rate of events in these women by including large number of women using POCs and enough cases of interest, which would provide us with more firm evidence on potential associations between POCs use and CV risk.

Estrogen-like Compounds and Metabolic Risk in Women

Due to the fear of potential negative health consequences (e.g. increased risk of CVD and breast cancer) that have been reported with HT use, increasing number of women use plant-based formulations to relieve menopausal symptoms [62]. Phytoestrogens, nonsteroidal plant-derived compounds with estrogen-like biological activity, are commonly used to improve menopausal symptoms and might have various beneficial health effects[63]. Phytoestrogens are so-called "selective estrogen receptor modulators", and may have organ-specific estrogenic and antiestrogenic effects depending on the circulating estrogen level and the target tissue[64,65]. Although dietary phytoestrogens are supposed to be advantageous in obesity and MS[66], emerging evidence has indicated that higher estradiol may increase the risk of diabetes in postmenopausal women[40]. This raises a concern that phytoestrogens may have similar effects due to their structural similarity to estradiol. The evidence of the association between phytoestrogens and glucose homeostasis and T2D risk is inconsistent, with some studies reporting adverse effects [67], some no association [68], while others reported a beneficial effect [69]. Also, phytoestrogens have been suggested to cause modest improvements in body weight and other parameters of body composition[70-72] which may also contribute to a decrease of diabetes risk. However, a few studies reported adverse body composition changes, such as an increase in weight[73-76] and body mass index (BMI)[75,77-79] with phytoestrogen use, raising a concern regarding potential cardiometabolic consequences. Also, there is no firm evidence on how phytoestrogen supplementation in combination with a regular diet (without calorie intake restriction) may affect body weight and the other parameters of body composition in postmenopausal women. In this population this is of high importance, as these women already have an increased risk of developing obesity due to hormonal disturbances that occur in menopausal transition[47].

Objectives of This Thesis

Serum levels and actions of sex hormones, in particular androgens and estrogens differ between men and women and have been shown to affect the cardiovascular system, and to determine sex differences in CVD. Physiologic fluctuations in concentrations of sex hormones over the course of life are more prominent in women, and are mainly observed during menstrual cycles, pregnancy and menopause[80]. Use of contraceptive or hormone therapy in women can further affect the levels of serum sex steroid concentrations (e.g. in healthy postmenopausal women, circulating levels of estrogens and SHBG are elevated by two- to four-fold with use of either estrogen alone or combined estrogen plus progesterone)[81]. Also, phytoestrogens, selective estrogen receptor modulators, may change estrogen concentrations and may modify estrogen-dependent signalling pathways causing estrogenic or anti-estrogenic effects. Therefore, the first objective of this thesis was to study the associations between endogenous sex hormones and cardiometabolic risk and to explore potential sex differences in stroke and diabetes (**Chapter 2**). The second objective was to summarize the existing literature on hormone therapy use and their potential adverse effects on cardiometabolic health in women (**Chapter 3**). Finally, the third aim was to summarize the evidence on associations between phytoestrogen dietary intake/supplementation and metabolic risk in adult women (**Chapter 4**). The overview of objectives of studies included in this thesis is presented in **Figure 2**.

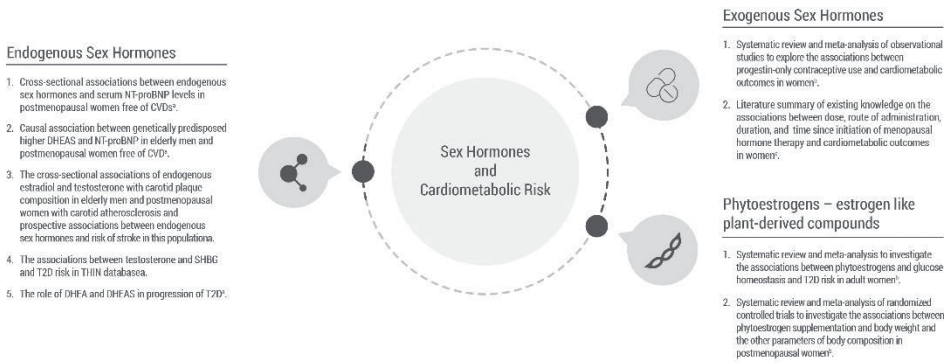


Figure 2. Thesis summary.

Study Design

The original studies presented in this thesis were embedded in the Rotterdam study and The Health Improvement Network (THIN) database.

Rotterdam Study

The studies presented in the **Chapter 2.1, 2.2, 2.3** and **2.5** were carried out within the framework of the Rotterdam Study (RS), a prospective, population-based cohort study among individuals aged ≥ 45 in Ommoord municipality of Rotterdam, The Netherlands. The rationale and design of RS is described in detail elsewhere [82]. In brief, all inhabitants of the Ommoord district aged 55 years or older were invited to participate (n =10,215). There were no eligibility criteria to enter the Rotterdam Study cohorts except the minimum age and residential area based on zip codes. At baseline (1990-1993), 7,983 participants were included (RS-I). In 2000, all persons living in the study district who had become 55 years of age (n=3011) were additionally enrolled (RS-II). A second extension of the cohort was initiated in 2006, in which 3,932 participants aged 45 years or older were included (RS-III). Follow-up visits were held every 3-5 years. The Rotterdam Study has been approved by the Medical Ethics Committee according to the Wet Bevolkingsonderzoek: ERGO (Population Study Act: Rotterdam Study), executed by the Ministry of Health, Welfare and Sports of The Netherlands. All participants gave informed consent to participate in the study and to obtain information from treating physicians and pharmacies, separately.

The Health Improvement Network (THIN) database

The study presented in the **Chapter 2.4** is carried out within the Health Improvement Network (THIN) database. THIN data base is a large primary care database in the UK with contribution from over 700 general practices (14 million patients), which was utilized for this study. Data from practices that use VISION Electronic Medical Record (EMR) are gathered, anonymized and released for research purpose[83]. The resulting database, The Health Improvement Network (THIN) database holds data on demographic characteristics, clinical diagnosis, physical measurement, laboratory results and prescriptions. The THIN is generalizable to the UK for demographics, major condition prevalence and death rates adjusted for demographics and deprivation[84].

Systematic Reviews and Meta-analyses

The research described in the **Chapter 3** and **4** of this thesis are systematic reviews and meta-analyses of the existing literature. The Cochrane Handbook for Systematic Reviews of Interventions and PRISMA Statement were used to guide the conduct and reporting of the reviews[85]. We systematically searched the electronic medical databases (Medline via Ovid, EMBASE, Web of Science Core Collection, Cochrane CENTRAL via Wiley, PubMed and Google Scholar) in order to collect relevant articles to answer specific research questions of interest. In order to identify additional relevant studies, the reference lists of the included studies and relevant reviews were screened as well. We sought to pool the results from individual studies using random-effects meta-analysis model when feasible. In each of the reviews we used individualized approach and methodology to best address the research questions. The details on the methodologies and specific approaches used can be found in **Chapters 3** and **4**.

The Outline of This Thesis

In **Chapter 2** we investigated the associations between endogenous sex hormones and cardiometabolic risk in men and women. In particular, we discuss the associations between sex hormones and NPs levels (*Chapter 2.1 and 2.2*), sex differences in plaque composition and risk of stroke (*Chapter 2.3*) and the role of androgen sex hormones in T2D risk (*Chapter 2.4*) and its complications (*Chapter 2.5*). In **Chapter 3** we summarized the existing knowledge on the role of exogenous hormones (menopausal hormone therapy and contraceptives) and cardiometabolic risk in women (*Chapter 3.1 and 3.2*). In **Chapter 4**, we discuss the potential role of estrogen-like compounds (phytoestrogens) in glucose homeostatic and diabetes risk in adult women, but also, their role in modifying body composition in postmenopausal women based on two comprehensive reviews of the literature (*Chapter 4.1 and 4.2*). Finally, in **Chapter 5** we discuss the implications of our findings, strengths and limitations of methodological approaches used and we give the directions for future research.

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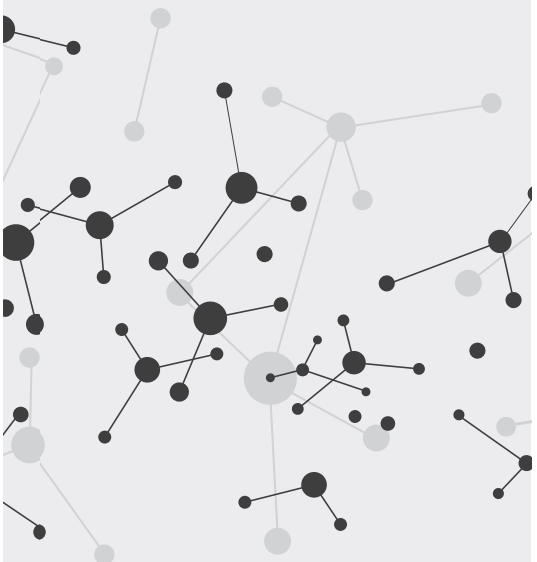
Chapter 2

Endogenous Sex Hormones and Cardiometabolic Risk



2.1

Sex steroids, sex hormone-binding globulin and levels of N-terminal pro-brain natriuretic peptide in postmenopausal women

2.2

Mendelian randomization provides evidence for a causal role of dehydroepiandrosterone sulfate in decreased NT-proBNP levels in a Caucasian population

2.3

Endogenous estradiol increases the risk of vulnerable carotid plaque composition and risk of stroke in postmenopausal women

2.4

Serum testosterone and sex-specific risk of incident type 2 diabetes: a longitudinal UK primary care database study

2.5

Prognostic value of dehydroepiandrosterone in type 2 diabetes: The Rotterdam Study

CHAPTER 2.1

Sex hormones affect serum N-terminal pro-brain natriuretic peptide in postmenopausal women

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Int J Cardiol. 2018 Jun 15;261:189-195

ABSTRACT

BACKGROUND: Amino-terminal pro-B-type natriuretic peptide (NT-proBNP) has a well-documented prognostic value for cardiovascular disease and sex-hormones are suggested to modulate NT-proBNP levels.

OBJECTIVE: To examine whether endogenous sex-hormones and sex hormone-binding globulin (SHBG) are associated with NT-proBNP levels in postmenopausal women free of clinical cardiovascular diseases.

METHODS: Total estradiol (E2), total testosterone (TT), androstenedione (AD), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), sex hormone-binding globulin (SHBG) and NT-proBNP were assessed in 4112 postmenopausal women free of cardiovascular diseases from the prospective population-based Rotterdam Study. Free androgen index (FAI) was calculated as ratio of TT to SHBG concentration. TT, AD, DHEA(S), SHBG, FAI and NT-proBNP were natural log transformed. Regression coefficients and 95% Confidence Intervals (CI) were calculated using multivariable linear regression models adjusting for confounders.

RESULTS: In models adjusted for multiple confounders (age, reproductive, life style and cardiovascular risk factors) higher SHBG (per 1 SD increase, β = 0.15, 95% CI = 0.12, 0.18), and lower levels of TT (per 1 SD increase, β = -0.05, 95%CI = -0.08, -0.02), FAI (per 1 SD increase, β = -0.13, 95%CI = -0.15, -0.09), DHEAS (per 1 SD increase, β = -0.06, 95% CI = -0.09, -0.04) and DHEA (per 1 SD increase, β = -0.06, 95%CI = -0.09, -0.04) were associated with higher levels of NT-proBNP. However, no consistent association was found between E2 and AD and NT-proBNP levels. Additionally, stratification by BMI did not affect any of observed associations.

CONCLUSION: Our findings support the hypothesis that higher androgens might be associated with lower natriuretic peptide levels in postmenopausal women.

INTRODUCTION

After menopause, sex differences in coronary heart disease (CHD) risk gradually disappear resulting in a similar incidence of CHD by the sixth decade in women as compared to men [1]. Accordingly, differences in sex and menopause status have been observed in the levels of N-terminal pro b-type natriuretic peptide (NT-proBNP) [2], which has prognostic value in CHD [3,4] and it has potential beneficial role in the etiology of diabetes mellitus type II [5]. Accumulating evidence suggests that women present with consistently higher levels of circulating NT-proBNP than men, reaching the values in healthy premenopausal women about 2-fold higher than men at the same age [2,6]. Also, in women, NT-proBNP levels change by menopause status, with women after menopause having lower levels of NT-proBNP [4,6-8]. The mechanisms underlying the sex and menopause related difference in circulating NT-proBNP have not been established yet. However, evidence suggested that sex hormones play an important role in the regulation of natriuretic peptides[9]. Before menopause, women have higher levels of estradiol (E_2) and lower levels of androgens than men, while after menopause, there is a decline in endogenous estradiol levels and a period of relative androgen excess[4,10]. Recently, estrogen receptors, which mediate estrogen actions, have been reported to be involved in atrial natriuretic peptide synthesis in the heart of mouse[11]. Also, studies in postmenopausal women show exogenous estradiol to increase levels of NT-proBNP, but findings are not consistent[6,7]. No study to date has examined the influence of endogenous estrogens on circulating NT-proBNP levels in postmenopausal women. In contrast, studies in young women showed that testosterone is independently and inversely associated with BNP, but there is uncertainty whether this effect persists after menopause [6]. Evidence from animal studies show that dehydroepiandrosterone (DHEA) significantly inhibit BNP mRNA levels [12]. Nevertheless, there is a lack of studies examining the associations of DHEA and its derivatives with NT-proBNP levels in humans, and in particularly in women. Furthermore, sex hormone-binding globulin (SHBG) is associated with cardiovascular risk in both pre-and postmenopausal women, as well as with BNP levels in pre-menopausal women[6,13,14]. It remains unclear, however, whether SHBG is associated with levels of NT-proBNP in older women.

Therefore, we aimed to investigate whether endogenous sex-hormones and SHBG levels associated with NT-proBNP in postmenopausal women free of clinical cardiovascular diseases.

METHODS

Study population

The Rotterdam Study (RS) is a population-based cohort study of individuals 45 years and over living in the Ommoord district of Rotterdam, the Netherlands. The rationale and design of RS is described elsewhere [15]. The Rotterdam Study has been approved by the Medical Ethics Committee according to the Wet Bevolkingsonderzoek: ERGO (Population Study Act: Rotterdam Study), executed by the Ministry of Health, Welfare and Sports of The Netherlands. All participants gave informed consent to participate in the study and to obtain information from treating physicians and pharmacies, separately.

Population for Analyses

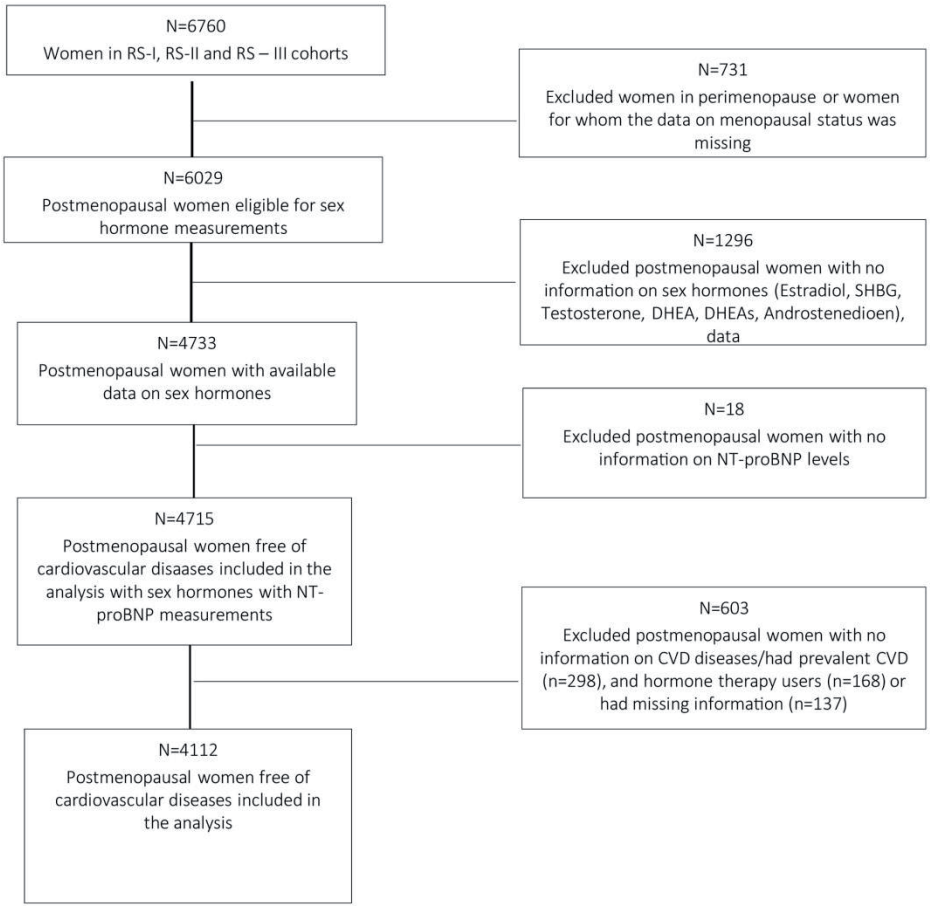
The present study includes data from postmenopausal women from the third visit of the first cohort of the RS (RS I-3), and from the first visits of the second (RSII-1) and the third cohort (RSIII-1). There were 6,760 women eligible for the analysis. Of those, 2,233 women were excluded because (i) they were non-postmenopausal or due to no information on their menopausal status (n=731); (ii) they did not have information on sex steroids (n=1296) or on NT-proBNP levels (n=18); (iii) had prevalent cardiovascular disease (CHD, stroke or heart failure) (n=267); (iv) there was no available information on the presence of cardiovascular disease (n=31), (v) used postmenopausal hormone therapy (n=168) or (vi) there was no available information on hormone therapy use (n=137) (**Figure 1**). Therefore, 4112 postmenopausal women were included in the final analysis.

Assessment of Exposure, Outcome and Covariates

NT-proBNP Measurement

Levels of NT-proBNP were obtained from serum. After blood collection, the samples were left to clot for 30 minutes and then centrifuged for 20 minutes at 3000 rotations per minute at 4º C. The serum was stored at -80°C. NT-proBNP was measured using a commercially available electrochemiluminescence immunoassay (Elecsys proBNP, F. Hoffman-La Roche Ltd., Basel, Switzerland) on an Elecsys 2010 analyzer [4]. The precision, analytical sensitivity and stability characteristics of the system have previously been described [16]. NT-proBNP levels are reported in pmol/L.

Figure 1. Flowchart for selection of study participants



Sex hormones Measurement

Sex steroid measurements and sex hormone-binding globulin

17β-estradiol (E_2) levels were measured with a radioimmunoassay and SHBG with the Immulite platform (Diagnostics Products Corporation Breda, the Netherlands). The minimum detection limit for estradiol was 18.35 pmol/liter. Undetectable estradiol was scored as 18.35. Serum levels of total testosterone (TT) were measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS). The corresponding interassay coefficients of variations for TE, SHBG and TT are <7%, <5%, and <5%. Free androgen index (FAI), calculated as (T/SHBG)*100 is used as a surrogate measure of bioavailable testosterone (BT). Androstenedione, dehydroepiandrosterone sulfate (DHEAS) and dehydroepiandrosterone (DHEA), were measured on a Waters XEVO-TQ-S system (Waters, Milford, MA, USA) using the CHS™ MSMS Steroids Kit (Perkin Elmer, Turku, Finland). The inter-assay coefficients of variation of androstenedione, DHEAS and DHEA were <6.5%.

Prevalent cardiovascular diseases assessment

Prevalent cardiovascular diseases were defined as a history of coronary heart diseases (myocardial infarction, revascularization, coronary artery bypass graft surgery or percutaneous coronary intervention), heart failure and stroke, and were verified from the medical records of the general practitioner.

Assessment of Covariates

At baseline, an interview was performed to obtain information on current health status, medical history, medication use, menopausal status, alcohol intake and smoking. Blood pressure was measured in the sitting position on the right upper arm with a random-zero sphygmomanometer. Diabetes mellitus type 2 diagnosis was considered present if a participant used glucose lowering drugs or in case a non-fasting random serum glucose level was found to be ≥11 mmol/L. Data on age at menarche were collected by asking women, “How old were you when you had your first menstrual period?”, The retrospective data on self-reported number of pregnancies of at least 6 months and use of hormonal replacement therapy, antihypertensive or antidiabetic therapy and statins were collected by a questionnaire during the home interview. Smoking status was assessed asking participants

whether they were current smokers of cigarettes, cigars, or pipe. Glomerular filtration rate (eGFR) was estimated using the simplified Modification of Diet in Renal Disease (MDRD) equation [17]. Physical activity was assessed with an adapted version of the Zutphen Physical Activity Questionnaire [18]. Every activity mentioned in the questionnaire was attributed a MET-value according to the 2011 Compendium described in details elsewhere [19]. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). All biochemical parameters were assessed in fasting serum. Thyroid stimulating hormone (TSH) was measured on the Vitros Eci (Ortho Diagnostics). Insulin, glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triacylglycerol (TG) and C-reactive protein (CRP) were measured on the COBAS 8000 Modular Analyzer (Roche Diagnostics GmbH). The corresponding interassay coefficients of variations are the following: TSH<13.2%, insulin <8%, glucose <1.4%, lipids <2.1% and CRP <16.9%. LDL-cholesterol level was estimated indirectly from measurements of total cholesterol, HDL and triglycerides by means of the Friedewald equation [20] .

Statistical analyses

Continuous variables are reported as mean ± standard deviation (SD) unless stated otherwise and categorical variables were presented as percentages. Correlations between endogenous sex hormones and SHBG were assessed by a non-parametric test (Spearman, Rs). To achieve approximately normal distribution, skewed variables (NT-proBNP, SHBG, testosterone, FAI, DHEA, DHEAS, androstenedione, triglycerides, insulin, C-reactive protein, and thyroid-stimulating hormone) were natural log transformed. Regression analysis was used to evaluate whether sex steroids and SHBG were associated with NT-proBNP. All sex hormones variables were assessed continuously in separate models. In the basic model (Model 1), we adjusted for age, age at onset of menopause, body mass index (BMI) and RS cohort (I, II and III), glucose (continuous), insulin (continuous), physical activity (continuous), total serum cholesterol (continuous), statin use (yes vs. no), smoking status (yes vs. no) and alcohol consumption (continuous), prevalent diabetes mellitus (yes vs. no), systolic blood pressure (continuous), antihypertensive medication (yes vs. no), glomerular filtration rate (eGFR) (continuous), C-reactive protein (CRP) (continuous). We also controlled (in Model 2) for upstream precursor hormones (**Supplemental Figure 1**) which may have acted as confounders. Collinearity analysis demonstrated high correlation between DHEA and DHEAS

(variance inflation factor, VIF>3), and therefore, when applicable, we did not adjust for DHEA in model 2, but only for DHEAS. There were missing values on one or more covariates (**Table 1**). Because the missing values were likely to be missing at random and for avoidance of loss in efficiency, missing values were imputed using a multiple imputation technique (5 imputation sets). Rubin's method was used for the pooled coefficients (β) and 95% Confidence Intervals [21]. In total, 21 variable has been imputed, percentage of missing values for the majority of imputed variables (n=19) was below 5%, we have imputed 28.4% of missing data on alcohol consumption and 11.2 of missing data on physical activity (**Supplemental table 1**). A P-value lower than 0.05 was considered as statistically significant, but as sensitivity analysis, to account for multiple testing, we adjusted the p-value from 0.05 to 0.007 by applying the Bonferroni correction for the number of exposures studied (N=7). All analyses were done using SPSS statistical software (SPSS, version 21.0; SPSS Inc, Chicago, Illinois).

Sensitivity Analysis

We performed a series of sensitivity analyses using imputed data. Firstly, we compared baseline characteristics of postmenopausal women who were not included in our study due to missing data on exposure and outcome (n=922) with women included in analyses. Since waist circumference is a better measure of visceral adiposity, an important determinant of sex steroid levels and SHBG after menopause, and because NT-proBNP levels correlate with abdominal fat, we performed a sensitivity analysis substituting BMI with waist circumference. To account for the specific effects of lipid particles on NT-proBNP levels, we substituted total cholesterol (TC) with high-density lipoprotein cholesterol (HDL-C), triacylglycerol (TG) and low-density lipoprotein L (LDL) . Thyroid stimulating hormone (TSH), physical activity, number of pregnancies, age of menarche and type of menopause (non-natural vs. natural) are associated with sex hormone levels, therefore, the models were further adjusted for these factors. Since DHEA showed collinearity with DHEAs, we performed a sensitivity analysis substituting DHEAs with DHEA. Furthermore, we restricted the analysis among women (i) who had NT-proBNP levels within age specific value for the diagnosis of heart failure as proposed by Januzzi et al, i.e. 50 to 75 years, 108 pmol/L; >75 years, 216 pmol/L) [22] and (ii) who had NT-proBNP levels > 125 pg/ml/14.78 pmol/l (30.7 % of our population) because this portion of women might have heart failure with preserved

ejection fraction (HFpEF) or heart failure with mid-range ejection fraction (HFmrEF) according to the 2016 ESC Guidelines [23]. Also, to explore whether the associations were independent of downstream hormones, we further adjusted for hormones including the downstream metabolites that might be casual intermediates. Effect modifications of sex hormones by BMI, age and years since menopause were tested by adding an interaction term in the final multivariable model in addition to performing stratified analysis. We preformed stratified analysis excluding women who had diabetes or were on antihypertensive therapy. Furthermore, to show the clinical relevance, we showed the associations of endogenous sex hormones and SHBG with NT-proBNP levels in tertiles for the 2nd Model. To study the relations across increasing tertiles, trend tests were computed by entering the categorical variables as continuous variables in the linear regression models.

RESULTS

The mean age of the study population was 65.9 years (SD 9). Women were on average 17.2 years (SD 10.2) into menopause, and the majority of women (68.2%%) experienced menopause of natural origin (**Table 1**). There was strong positive correlation between DHEA and DHEAS (Rs=0.73) and between DHEA and androstenedione (Rs=0.67), and moderate negative correlation between FAI and SHBG (Rs=-0.58), FAI and TT (Rs=0.62), and FAI and androstenedione (Rs=0.41) (**Supplemental Table 2**). In addition, there was a weak negative correlation between SHBG and E2 (r=-0.15) and a weak positive correlation between SHBG and testosterone (Rsr=0.21).

Estradiol and NT-proBNP levels

After adjusting for potential confounders and intermediate factors (Model 2) we did not observe significant association between E_2 and NT-proBNP levels (per 1 SD increase in estradiol levels, β= 0.014, 95% CI=-0.013, 0.040) **(Table 2)**.

Table1. Characteristics of the Study Population

Age at baseline, mean (SD), y	65.9±9	Serum lipid lowering medication, yes	610 (14.8)
Education		Prevalent diabetes mellitus	426 (10.4%)
Primary	612 (14.9%)	CRP mg/l	1.6 (0.7-3.4)
Lower/intermediate or lower vocational	2143 (52.8%)	eGFR	77.1±15
Intermediate vocational or higher general	912 (22.2%)	Physical activity, total MET hours	78.5 (53-111)
Higher vocational or university	431(10.5%)	**Hormones**	
BMI, kg/m²	27.5 ±4.6	Estradiol, pmol/l	31.4 (18.4-56.5)
Waist to hip ratio	0.7 (0.9)	Testosterone, nmol/l	0.8 (0.6-1.1)
Smoking		SHBG, nmol/l	57.5 (41.6-79.4)
yes	568 (13.8%)	FAI	1.4 (0.9-2.1)
no	3526 (85.8%)	DHEA, nmol/l	9.5 (6-.14.7)
Alcohol intake g/day	2.1 (0.01-11.4)	DHEAS, nmol/l	1669.7 (1025.8-2582.3)
Health indicators		Androstenedione nmol/l	2.3 (1.7-3.2)
Systolic BP, mmHg	139.5±21.8	TSH mU/l	2 (1.3-3)
Diastolic BP, mmHg	77.9 ±11.3	NT-proBNP, pmo/l	9.2 (5.3-16.8)
Antihypertensive therapy with indication, yes	1022 (24.9%)	NT-proBNP, ng/l	77.8 (44.8-142.1)
Total cholesterol, mmol/l	5.9 ± 0.9	**Women-specific variables**	
LDL, mmol/l	4.1 (0.9)	Age at menopause, years[1]	48.7±5.6
HDL, mmol/l	1.5 ±0.4	Years since menopause	17.2±10.2
Triglycerides, mmol/l	1.3 (1-1.8)	Menopause type, natural menopause[1]	2803 (68.2%)
Fasting blood glucose, mmol/l	5.7±1.4	Age at menarche, years	13.4 ±1.7
Insulin, pmol/l	72 (51-104)	Number of pregnancies	2 (1-3)

Values are reported as number 9percentage) for categorical variables, and mean ± SD or median (25^{th}-75^{th} quartile) for continuous variables; [1]Age at menopause and type of menopause were not available for all women, the present values are based on 4425 and 4497 respectively; * body mass index (BMI), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), glomerular filtration rate (eGFR), free androgen index (FAI), High-density lipoprotein L (HDL), low-density lipoprotein L (LDL), amino-terminal pro-brain natriuretic peptide (NT-proBNP), sex hormone binding globulin (SHBG), thyroid stimulating hormone (TSH), high-sensitivity C reactive protein (CRP)

Androgens, Sex hormone-binding globulin and NT-proBNP levels

After adjustments for multiple confounders (Model 1), lower levels of TT (per SD increase in natural log transformed variable, β= -0.03, 95%CI=-0.054, -0.005), FAI (per unit increase in natural log transformed variable, β= -0.115, 95%CI=-0.141, -0.09), DHEAS (per SD increase in natural log transformed variable, β= -0.066, 95%CI=-0.092, -0.041), androstenedione (per SD increase in natural log transformed variable, β= -0.026, 95%CI=-0.05, 0.002) and DHEA (per SD increase in natural log transformed variable, β= -0.053, 95%CI=-0.092, -0.041), and higher levels of SHBG (per SD increase in natural log transformed variable, β= 0.144, 95%CI=0.115, 0.172) were associated with higher levels of NT-proBNP. Further adjustment for upstream sex steroids did not affect the associations of TT, FAI, DHEA, DHEAs and SHBG with NT-proBNP, but abolished the association between androstenedione and NT-proBNP levels (**Table 2**).

Table 2. Associations of androgens, estrogen, and sex-hormone binding globulin with the level of serum NT-proBNP in postmenopausal women free of CVD, the Rotterdam Study (N=4112)

Model	Sex-hormone binding globulin	Total testosterone	Free androgen index	Total estradiol	DHEA	DHEAS	Androstenedione
1	**0.144**	**-0.03**	**-0.115**	0.017	**-0.053**	**-0.066**	**-0.026**
	(0.115;0.172)*	**(-0.054;-0.005)**	**(-0.141;-0.090)***	(-0.009;0.044)	**(-0.079;-0.028)***	**(-0.092;-0.041)***	**(-0.05;-0.002)**
2	**0.152**	***-0.053**	**-0.125**	0.014	**-0.060**	**-0.062**	0.005
	(0.123;0.181)*	**(-0.083;-0.024)***	**(-0.154;-0.097)***	(-0.013;0.040)	**(-0.085;-0.035)***	**(-0.087;-0.037)***	(-0.023;0.033)

Model 1: Age, age at menopause, Rotterdam Study cohort, BMI, physical activity, smoking, alcohol, cholesterol, statin use, glucose, insulin , systolic blood pressure, antihypertensive therapy, diabetes mellitus type II, eGFR, CRP

Model 2: Model 1+ adjusting for upstream hormones DHEAS, SHBG, total testosterone, androstenedione for estradiol, SHBG, DHEAS and androstenedione for total testosterone, estradiol, total testosterone, DHEAS and androstenedione for SHBG, estradiol, DHEAS and androstenedione for free androgen index, SHBG and DHEAS for androstenedione, and SHBG for DHEAs and DHEA.

Values shown in the table are continuous per 1 SD; Significant results are bold (p<0.05); *results remain significant after Bonferroni correction p<0.007;
Abbreviations: CRP: c-reactive protein, DHEA: dehydroepiandrosterone , DHEAS: dehydroepiandrosterone sulphate, eGFR: glomerular filtration rate, FAI: free androgen index, SHBG: sex hormone binding globulin

Sensitivity Analysis

The associations between TT, SHBG, FAI, DHEA and DHEAS and NT-proBNP levels remained significant after we applied the Bonferroni correction (p<0.007). There were significant differences in age, systolic blood pressure, BMI, hsCRP, prevalent T2D, statin and alcohol use, and smoking status among women included in our analysis and women that were excluded because of incomplete data on sex hormones and NT-proBNP (**Supplemental table 3**). In sensitivity analyses, substituting BMI with waist circumference as a measure of adiposity, total cholesterol for other blood lipids, adjustment for DHEAs with DHEA in Model 2, adjusting further for serum TSH, number of pregnancies, age of menarche and type of menopause, or further adjustment for downstream sex hormones and excluding

women who reported use of HRT, or exclusion of women who came non-fasting in the visit center did not affect the associations of sex steroid and SHBG with NT-proBNP levels. Also, the results did not change after exclusion of (i) 329 postmenopausal women with an NT-proBNP level above the age-specific cutoff value for the diagnosis of heart failure; (ii) 1234 women who had NT-proBNP levels > 125 pg/ml/14.78 pmol/l, (iii) 426 women who had diabetes; and (iv) 1022 women who used antihypertensive medications (**Supplemental table 4**). In the stratified analysis, no significant interactions were found for sex steroids and SHBG with BMI, age or years since menopause (**Supplemental table 5**). The results of endogenous sex hormones and SHBG in tertiles provided same conclusions as the analysis of sex steroids and SHBG as continuous variables (**Figure 2A and Figure 2B**).

Figure 2A. Association of NT-proBNP and hormone-specific tertiles sex-hormone binding globulin (SHBG), Estradiol, Testosterone, and free androgen index
Figure 2B. Association of NT-proBNP and hormone-specific tertiles sex-hormone binding globulin (SHBG), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS) and

DISCUSSION

In this large population-based study of postmenopausal women free of clinical CVDs, lower levels of androgens (TT, FAI, DHEA and DHEAS) and higher level of SHBG were associated with higher levels of serum NT-proBNP, irrespective of known confounders (**Figure 3**). However, no consistent association was found between E_2, AD and NT-proBNP levels. Androgens (testosterone, DHEA, DHEAS, FAI) were negatively associated with NT-proBNP levels (lower levels of androgens higher NT-proBNP levels), SHBG was positively associated with NT-proBNP (higher levels of SHBG, higher levels of NT-pro-BNP), Estradiol was

positively associated with NT-pro-BNP, however, the association was not statistically significant.

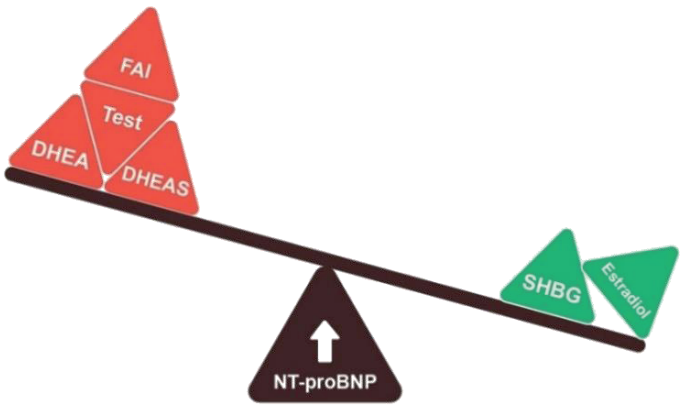


Figure 3. Summary of study results

Androgens and NT-proBNP

Several studies suggest that testosterone has suppressive effect on natriuretic plasma levels. In line with our results on an inverse association between androgens and NT-proBNP, in a clinical trial of 51 women with hypoandrogenemia due to hypopituitarism, levels of NT-proBNP decreased after transdermal application of testosterone [24]. Also, Chang et al., in a study of 682 young adult women age 35 to 49 years reported that free testosterone was inversely associated with NT-proBNP levels [6]. Furthermore, Lam et al. in a study of 1798 premenopausal women and 181 postmenopausal women reported inverse associations between FAI and NT-proBNP [9]. Unlike previous population based-studies, which were included mainly young adult women, our study extends these findings to postmenopausal women and shows that the association between testosterone and NT-proBNP is independent of other sex steroids, including estradiol which in a downstream hormone and might be in the pathway between testosterone and NT-proBNP.

We show that other androgens such as DHEA and its derivate DHEAs are inversely associated with NT-proBNP levels in postmenopausal women, supporting the hypothesis that androgens have inhibitory effect on NPs in older women. In a neonatal rat cardiocyte culture system, DHEA significantly inhibited BNP mRNA levels [12]. Similarly, studies in men have reported inverse correlations between DHEAs and BNP. DHEA and its sulfate conjugate DHEAS are the major secretory steroidal products of the human adrenal glands [25]. In either gender, serum level peaks of DHEA and DHEAs occur by the second decade and then declines steadily by an average of about 10%/decade [26]. Mechanisms of action of DHEA are still to be described. DHEA(S) is converted to testosterone or 17β-estradiol and therefore it is unclear whether DHEA directly exerts its effects or if it acts after conversion to these hormones. However, in our study we corrected for levels of estradiol and testosterone, supporting an independent role of DHEA on NT-proBNP levels. Recent evidence shows that there are specific DHEA-bindings sites in the cardiovascular system, including the heart tissue [27,28]. Therefore, DHEA might have a direct effect in the vascular system, and might play a role in the development of cardiovascular disease independent of its derivate. However, to date, little is known about the role of DHEA and DHEAs in the risk cardiovascular disease, including the risk of developing heart failure. In a sample of 942 postmenopausal women, although higher DHEAS levels were associated with several major cardiovascular risk factors, such as elevated total cholesterol and blood pressure, they were unrelated to the risk of fatal cardiovascular disease [28]. Additional studies should be undertaken to further elucidate the exact mechanisms of how DHEA(S) might affects the levels of NT-proBNP and the risk of developing heart failure.

SHBG and NT-proBNP

We found positive association between SHBG and NT-proBNP levels, independent of potential confounding factors. The main role of SHBG is sex steroids transport within the blood stream to extravascular target tissues. Testosterone have higher SHBG binding affinity than estradiol, thus SHBG regulates balance between bioavailable testosterone and estrogens. It has been hypothesized that SHBG plays an indirect role in rising NT-pro BNP levels, by binding more testosterone which have negative effect on natriuretic peptides. Findings from Framingham Heart Study showed that each unit increase in log SHBG was associated with a 19% increase in NT-proBNP among men, and a 40% increase in NT-

proBNP among young adult women, adjusting for clinical covariates [9] . However, in our study, positive association between SHBG and NT-proBNP remains significant after adjustment for potential confounders but also for TT, DHEA, DHEAS and E_2, implicating that SHBG does not modify only the balance between circulating steroids, but might directly influence NT-proBNP levels. Indeed, in recent years, it has been shown that SHBG may directly mediate cell-surface signaling, cellular delivery and biologic action of sex hormones via activation of a specific plasma receptor [29-31].

Low levels of SHBG have been associated with increased cardiovascular risk in women, irrespective of menopause status [13,14]. Our data and other evidence show that lower SHBG levels are associated with lower NT-proBNP levels in both pre- and postmenopausal women (2). Natriuretic peptides have antiproliferative and vasodilatory effects, as well as antagonism of the renin-angiotensin- aldosterone and adrenergic axes [32]. Therefore, given the cardioprotective effects on NPs, future studies should explore whether lower natriuretic peptide concentrations may explain, in part, the excess cardiovascular risk associated with low SHBG concentrations.

Estradiol and NT-proBNP

This is the first study to examine the association between endogenous estradiol levels and NT-proBNP in postmenopausal women, showing no association. To our knowledge, no study has examined whether endogenous estradiol levels are associated with NT-proBNP in menopausal women, which would shed more light whether the decline in estrogen levels after menopause would be, in part, responsible for the increased risk of cardiovascular disease observed after menopause. Female hormones are considered as important determinants of the lower risk of CVD observed in premenopausal women, while lack of estrogens disadvantages men with regard to CVD risk. Therefore, menopause and drop in endogenous estrogens, suggested that HRT might have an important cardio protective role in women [33].

In line with this hypothesis and in contrast with our findings, a clinical trial of 22 healthy postmenopausal women, reported that administration of hormone replacement therapy with transdermal estradiol produced a rise in plasma levels of BNP [34]. Also, a study conducted in female rats reported that treatment with estradiol and progesterone stimulated atrial NP gene expression[35]. Oral estrogen leads to an increase in SHBG, which

binds more testosterone and therefore leads to an increase of NT-proBNP levels [4]. In contrast to this, a population based study in 682 women showed no association between oral estrogen use and NT-proBNP levels [6]. Also, results of Women's Health Initiative showed that HRT in postmenopausal women was not cardio protective[7].

Body composition, sex steroids and NT-proBNP

Sex differences in body composition were identified as major determinant of metabolic profile and CVD risk differences among genders [2]. Also, it is suggested that endocrine cardiac function is regulated by sex steroids. BNP/NT-proBNP levels are constantly higher in women than in men, while after menopause sex differences in NPs tend to decrease [2]. Several studies reported negative correlation between NT-proBNP and BMI values, in healthy subjects, and also in subjects with heart failure [2]. The majority of postmenopausal women enrolled in our study were overweight (median BMI 27.5 kg/m² (SD 4.6)), therefore, we performed sensitivity analysis by stratifying the analysis across the 3 categories of BMI, (BMI<25, BMI 25-29.9, BMI ≥30 kg/m²), but the results were similar across strata of BMI.

Strengths and limitations

To the best of our knowledge, this is the first and most comprehensive study to examine the associations of estradiol, androgens and SHBG with NT-proBNP levels in a large sample of postmenopausal women, with consistent findings; our results remained significant at more conservative level ($p<0.007$) set by the Bonferroni correction. Also, androgens are measured using chromatography-tandem mass spectrometry, which is at the moment considered to be a gold standard method [36]. However, there are several limitations that need to be taken into account. First, the cross-sectional design does not allow us to address the temporality of the observed associations. Therefore, we cannot draw any conclusions with regard to the causality of the observations. Second, we did not have measures of bioavailable estradiol in the RS, which could have strengthened our results. Also, E_2 was measured using an immunoassay with a detection limit of 18.35 pmol/L, which is considered suboptimal, particularly in postmenopausal women. In our population 1502 women (33.18 %) had values of E_2 lower than 18.35pmol/l. However, we performed sensitivity analysis using E_2 tertiles instead of E_2 as continuous variable, which provided similar results. Third, free T levels were not measured directly in the blood and therefore

have to be interpreted with caution. Nevertheless, free T levels in this study were derived from the ratio of T to SHBG, which is considered a precise proxy for bioavailable T [37]. Furthermore, NT-proBNP is hormonally inactive N-terminal portion of its pro-hormone, and we do not have measurement of BNP which is the active hormone. However, recent systematic reviews and meta-analyses demonstrated that both BNP and NT-proBNP have similar diagnostic and prognostic accuracy in CVDs [2]. Finally, we found differences in baseline characteristics between participants included in our analysis and participants that were excluded because of missing data on exposure and outcome. However, in cohort studies, baseline selection of participants may affect the study validity when the confounding effect of an unknown or unmeasured disease risk factor is larger in the selected sample than in the general population. Yet, it has been shown that using a selected source population for a cohort study usually leads to bias toward the null, but may affect the generalizability of our results regarding mean sex hormone levels and NT-proBNP [38].

In summary, our findings support the hypothesis that higher androgens might be responsible for lower natriuretic peptides levels in postmenopausal women. Given the known cardioprotective effect of NPs, future studies should elucidate mechanisms of actions and to examine whether androgens levels are prospectively associated with NT-proBNP and risk of CVD, in particularly of heart failure, in postmenopausal women.

Clinical Perspectives

Findings from our study support the hypothesis that higher androgens, and not estradiol, might be responsible for lower natriuretic peptides levels in postmenopausal women. Considering that NT-proBNP levels are associated with risk of type 2 diabetes and CVD, our results may also suggest that androgens might be responsible for the change in risk of developing cardiometabolic outcomes after menopause. This rise a question, whether menopausal women might benefit more from androgen agonists/inhibitors than from oral estrogens. Future studies should elucidate mechanisms of actions and to examine whether androgens levels are prospectively associated with NT-proBNP and risk of CVD, in particularly of heart failure, in postmenopausal women. Also, future studies should examine

whether free estradiol, the active form of the hormone, is associated with NT-proBNP levels and risk of CVD in women.

Supplement available online at:
https://www.sciencedirect.com/science/article/pii/S0167527317334265?via%3Dihub#s0095

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CHAPTER 2.2

Mendelian randomization provides evidence for a causal role of dehydroepiandrosterone sulfate in decreasing NT-proBNP levels in a Caucasian population

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Manuscript under review

ABSTRACT

BACKGROUND: Observational evidence indicates an inverse association between the levels of the most abundant hormones in the human body, dehydroepiandrosterone and its sulfate ester – DHEA and DHEAs and N-terminal pro B-type natriuretic peptide (NT-ProBNP). We aimed to generate estimates of the associations of DHEA and DHEAs (exposures) with NT-proBNP that were free from confounding and reverse causation, and thus to assess the causal role of these endogenous sex hormones.

METHODS: Serum DHEA, DHEAs and NT-proBNP were assessed in 7,390 men and women free of cardiovascular diseases from the prospective population-based Rotterdam study. DHEA, DHEAS and NT-proBNP were naturally log transformed. Regression coefficients and 95% confidence intervals (CI) were calculated from multivariable linear regression models adjusting for confounders to explore the cross-sectional association of DHEA and DHEAs with NT-proBNP. To investigate the causal association between DHEAs and NT-proBNP, we applied the two-stage least squares (2SLS) method using genetic risk score associated with DHEAs (DHEAs GRS) as an instrumental variable (IV). DHEAs GRS was calculated using nine SNPs previously reported from genome wide-association studies to have an association with DHEAs. No genome-wide associations have been described for DHEA in literature, and therefore we could not run the MR analysis for this hormone.

RESULTS: In models adjusted for multiple confounders (age, sex, lifestyle and cardiovascular risk factors), high levels of DHEA (β=-0.146, 95%CI: -0.190; -0.101, p<0.001) or DHEAs (β=-0.214, 95%CI: -0.262; -0.166, p<0.001) were associated with lower levels of NT-proBNP. Genetic risk score of DHEAs explained 29.39% variance of the circulating levels of NT-proBNP. The Mendelian Randomization analysis showed evidence for a causal association between DHEAs and NT-proBNP, with a causal coefficient of -0.450 (95% CI: -0.792; -0.107, p=0.010). When stratified by sex, although, the directions of associations were in line with overall findings, results were statistically significant only in women, which may be due to low power, as we confirmed in the power calculation analysis.

CONCLUSIONS: The causal association between DHEAs and NT-proBNP observed in this study suggests a new metabolic pathway linking DHEAs with NT-proBNP. Our results should stimulate future research to evaluate the potential role of DHEAs in prevention and management of chronic heart failure.

INTRODUCTION

Dehydroepiandrosterone (DHEA) and its sulfate conjugate (DHEAs) are the most abundant sex hormones with serum concentrations up to 20-fold higher than the other sex steroids[1]. Plasma levels of DHEAs increase after birth reaching the peak by the second decade of life, afterwards serum levels of DHEAs have a stable decline so by the age of 80 concertation drops to 10-20% of peak levels[1]. Emerging evidence indicates an association between low DHEAs, impaired longevity and common age-related diseases, including cardiovascular disease (CVD)[2]. Pooled estimates from several studies showed low DHEAs to be associated with a 47% higher risk of future CVD mortality events[2]. Furthermore, plasma levels of DHEAs are also decreased in proportion to the severity of heart failure (HF), which is the final common pathway of the majority of CVD[3]. B-type natriuretic peptide (BNP) and its hormonally inactive N-terminal portion (NT-proBNP) are sensitive biochemical markers of HF, particularly of left ventricular dysfunction and have similar diagnostic and prognostic accuracy in CVDs[4]. BNP is released from the myocardium in response to increased mechanical stress in order to maintain cardiac function by mediating vasodilation, natriuresis, and via its anti-fibrotic effects[5].

Emerging evidence showed that endogenous sex hormones levels play a role in the regulation of natriuretic peptides (NP); estrogens may exert a stimulating effect on the NP system, while androgens may exert an inhibitory effect on the NP system[6]. In line with previous evidence from observational studies, we have showed in the Rotterdam Study inverse associations of DHEA and DHEAs with serum NT-proBNP levels in postmenopausal women without CVD[7]. Similarly plasma level of DHEAs was significantly inversely correlated with plasma levels of BNP independently of age and other clinical variables in subjects with HF[1]. In line with this, the experimental evidence from human heart showed that cardiac production of DHEA was suppressed in the failing heart[3]. Evidence from animals showed that DHEA significantly inhibited BNP mRNA levels in a neonatal rat cardiocyte culture system[3].

However, due to observational nature of previous studies affected by the possibility of confounding and reverse causation, it is not possible to draw conclusion regarding the causal association between DHEAs and NPs. Mendelian randomization (MR) method may be used to study the causal associations in presence of such limitations. The method is considered as a 'natural' randomized control trial since it uses selected common genetic

variants related to a specific exposure of interest as an instrumental variable to evaluate causality between exposure and outcome. Since genotypes are assorted randomly during meiosis, MR avoids the issue of reverse causality. In addition, the distribution of genetic variants is thought to be unrelated to confounders, a common source of false positives in epidemiological studies[8]. Although, the physiological function of DHEAs and its importance in maintaining health are poorly understood, several common single nucleotide polymorphisms (SNPs) were associated with changes in gene expression levels, and the related genes are connected to biological pathways linking DHEAs with ageing[9,10].

Therefore, we aimed to study the causal association between serum DHEA(s) and NT-proBNP, in subjects free of cardiovascular diseases, using the MR approach of identified genetic variants combined into genetic risk score (GRS) as an instrumental variable.

METHODS

Study Population

This study was conducted among participants of the prospective population-based Rotterdam Study (RS)[11]. RS is a study of individuals aged 45 and over living in the Ommoord district of Rotterdam, the Netherlands. The rationale and design of RS have been described previously. In brief, all residents of the Ommoord district aged 55 or older were invited to participate (n=10,215). At baseline (1990-1993), 7,983 participants were included (RS-I). In 2000, an additional 3,011 participants were enrolled (RS-II), consisting of persons living in the study district who had become 55 years of age. A second extension of the cohort was initiated in 2006, in which 3,932 participants aged 45 or older were included (RS-III). Follow-up visits were held every 3-5 years, with follow-up for a variety of diseases. The RS has been approved by the medical ethics committee according to the Population Study Act Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. Written informed consent was obtained from all participants[11].

Population for Analyses

The present study includes data from individuals from the third visit of the first cohort of the RS (RS I-3), and from the first visits of the second (RSII-1) and the third cohort (RSIII-1). There were 11,732 subjects eligible for the analysis. Of those 4,342 participants were excluded because (i) information was not available on NT-proBNP (n=814), DHEAs (n=205)

or on the genetic risk score (n=2,318); (ii) they had prevalent cardiovascular disease (coronary heart disease, stroke or HF) (n=947); and (iv) there was no information on history of cardiovascular disease (n=58). Finally, there were 7,390 participants left for the analysis (Figure 1).

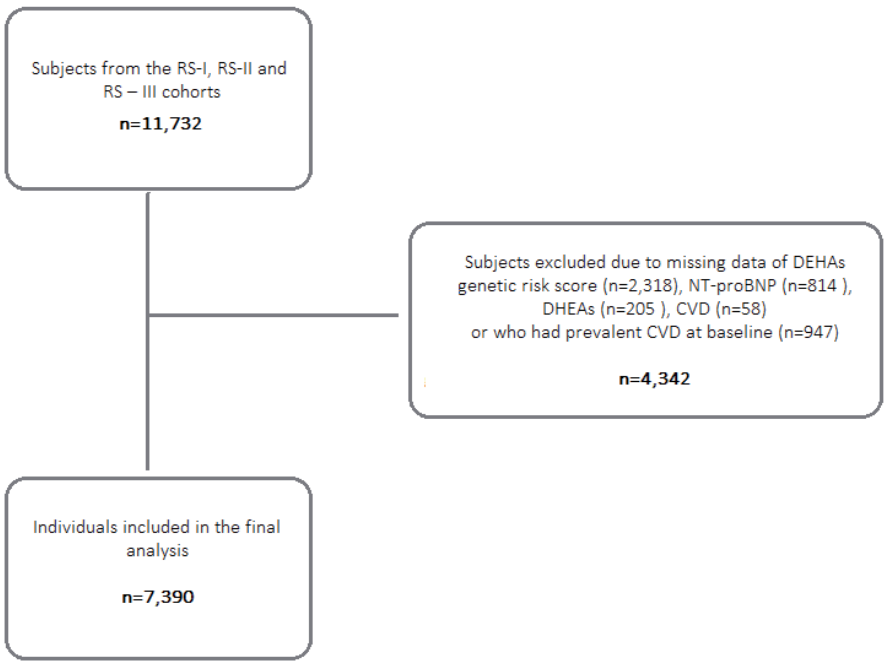

Figure 1. Flowchart for selection of study participants (n=7,390)

Exposure and Outcome Measurement

The exposure (serum DHEA and DHEAs) was measured on a Waters XEVO-TQ-S system (Waters, Milford, MA, USA) using CHS™ MSMS Steroids Kit (Perkin Elmer, Turku, Finland). Inter-assay coefficients of variation of androstenedione, DHEAs and DHEA were <6.5%. NT-proBNP levels were obtained from serum. After blood collection, samples were left to clot for 30 minutes and then centrifuged for 20 minutes at 3000 rotations per minute at 4ºC. Serum was stored at -80°C. NT-proBNP was measured using a commercially available electrochemiluminescence immunoassay (Elecsys proBNP, F. Hoffman-La Roche Ltd., Basel,

Switzerland) on an Elecsys 2010 analyser. Precision, analytical sensitivity and stability characteristics of the system have been previously described[12].

Assessment of Covariates

At baseline interview, all participants provided information on current health status, medical history, medication use, alcohol intake, smoking and physical activity. History of cardiovascular disease was defined as the history of coronary heart disease (myocardial infarction, revascularization, coronary artery bypass graft surgery or percutaneous coronary intervention) and was verified from the medical records of the general practitioner. Diabetes mellitus was defined as the use of blood glucose–lowering medications or a random non-fasting glucose >11.1 mmol/L[13]. Antihypertensive or antidiabetic therapy and statins were collected by questionnaire during home interview. Alcohol intake was assessed in grams of ethanol per day and grouped into 4 categories (0-0.99, 1-19.9, 20-39.9 and ≥40 g/day); smoking status was assessed by asking participants whether they were current smokers of cigarettes, cigars, or pipe and were classified (yes/no). Physical activity was assessed with adapted version of the Zutphen Physical Activity Questionnaire[14]. Every activity mentioned in the questionnaire was attributed a MET-value according to the 2011 Compendium described in detail elsewhere[15]. Blood pressure was measured in sitting position on the right upper arm with a random-zero sphygmomanometer. Body mass index (BMI) was calculated as weight (kg) divided by height square (m^2). Glomerular filtration rate (eGFR) was estimated using the simplified Modification of Diet in Renal Disease (MDRD) equation[16]. Thyroid stimulating hormone (TSH) was measured on the Vitros Eci (Ortho Diagnostics). Insulin, glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triacylglycerol (TG) and C-reactive protein (CRP) were measured on the COBAS 8000 Modular Analyzer (Roche Diagnostics GmbH). Corresponding interassay coefficients of variations are as follows: TSH<13.2%, insulin <8%, glucose <1.4%, lipids <2.1% and CRP <16.9%. LDL-cholesterol level was estimated indirectly from measurements of total cholesterol, HDL and triglycerides by means of the Friedewald equation[17]. Total estradiol (TE) levels were measured with a radioimmunoassay and sex hormone binding globulin (SHBG) by means of the Immulite platform (Diagnostics Products Corporation Breda, the Netherlands). Minimum detection limit for estradiol was 18.35pmol/l. Undetectable estradiol was scored as 18.35pmol/l. Serum levels of total testosterone (TT) were measured

with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Corresponding interassay coefficients of variations for TE, SHBG and TT are <7%, <5%, and <5%. Free androgen index (FAI), calculated as (T/SHBG)*100 is used as a surrogate measure of bioavailable testosterone (BT). All biochemical parameters were assessed in fasting serum.

Genotyping

Genotyping was conducted in all three cohorts using the Illumina Infinium HumanHap550K Beadchip in RS-I and RS-II and the Illumina Infinitum HumanHap 610 Quad chip in RS-III at the Genetic Laboratory of the Erasmus MC, Department of Internal Medicine, Rotterdam, The Netherlands. Participants were excluded if they had excess autosomal heterozygosity, mismatch between called and phenotypic sex, or recognized as being outlier with identical-by-state clustering analysis. Moreover, SNPs with allele frequency ≤1%, Hardy–Weinberg equilibrium p<10–5, or SNP call rate ≤90% were excluded. Imputation was done with reference to HapMap release 22 CEU (Utah residents of northern and western European ancestry) using the maximum likelihood method implemented in Markov Chain based haplotyper (version 1.0.15).

Construction of DHEAs Genetic Risk Score (GRS)

We searched GWAS catalog, Genome-Wide Repository of Associations Between SNPs and Phenotypes (GRASP) and Cardiovascular Disease Knowledge Portal and identified two large genome-wide association studies conducted on >14,846 individuals of European descent[9,10]. Nine SPNs identified from these GWAS were used to build the genetic risk score of DHEAs (rs148982377, rs11761528, rs2637125, rs7181230, rs2497306, rs2185570, rs740160, rs17277546 and rs6738028) **(Supplemental Table 1).** The effect allele (coded 0–2) was the DHEAs raising allele. A weighted GRS was calculated by multiplying the number of risk alleles at each locus by the corresponding reported β coefficient from the previous GWAS and then summing the products[18]. The total score was then divided by the average effect size multiplied by 100 to rescale the scores to a range between 0 and 100. We could not identify genome-wide association studies published for DHEA, and therefore we could not build a genetic risk score for this hormone.

Statistical Analyses

Cross-sectional Analyses

DHEA, DHEAs, NT-proBNP, hsCRP, 17-hydroxyprogesterone and cortisol levels were log-transformed using a natural log to obtain normal distribution. Cross-sectional association between log transformed continuous DHEA/DHEAs and NT proBNP was assessed using ordinal linear regression (OLR) models. Betas were calculated after adjusting for age, sex, interaction term between sex and DHEA/DHEAs (sex*DHEAs p=0.000 and sex*DHEA p=0.002), RS cohort, BMI, physical activity, smoking, alcohol, cholesterol, statin use, glucose, systolic blood pressure, antihypertensive therapy, type 2 diabetes (T2D), eGFR, hsCRP, 17-hydroxyprogesterone and cortisol. Additionally, to explore potential sex differences we run the analysis stratified by gender. A multiple imputation (chained equations method) was applied for missing data. For the most of most baseline clinical variables, less than 2% of values were missing, whereas this was up to 12% and 26% for self-reported variables such as physical activity and alcohol intake, respectively.

Association of DHEAs Genetic Risk score and NT-proBNP

The MR approach is used to investigate the causality of associations between DHEAs and NT-proBNP. Since no SNPs genome-wide significant for DHEA have been published, we could not assess the causality between DHEA and NT-proBNP. In the current study we used the genetic risk score (GRS) of DHEAs (calculated based on nine publically avaiable SNPs) as an instrumental variable (IV). Valid instrumental variable is a factor that is associated with the exposure, but is not associated with any confounder of the exposure–outcome association, nor is there any pathway by which the IV can influence the outcome other than via the exposure of interest/no pleiotropy[19] **(Figure 2)**. Given a continuous outcome (NT-proBNP) and assuming the linear associations between DHEAs and NT-proBNP without interaction, we estimated the casual association between GRS of DHEAs and serum NT-proBNP through a 2-stage least squares (2SLS) regression[20]. The 2SLS estimation proceeds by first fitting the regression of DHEAs (exposure) on the GRS of DHEAs (instrument), and the second step assesses the association of DHEAs with NT-proBNP (outcome) on the fitted values from the first-stage regression. Within these models, age, sex, RS cohort, BMI, physical activity, smoking, alcohol, total cholesterol, statin use, glucose, systolic blood pressure, antihypertensive therapy, T2D, glomerular filtration rate (eGFR), hsCRP, 17-

hydroxyprogesterone and cortisol were included as covariates in order to generate estimates from the IV analyses that were comparable to those from the observational regressions. We also evaluated the instrument strength using F-statistics from the first-stage regressions, where F-statistics >10 has been used to indicate sufficient strength, and by R^2 (%) as a measure of the percentage contribution of GRS to the variation of NT-proBNP levels. Standard MR analysis assume that genetic instruments only influence the outcome (i.e. NT-proBNP) through the exposure of interest (serum DHEAs), however, DHEAs associated SNPs may influence serum NT-proBNP through pathways other than serum DHEAs concertation. We therefore tested the robustness of our findings by MR Egger regression which helps to control for biases though horizontal pleiotropy. The slope of the weighted regression line provides an estimate of the causal effect of the exposure on the outcome free from the effects of horizontal pleiotropy. While the intercept in the regression is a function of extent of directional pleiotropy in the data aggregated across all the different variants used in the analysis, and statistical tests of the degree to which the intercept differs from zero are testing for the overall presence of directional pleiotropy in the data[21]. In case of significant intercept (and therefore evidence of directional effects) the estimate from the Eggers would be a better estimate, however, if no evidence of directional pleiotropy then the 2SLS is better powered. All statistical analyses were carried out using Stata/IC statistical Software, version 15 and MR package of R software.

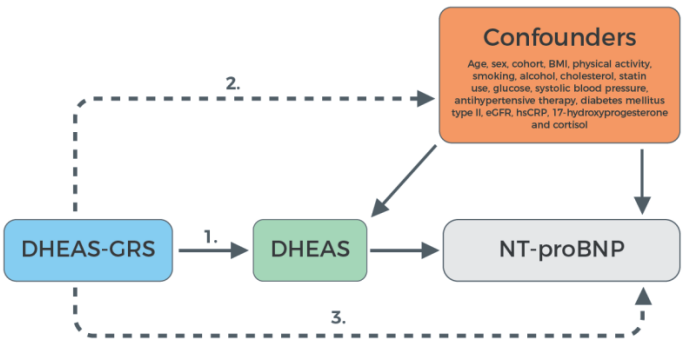

Figure2. Assessing the causality of DHEAS and NT-proBNP levels

(1) GRS of DHEAS is associated with DHEAS (exposure); (2) GRS of DHEAS is not associated with confounding variables; (3) GRS of DHEAS is only associated with NT-proBNP (outcome) through the exposure

RESULTS

Baseline characteristics of the population used for analysis are shown in **Table 1**. Median age (Q1-Q3) of participants was 63 (58-71) years, and 59.9% of included subjects were women. The median levels of NT-proBNP were 7.9 pmol/l (Q1=4.3; Q3=14.9), DHEA 9.6 nmol/l (Q1=6.2; Q3=15.0), DHEAs 2,099nmol/l (Q1=1,257; Q3=3,365) and DHEAs GRS 48.2 (Q1=45.1; Q3=50.8).

Table 1. Characteristics of study population.

Characteristics	Median (Q1-Q3)/ n (%)	Characteristics	Median (Q1-Q3)/ n (%)
Age (years)	63 (58-71)	**Health indicators**	
Sex (female)	4,431 (59.96)		
Health behaviours			
Body mass index (kg/m^2)	27 (24-29)	Systolic blood pressure	138 (125-152)
Smoking (yes)	1,528 (20.68)	(mmHg)	79 (71-86)
Alcohol intake (g/day)		Diastolic blood pressure	5.8 (5.1-6.4)
0-0.99	2,541 (34.38)	(mmHg)	1.4 (1.1-1.7)
1-19.9	2,559 (34.63)	Total cholesterol (mmol/l)	1.3 (1.0-1.8)
20-39.9	1,890 (25.58)	HDL-C (mmol/l)	5.5 (5.1-6.0)
≥40	400 (5.41)	Triglycerides in serum	71 (50-103)
Physical activity (total MET	70 (41-103)	(mmol/l)	1.6 (0.6-3.5)
hours)		Fasting blood glucose (mg/dl)	
		Insuline (pmol/l)	
		hs-CRP (mg/ml)	
Hormones		eGFR (mL/min/1.73m^2)	80 (69-90)
Estradiol (pmol/l)	63 (25-103)	Antihypertensive use (yes)	2,085 (28.21)
Testosterone (nmol/l)	1.3 (0.7-15.1)	Serum lipid lowering	980 (13.26)
SHBG (nmol/l)	52 (38-73)	medication (yes)	781 (10.57)
DHEA (nmol/l)	9.6 (6.2-15.0)	Prevalent diabetes mellitus	
DHEAs (nmol/l)	2,099 (1,257-		
DHEAs GRS	3,365)		
Androstenedione	48.2 (45.1-50.8)		
17-hydroxyprogesterone	2.7 (2.0-3.6)		
(nmol/l)	1.5 (0.8-2.7)		
NT-proBNP	7.9 (4.3-14.9)		

Values are presented absolute value and percentage for categorical variables, and median (25th-75th quartile) for continuous variables.

Abbreviations: HDL=high density lipoprotein colesterol; hs-CRP=high-sensitivity C reactive protein; eGFR=glomerular filtration rate; SHBG= sex hormone binding globulin; DHEA=dehydroepiandrosterone; DHEAs=dehydroepiandrosterone sulfate; DHEAs GRS= dehydroepiandrosterone sulfate genetic risk score; TSH=thyroid stimulating hormone; NT-proBNP=amino-terminal pro-B-type natriuretic peptide.

Observational associations between DHEA, DHEAs and NT-proBNP

Based on 7,390 subjects, we observed an inverse association between serum DHEAs levels and NT-proBNP levels. In crude model for each one-point increase in levels of natural log transformed DHEAs, NT-proBNP levels decreased -0.395 (β; 95%CI: -0.423; -0.366; p<0.001). In multivariable linear regression model (adjusted for age, sex, interactions of DHEAs*sex, RS cohort, BMI, physical activity, smoking, alcohol, cholesterol, statin use, glucose, systolic blood pressure, antihypertensive therapy, diabetes mellitus type 2, eGFR, hsCRP, 17-hydroxyprogesterone and cortisol) for each one-point increase in levels of natural log transformed of DHEAs, NT-proBNP levels decreased -0.214 (β; 95%CI: -0.262; -0.166; p<0.001) (**Table 2**). Stratification by gender did not materially change the results. Among both, men and women in fully adjusted models, high levels of serum DHEAs were associated with low levels of NT-proBNP levels (**Supplemental table 2**). Furthermore, in fully adjusted model for each one-point increase in levels of natural log transformed DHEA, NT-proBNP levels decreased by -0.146 (β; 95%CI: -0.190; -0.101; p<0.001), also, gender stratification did not yield any changes (**Supplemental table 3**). Assumptions of linearity, homoscedasticity and normality were assessed; but no major violations were observed.

Table 2. Summary statistics describing observational and causal relationship DHEAs and NT-proBNP (n=7,390)

Method	β	SE Error	95% CI	p-value	F-statistics	R^2
Overall Crude Model						
OLR	-0.395	0.014	-0.423; -0.366	0.000	----	0.0898
2SLS	-0.530	0.169	-0.863; -0.198	0.002	56.02	0.0075
Overall Adjusted Model						
OLR	-0.214	0.024	-0.262; -0.166	0.000	----	0.3252
2SLS	-0.450	0.174	-0.792; -0.107	0.010	58.28	0.2939

Coefficients represent the decrease in log-NT-proBNP for each unit increase in log-DHEAs.

Adjusted model: age, sex, sex*DHEAs, cohort, BMI, physical activity, smoking, alcohol, total cholesterol, statin use, glucose, systolic blood pressure, antihypertensive therapy, diabetes mellitus type 2, glomerular filtration rate (eGFR), hsCRP, 17-hydroxyprogesterone and cortisol; DHEAs, NT-proBNP, hsCRP, 17-hydroxyprogesterone and cortisol levels were log-transformed using a natural-log.

NOTE: in 2SLS method the analysis was not adjusted for sex*DHEAs interaction term

Abbreviations: OLR= Ordinal lineal regression (Observational analysis); 2SLS=Two-stage least squares regression; DHEAs=dehydroepiandrosterone sulfate; NT-proBNP=amino-terminal pro-B-type natriuretic peptide; BMI=body mass index; eGFR: glomerular filtration rate; hs-CRP=high-sensitivity C reactive protein.

Causal estimates for the effect of DHEAs genetic risk score on NT-proBNP levels

A weighted gene score composed of 9 genetic variants for elevating DHEAs was used as the genetic instrument. DHEAs GRS was strongly associated with circulating NT-proBNP levels, explaining on average 29.39% of NT-proBNP variation with F-statistics=58.26 (**Table 2**), indicating that GRS is unlikely to be affected by weak instrument bias. Neither individual genetic variants nor the gene score were associated with potential confounders including sex, age and BMI **(Supplemental Table 4)**. Also, using ordinal lineal regression model adjusted by age, sex and RS cohort, we investigated the association between individual DHEAs SNPs and NT-proBNP and none of the SNPs was statistically significant at Bonferroni corrected p-value of 0.0055 **(Supplemental Table 5)**. In the MR analysis, applying 2SLS approach in the entire study group, using DHEAs genetic risk score as the instrumental variable, significant causal association was observed between DHEAs and NT-proBNP levels, either in the crude or adjusted analysis. Genetically predisposed higher levels of DHEAs were associated with decreased serum NT-proBNP levels [crude model β=-0.530 (95%CI:-0.863; -0.198; p=0.002) and adjusted model β=-0.450 (95%CI:-0.792; -0.107; p=0.010)] **(Table 2).** As in the observational analysis, we found significant interaction between DHEAs and sex we run the 2SLS analysis separate for men and women. After stratification by sex, in both men and women, results were similar to overall findings, however, the estimates did not reach statistical significance (**Supplemental table 2**). However, in men the value of F statistics the GRS of DHEAs was close to value considered as a weak instrument (F <10), which could be due to low power, as we confirmed in the power calculation analysis (**Supplemental table 2**). We applied an extension of MR, Eggers regression to test for horizontal pleiotropy. The intercept of the MR-Egger regression captures the average pleiotropic effect across all genetic variants. None of the analyses performed had a significant intercept indicating no directional pleiotropy (**Supplemental table 6**).

DISCUSSION

Overall, in this large population-based study among individuals free of CVD we found statistically significant inverse associations between DHEA and DHEAs and serum NT-proBNP. In Mendelian randomization approach genetically predisposed higher levels of DHEAs were associated with lower NT-proBNP concentrations; therefore, providing some evidence for potential causal, inverse association between DHEAs and NT-proBNP.

Our findings complement the preceding publication from the RS, where cross-sectional data from postmenopausal women free of CVD disease, have shown inverse association between DHEA and DHEAs and serum NT-proBNP[7]. Also, our results are in line with previous observational data. Several epidemiological studies have demonstrated an association between low serum levels of DHEAs with elevated CVD risk[22,23], cardiovascular morbidity[24-26], coronary artery disease [27,28] and vascular atherosclerotic disease[29]. Moriyama et al. reported positive association between DHEAs levels and left ventricular ejection fraction (LVEF), as well as inverse association with BNP levels in an Asian population, independently of age and other clinical variables[1]. Also, Kawano et al. showed DHEAs levels to increase upon improvement of ventricular function in patients undergoing HF treatment[30]. It has also been reported that DHEAS can be produced in cardiomyocytes of structurally healthy hearts, but not in failing hearts[31].

Despite increasing evidence suggesting its beneficial cardiovascular effects, an intracellular steroid hormone receptor for DHEA has not been identified[3]. Recent reports suggested specific DHEA-binding sites in cardiovascular tissue[32,33], and that this putative receptor is present in the rat heart[33]. However, it is unclear if DHEA directly exerts its effects or if it acts after conversion to testosterone/17β-estradiol, via binding specific receptors for testosterone and 17β-estradiol that are present in the heart[34]. The inverse association between DHEAs and NT-proBNP can be explained by the opposite biological effect they produce. DHEA and DHEAs may play a beneficial role in cardiovascular system through modulation of several processes such as nitric oxide production stimulation, oxidation stress inhibition, prevention of vascular remodelling, stimulated vasodilation[35]. Conversely, increased NPs production at both auricular and ventricular level, and progressively according to ventricular dysfunction, has been previously evidenced in patients with HF, which is in turn associated with increased oxidative stress, that might alter the electron transport mechanism at P450C17 cytochrome level, selectively suppressing 17,20 lyase enzyme activity, resulting in decreased DHEAs serum levels [22].

Recently, nine common genetic variants were associated with serum DHEAs, suggesting its key role in aging mechanisms[36]. Genes at or near these genetic variants include BCL2L11, ARPC1A, ZKSCAN5, ZNF789, TRIM4, HHEX, CYP2C9, BMF and SULT2A1.

These genes have various associations with steroid hormone metabolism co-morbidities of ageing including type 2 diabetes, lymphoma, actin filament assembly, drug and xenobiotic metabolism, and zinc finger proteins—suggesting a wider functional role for DHEAs than previously thought. Using DHEAs genetic risk score as an IV, our findings suggest that genetically predisposed higher DHEAs concentrations are inversely associated with NT-proBNP levels. Therefore, there might be a causal association between DHEAs and NPs. Still, the common biochemical pathways that link the metabolism of these two hormones are largely unknown, and should further be investigated.

Strengths and limitations

To the best of our knowledge, this is the first study to examine the causal association between DHEAs and NT-proBNP levels in a large population based sample of CVD free men and women. Also, DHEA and DHEAs are measured using chromatography-tandem mass spectrometry, which is at the moment considered to be a gold standard method[37]. Although MR is considered as a flexible and robust statistical method, there is a number of MR limitations which need to be considered, also, the limitations of the observational part of our analysis merits further discussion. First, in the RS, serum BNP levels were not measured, but solely its inactive precursor NT-proBNP. However, recent systematic reviews and meta-analyses demonstrated that both BNP and NT-proBNP have similar diagnostic and prognostic accuracy in CVDs[4]. Second, there were no publically available SNPs on DHEA, therefore, we were not able to calculate GRS of DHEA and we could not study the causal association between DHEA and NT-proBNP. Also, within the RS we did not identify any SNPS associated with serum DHEA. However, DHEAs is more stable sulfate ester of DHEA, and it can be converted back to DHEA by steroid sulfatase, which can be considered a good proxy of the association between DHEA and NT-proBNP as confirmed in our regression analysis (cross-sectional associations between DHEA and DHEAs and NT-proBNP were in line)[38]. Third, calculation of allele score is considered to be a good approach to avoid weak IV bias for reasons and also may increase the power and simplicity[18]. However, due to complex biology, the effects of all the variants in an allele score may not be well known, the instrumental variable assumptions may not be satisfied for all the variants[18]. Weakly associated instruments (F statistics < 10) can bias causal estimates towards the observational estimate for one-sample MR. Indeed, the strength of the GRS as an

instrument, measured by the F statistic was satisfactory overall, and in females, but in men F statistics was close to 10 indicating that in males, DHEAS GRS might be a weak instrument. However, we consider this could be due to low power, as we confirmed in the power calculation analysis. Fourth, an important assumption of Mendelian randomization is that the genetic variant must mediate its effect on outcome only via the risk factor, i.e., the genetic variant shows no pleiotropic effects. Therefore, this assumption cannot be proven formally in practice because of incomplete knowledge of the underlying biology. However, we applied an extension of MR approach: MR Egger regression, to test for the causal effect free of pleiotropy. In simple words, provided the underlying assumptions are met, the slope of the MR Egger regression analysis should yield an estimate of the causal effect of DHEA on NT-proBNP that is free from any confounding effects due to horizontal pleiotropy. However, it is important to mention that the validity of MR Egger regression rests on the 'INSIDE assumption' (INstrument Strength is Independent of Direct Effect) which states that across all instruments there should be no correlation between the strength with which the instrument proxies the exposure of interest, and its degree of association with the outcome via pathways other than through the exposure[39]. This is a weaker requirement than the exclusion restriction criterion in normal MR which postulates that SNPs may only affect the outcome (NT-proBNP) through the exposure of interest (serum DHEAs), and so MR Egger regression is likely to be more robust to horizontal pleiotropy than standard MR approaches, although this appears to come at the cost of decreased power to detect a causal effect in one sample MR, therefore, we decided not to use this approach to assess the causality[39]. However, the MR-Egger intercept indicated no presence of horizontal pleiotropy.

Clinical implications

In cross-sectional analysis DHEA and DHEAs were significantly inversely associated with serum NT-ProBNP levels. Causal association we have observed between DHEAs and NT-proBNP suggests a new metabolic pathway linking DHEAs with NT-proBNP, which merits detailed experimental investigation in the future. Altering the serum DHEAs might play an important role in prevention and management of chronic heart failure, therefore, after exploring the biology behind our findings; clinical studies shall address health benefit of modifying serum DHEAs in subjects with heart failure.

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Supplemental Material

Supplemental Table 1. Genetic variants and alleles used in the genetic risk score of DHEAs

SNP	Gene	Coded	Beta
rs148982377	ZNF789	C	0.255
rs11761528	ZKSCAN5	T	-0.16
rs2637125	SULT2A1	A	-0.09
rs7181230	BMF	G	0.05
rs2497306	HHEX	C	-0.04
rs2185570	CYP2C9	C	-0.06
rs740160	ARPC1A	T	0.15
rs17277546	TRIM4;CYP3A43	A	-0.11
rs6738028	BCL2L11	G	-0.04

Supplemental table 2. Summary statistics describing observational and causal relationship DHEAs and NT-proBNP stratified by gender (n=7,390)

Method	β	SE Error	95% CI	p-value	F-statistics	R^2
Women Crude Model						
OLR	-0.268	0.017	-0.302; -0.234	0.000	----	0.0515
2SLS	-0.424	0.178	-0.773; -0.075	0.017	43.05	0.0096
Women Adjusted Model						
OLR	-0.088	0.016	-0.121; -0.055	0.000	----	0.2722
2SLS	-0.388	0.165	-0.714; -0.063	0.019	48.91	0.1911
Men Crude Model						
OLR	-0.494	0.029	-0.551; -0.436	0.000	----	0.0871
2SLS	-0.729	0.489	-1.688; 0.229	0.136	10.96	0.0037
Men Adjusted Model						
OLR	-0.145	0.029	-0.202; -0.088	0.000	----	0.3382
2SLS	-0.643	0.467	-1.560; 0.273	0.169	12.48	0.2588

Coefficients represent the decrease in log-NT-proBNP for each unit increase in log-DHEAs.
Adjusted model: age, cohort, BMI, physical activity, smoking, alcohol, total cholesterol, statin use, glucose, systolic blood pressure, antihypertensive therapy, diabetes mellitus type 2, glomerular filtration rate (eGFR), hsCRP, 17-hydroxyprogesterone and cortisol.
DHEAs, NT-proBNP, hsCRP, 17-hydroxyprogesterone and cortisol levels were log-transformed using a natural-log.
Abbreviations: OLR=Ordinal lineal regression (Observational analysis); 2SLS=Two-stage least squares regression; DHEAs=dehydroepiandrosterone sulfate; NT-proBNP=amino-terminal pro-B-type natriuretic peptide; BMI=body mass index; eGFR=glomerular filtration rate; hs-CRP=high-sensitivity C reactive protein.

Supplemental table 3. Associations between DHEA with the level of serum NT-proBNP in people free of CVD, the Rotterdam Study (n=7,357)

	NT-proBNP				
	β	95% CI	SE	p-value	R^2
All	-0.146	-0.190; -0.101	0.022	0.000	0.3216
Women	-0.097	-0.137; -0.057	0.020	0.000	0.2712
Men	-0.086	-0.150; -0.023	0.032	0.008	0.3343

Models adjusted by age, sex, sex*DHEA, cohort, BMI, physical activity, smoking, alcohol, total cholesterol, statin use, glucose, systolic blood pressure, antihypertensive therapy, diabetes mellitus type 2, glomerular filtration rate (eGFR), hsCRP, 17-hydroxyprogesterone and cortisol. Models of women and men the same adjustment except sex and sex*DHEA interaction. DHEA, NT-proBNP, hsCRP, 17-hydroxyprogesterone and cortisol levels were log-transformed using a natural-log.

Abbreviations: DHEA=Dehydroepiandrosterone; NT-proBNP= Amino-terminal pro-B-type natriuretic peptide; BMI=Body mass index; eGFR=glomerular filtration rate; hs-CRP=high-sensitivity C reactive protein.

Supplemental Table 4. Analysis of association of DHEAs genetic risk score with tested confounders by linear regression

	Crude		Adjusted	
DHEAs Genetic Risk score	β	p-value	β	p-value
Age	-0.009	0.072	-0.002	0.759
Sex	-0.190	0.059	-0.177	0.080
Cohort	0.126	0.026	0.107	0.165
Body mass index	0.010	0.375	0.009	0.419
Physical activity	-0.001	0.149	-0.0008	0.380
Smoking	0.116	0.340	0.080	0.518
Alcohol	0.014	0.794	-0.013	0.810
Total cholesterol	-0.018	0.710	0.007	0.885
Statin Use	-0.166	0.253	-0.200	0.175
Glucose	-0.006	0.855	-0.005	0.879
Systolic blood pressure	-0.0007	0.739	0.00005	0.981
Antihypertensive therapy	0.149	0.173	0.201	0.073
Diabetes mellitus, type 2	0.017	0.915	0.022	0.891
Glomerular filtration rate	0.001	0.581	-0.003	0.361
High-sensitivity C reactive protein	-0.012	0.777	0.020	0.642
17-hydroxyprogesterone	-0.013	0.835	-0.176	0.044
Cortisol	-0.092	0.521	-0.068	0.633

Adjusted by age, sex and cohort. *Statistically significant p-value=0.0055

Supplemental Table 5. Association of individual DHEAs SNPs with NT-proBNP levels in the Rotterdam Study

NT-proBNP	β	p-value*
rs148982377	-0.013	0.744
rs11761528	-0.044	0.082
rs2637125	-0.028	0.143
rs7181230	0.018	0.217
rs2497306	-0.006	0.627
rs2185570	-0.013	0.520
rs740160	-0.050	0.150
rs17277546	0.001	0.953
rs6738028	-0.006	0.685

Adjusted by age, sex, cohort. *Statistically significant p-value=0.0055

Supplemental Table 6. MR-Egger intercept

Method	β	SE Error	95% CI	p-value
Overall Crude Model				
MR-Egger (intercept α)	-0.016	0.013	-0.041; 0.008	0.195
Overall Adjusted Model				
MR-Egger (intercept α)	-0.006	0.010	-0.025; 0.013	0.543
Women Crude Model				
MR-Egger (intercept α)	-0.016	0.020	-0.054; 0.022	0.409
Women Adjusted Model				
MR-Egger (intercept α)	0.002	0.017	-0.031; 0.035	0.900
Men Crude Model				
MR-Egger (intercept α)	-0.015	0.018	-0.051; 0.021	0.410
Men Adjusted Model				
MR-Egger (intercept α)	0.000	0.015	-0.029; 0.029	0.982

Models adjusted by age, sex, cohort, BMI, physical activity, smoking, alcohol, total cholesterol, statin use, glucose, systolic blood pressure, antihypertensive therapy, diabetes mellitus type 2, glomerular filtration rate (eGFR), hsCRP, 17-hydroxyprogesterone and cortisol. DHEA, NT-proBNP, hsCRP, 17-hydroxyprogesterone and cortisol levels were log-transformed using a natural-log.

Abbreviations: DHEA=Dehydroepiandrosterone; NT-proBNP= Amino-terminal pro-B-type natriuretic peptide; BMI=Body mass index; eGFR=glomerular filtration rate; hs-CRP=high-sensitivity C reactive protein.

CHAPTER 2.3

Endogenous estradiol increases the risk of vulnerable carotid plaque composition and risk of stroke in postmenopausal women

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Circ Res. 2018 Jan 5;122(1):97-105

ABSTRACT

BACKGROUND: Sex steroids may play a role in plaque composition and in stroke incidence.

OBJECTIVES: To study the associations of endogenous estradiol and testosterone with carotid plaque composition in elderly men and postmenopausal women with carotid atherosclerosis, as well as with risk of stroke in this population.

METHODS: Data of 1023 postmenopausal women and 1124 men (≥45 years) with carotid atherosclerosis, from prospective population-based Rotterdam Study, were available. At baseline, total estradiol (TE) and total testosterone (TT) were measured. Carotid atherosclerosis was assessed by ultrasound, whereas plaque composition (presence of calcification, lipid core and intraplaque hemorrhage) was assessed by MRI.

RESULTS: TE and TT were not associated with calcified carotid plaques in either sex. TE was associated with presence of lipid core in both sexes (in women odds ratio (OR), 95% CI: 1.48 [1.02, 2.15], in men OR, 95% CI: 1.23 (1.03, 1.46]), whereas no association was found between TT and lipid core in either sex. Higher TE (OR, 95% CI: 1.58 [1.03, 2.40]) and lower TT (OR, 95% CI: 0.82 [0.68, 0.98]) were associated with intraplaque hemorrhage in women but not in men. In women, TE was associated with increased risk of stroke (hazard ratio (HR), 95% CI: 1.98 [1.01, 3.88], whereas no association was found in men. TT was not associated with risk of stroke in either sex.

CONCLUSIONS: TE was associated with presence of vulnerable carotid plaque as well as increased risk of stroke in women, whereas no consistent associations were found for TT in either sex.

INTRODUCTION

Ischemic stroke, a major cause of death and long-term disability among men and women, inflicts a considerable economic burden to society [1]. Within the etiology of ischemic stroke, atherosclerotic disease of the carotid artery, and particularly plaque composition are important risk factors [2]. Carotid atherosclerotic plaque can be composed of various components, such as a lipid pool (with/without necrosis), calcification, and intraplaque hemorrhage [3]. Plaques that contain lipid deposits can lead to development of so-called vulnerable plaques in which hemorrhage can develop. This in turn can lead to plaque instability, further progress to rupture, ultimately precipitating embolism [4,5] and subsequently increase risk of stroke [2]. Sex differences have been observed in plaque composition as well as in stroke incidence [6]. Stroke incidence is about 30% higher in men than in women [6]. In line with this, women have higher rates of stable fibro-calcific atherosclerotic plaques, while plaques found in men tend to be more complex with higher rates of unstable lesions- intraplaque hemorrhage and presence of necrotic lipid core [7]. Nevertheless, in women the risk for stroke roughly doubles during the 10 years after menopause [8]. These sex- and menopause-differences in plaque composition and risk of stroke might be driven by endogenous sex hormones [9,10]. Experimental evidence suggests a direct action of estradiol on the vascular system, affecting many mechanisms that impact plaque composition and occurrence of atherothrombotic ischemic stroke, including lipid metabolism, inflammation, oxidative stress, fibrinolysis, and thrombosis [11]. Testosterone may slow down atherosclerosis through inhibiting carotid intima-media thickening , atheroma formation [12] and immunomodulation of plaque development and stability [13]. To date, no study has investigated the association between circulating estradiol, testosterone and plaque composition in human populations. Also, limited evidence exists on endogenous estradiol, testosterone and risk of stroke, and particularly in high risk populations such as subjects with presence of carotid atherosclerosis who are at increased risk of developing stroke [14].

The aim of our study was to investigate the associations of endogenous estradiol and testosterone with carotid plaque composition in middle-aged and elderly men and postmenopausal women with carotid atherosclerosis, as well as with risk of stroke in this population.

Methods and materials

Study Population

The Rotterdam Study (RS) is a prospective, population-based cohort study among individuals aged ≥ 45 in Ommoord municipality of Rotterdam, The Netherlands. The rationale and design of RS is described in detail elsewhere [15]. In brief, all inhabitants of the Ommoord district aged 55 years or older were invited to participate (n =10,215). At baseline (1990-1993), 7,983 participants were included (RS-I). In 2000, all persons living in the study district who had become 55 years of age (n=3011) were additionally enrolled (RS-II). A second extension of the cohort was initiated in 2006, in which 3,932 participants aged 45 years or older were included (RS-III). Follow-up visits were held every 3-5 years. The Rotterdam Study has been approved by the Medical Ethics Committee according to the Wet Bevolkingsonderzoek: ERGO (Population Study Act: Rotterdam Study), executed by the Ministry of Health, Welfare and Sports of The Netherlands. All participants gave informed consent to participate in the study and to obtain information from treating physicians and pharmacies, separately. The present study used data from the third visit of the first cohort (RSI-3, year 1997-1999) and the baseline examinations of the second (RSII-1, year 2000-2001) and third cohort (RSIII-1, year 2006-2008).

Population for Analyses

Sex steroids and Plaque Composition

All subjects (n=2,666) diagnosed with carotid atherosclerosis by carotid artery ultrasound (intima-media thickness > 2.0 mm in one or both carotid arteries) were invited to magnetic resonance imaging (MRI) of carotid arteries. Subjects were not examined due to various reasons (n=790): MRI contraindications (n=115), physical limitations (n=191), claustrophobia (n=163), refusal to participate (n=272), and loss to follow-up (n=49). Of the remaining 1876 participants, 95 were excluded due to poor image quality, 41 because of absence of carotid plaque bilaterally, ,215 subjects did not have information on TE, TT levels and 45 women previously used HRT or did not have information on HRT use. Therefore, 645 postmenopausal women and 835 men were included in final analysis for the association of sex steroids with plaque composition and subsequent carotid features (**Figure 1).**

Sex Steroids and Stroke Incidence

From 2,666 participants diagnosed with carotid atherosclerosis, 315 subjects were excluded due to missing information on TE and TT, and 84 subjects with prevalent stroke, 85 women who used HRT/did not have information on HRT use and 26 women who were not postmenopausal, leaving 2,147 subjects, 1,124 men and 1,023 women, for final analysis for the association of sex steroids and stroke incidence (**Figure 1**).

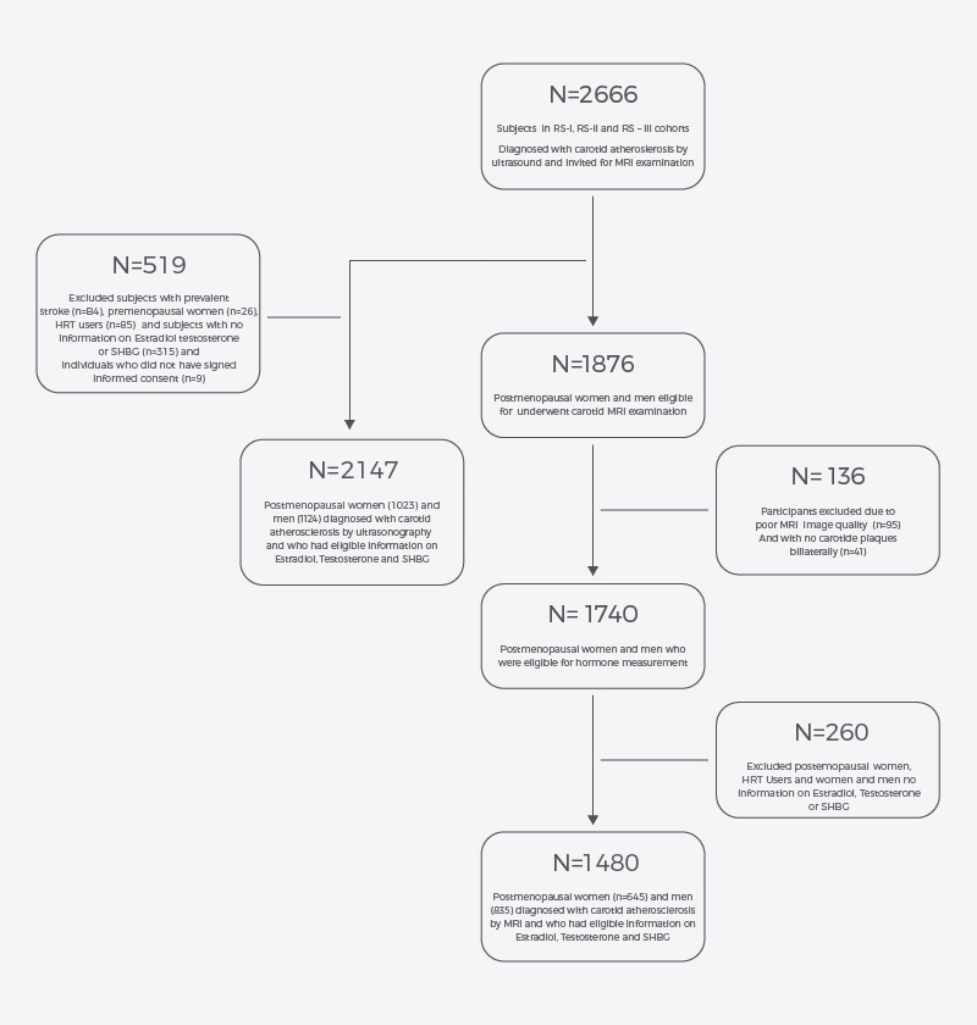

Figure 1. Flowchart of study participants

Sex steroids Measurement

Sex hormones serum levels were assessed at baseline visit (RSI-3, RSII-1 and RSIII-1). Serum levels of total testosterone (TT) were measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Total estradiol/17-β estradiol (TE) levels were measured with a radioimmunoassay and sex hormone-binding globulin (SHBG) with the Immulite platform (Diagnostics Products Corporation Breda, the Netherlands). The minimum detection limit for TE was 18.35 pmol/liter. Undetectable estradiol was scored as 18.35. The corresponding interassay coefficients of variations for TE, SHBG and TT are <7%, <5%, and <5%. Free androgen index (FAI), calculated as (T/SHBG)*100 is used as a surrogate measure of bioavailable testosterone.

Carotid scanning and analysis of plaque components

Ultrasonography of carotid arteries

At baseline (RSI-3, RSII-1 and RSIII-1), carotid intima-media thickness (CIMT) of the common carotid artery, carotid bifurcation, and internal carotid artery of the left and right carotid arteries was measured using Rotterdam Study ultrasound protocol. CIMT was measured as a distance between lumen-intima interface and media-adventitia interface, on a longitudinal, two-dimensional ultrasound image of the carotid artery [16]. Carotid atherosclerosis was defined as CIMT of >2.0 mm in one or both carotid arteries.

Carotid MRI Scanning

MRI has been implemented into the core protocol of the Rotterdam Study (RS I-5:2009-2011, RSII-2: 2005-2006 and RS III-1: 2006-2008) since 2005. Therefore, it is important to mention that there was a time difference between hormone measurement at baseline and MRI scan (men: mean= 7.30 ±SD=4.2 years and women: mean=7.43 ± SD=4.1 years). MRI imaging was performed on a 1.5 Tesla scanner (GE Healthcare, Milwaukee, WI, USA) with a dedicated bilateral phase-array surface coil (Machnet, Eelde, the Netherlands). High-resolution images were obtained using a standardized protocol, used with a total scanning time of approximately 30 minutes. Details of the review protocol, scan reading procedure, and reproducibility are described in detail elsewhere [17]. Plaque characteristics are assessed in all plaques with a maximum thickness of ≥2.0 mm on MRI. Plaque composition were

reviewed for the presence of three different components- presence of calcification, intraplaque hemorrhage, and lipid core. Plaque evaluation was done by one trained observer with 3 years of experience using standardized evaluation protocol. The observer was blinded to all participant characteristics. Intraplaque hemorrhage was defined as the presence of a hyperintense focus within the plaque on the 3-dimensional T1-weighted gradient radio echo sequence. Calcification was defined as a hypointense region in the plaque on all sequences. Finally, the presence of lipid core was defined as a hypointense region in the plaque on proton density weighted fast spin echo or proton density weighted echo planar image and T2-weighted echo planar image, or a region of relative signal intensity drop in the T2-weighted echo planar images compared with the proton density weighted echo planar image. Multiple components were permissible in one plaque [17].

Assessment of Stroke

History of stroke was assessed using home interviews and confirmed by reviewing medical records. Participants were continuously followed up for stroke through digital linkage of general practitioners' files with the study database. Furthermore, nursing home physicians' files and files from general practitioners of participants who moved out of the district were checked on a regular basis. Hospital discharge letters and information from general practitioners was collected for all potential strokes. Research physicians reviewed the information and an experienced neurologist (M.K.I.) verified the strokes. Follow-up for stroke was complete until January 1st 2012 for 96.3% of potential person-years [18]. In the Rotterdam study a stroke was subclassified as ischemic if a CT or MRI scan, made within 4 weeks after the stroke occurred, confirmed the diagnosis, or if indirect evidence (deficit limited to 1 limb or completely resolved within 72 hours, atrial fibrillation in absence of anticoagulants) pointed at an ischemic nature of the stroke. A stroke was sub classified as hemorrhagic if a relevant hemorrhage was shown on CT or MRI scan. Supratentorial hemorrhagic strokes were sub classified as deep or lobar based on neuroimaging. If we could not retrieve enough information to sub classify a stroke as ischemic or hemorrhagic, it was called unspecified[19].

Assessment of Covariates

The information on current health status, medical history, medication use, menopausal status, alcohol intake and smoking were obtained by an interview at baseline (RSI-3, RSII-1 and RSIII-1). Blood pressure was measured in the sitting position on the right upper arm with a random-zero sphygmomanometer. Cardiovascular disease was defined as a history of coronary heart diseases (myocardial infarction, revascularization, coronary artery bypass graft surgery or percutaneous coronary intervention), heart failure and stroke, and was verified from the medical records of the general practitioner. Diabetes mellitus type II diagnosis was considered present if a person used glucose lowering drugs or in case that a non-fasting random serum glucose level was ≥11 mmol/L. Postmenopausal women were defined as women who reported absence of menstrual periods for 12 months. Age at menopause was defined as self-reported age at the time of last menstruation. Data on age at menarche were collected by asking women, "How old were you when you had your first menstrual period"? Retrospective data on self-reported number of pregnancies of at least 6 months, use of hormone replacement therapy, as well as use of current antihypertensive medications and lipid-lowering medications were collected by a questionnaire during the home interview. Smoking status was assessed asking participants whether they were current smokers of cigarettes, cigars, or pipe. Alcohol intake was assessed in grams of ethanol per day. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). All biochemical parameters were assessed in fasting serum. Dehydroepiandrosteron (DHEA) was measured on a Waters XEVO-TQ-S system (Waters, Milford, MA, USA) using the CHS™ MSMS Steroids Kit (Perkin Elmer, Turku, Finland). Thyroid stimulating hormone (TSH) was measured on the Vitros Eci (Ortho Diagnostics). Insulin, glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triacylglycerol (TG) and C-reactive protein (CRP) were measured on the COBAS 8000 Modular Analyzer (Roche Diagnostics GmbH). The corresponding interassay coefficients of variations are the following: TSH<13.2%, insulin <8%, glucose <1.4%, lipids <2.1% and CRP <16.9% [15]. LDL-cholesterol level was estimated indirectly from measurements of total cholesterol, HDL and triglycerides by means of the Friedewald equation [20].

Statistical analyses

Main Analysis

Continuous variables are reported as mean ± standard deviation (SD) unless stated otherwise and categorical variables were presented as percentages. To achieve normal distribution, skewed variables (TT, SHBG, FAI, triglycerides, glucose, insulin, CRP, and TSH) were natural log transformed. All analyses were stratified by sex. Logistic regression models were used to evaluate whether sex steroids were associated with plaque composition. All sex hormones variables were assessed continuously (per SD increase) in separate models. As the minimum detection limit for TE was 18.35pmol/liter, and 32.7 percent of women had values of TE lower than 18.35, we also analyzed TE as dichotomized variable (18.35 pmol/liter as reference category) in women. Only 4 male participants had values of TE lower than 18.35, and therefore TE was analyzed (per SD increase) continuously in men [21]. In the basic model (Model 1), we adjusted for age at baseline, time from hormone measurement at baseline and MRI scan (men: mean= 7.80 ±4.20 years and women: mean=7.43 ± SD=4.13 years), BMI, and sex steroids for each other. The main role of SHBG is the transport of sex steroids within the blood stream to extravascular target tissues. By binding testosterone and estradiol, SHBG regulates the balance between bioavailable testosterone and estrogens, and thus, might act as a confounder in the associations of TT and TE with plaque composition and risk of stroke. Also, upstream hormones, but not downstream hormones (which might act as mediators) in the cascade of sex hormone synthesis may act as confounders (**Supplemental figure 1**). Therefore, for TT we adjusted for SHBG, for TE we adjusted for SHBG and TT, for FAI we adjusted for E and for SHBG we adjusted for TT and E). Model 2, was additionally adjusted for serum total cholesterol (continuous), statin use (yes vs. no), prevalent diabetes mellitus (presence versus absence), systolic blood pressure (continuous), antihypertensive medication (yes vs. no), prevalent cardiovascular disease at time of magnetic resonance imaging (yes vs no), smoking status (yes vs. no) and alcohol consumption (continuous). Cox proportional hazard modelling was used to evaluate whether TE and TT were associated with risk of stroke in subjects who were diagnosed with carotid atherosclerosis based on ultrasonography. Hazard ratios (HR) and 95% confidence intervals (95% CIs) were reported. We used same models as in the analysis for plaque

composition as outcome. However for these analyses, we corrected for age and prevalent cardiovascular disease (excluding stroke) at the time when sex steroid were measured.

There were missing values on one or more covariates. Because the missing values were likely to be missing at random and for avoidance of loss in efficiency, missing values were imputed using a multiple imputation technique (5 imputation sets). Rubin's method was used for the pooled coefficients (odds ratio (OR) or HR) and 95% CIs [22]. A *p*-value of less than 0.05 was considered as statistically significant. A P-value lower than 0.05 was considered as statistically significant, but to account for multiple testing, we adjusted the p-value from 0.05 to 0.025 by applying the Bonferroni correction for the number of exposures studied (N=2). All analyses were done using SPSS statistical software (SPSS, version 21.0; SPSS Inc, Chicago, Illinois).

Sensitivity Analysis

We performed a series of sensitivity analyses using imputed data. First, we repeated the analysis on sex hormones and plaque composition by using tertiles of endogenous sex hormone, and, to study the relations across increasing tertiles, trend tests were computed by entering the categorical variables as continuous variables in the logistic regression models. To account for the specific effects of lipid particles on carotid plaque composition and risk of stroke we substituted total cholesterol with HDL-C, TG, and LDL-C. We created additional models adjusting further for TSH (continuous), glucose and insulin (continuous), CRP (continuous), DHEA (continuous) and maximal carotid plaque thickness (continuous). Number of pregnancies, age of menarche and type of menopause (non-natural vs. natural) are associated with sex hormone levels, therefore, we built another model adjusting further for these factors in women. In both men and women, effect modifications of sex hormones by BMI, age and years since menopause (in women) were tested by adding an interaction term in the final multivariable model in addition to performing stratified analysis. Since there was a time difference between time of sex steroids measurements and MRI assessment, we stratified analysis by median time difference between the two assessments (men: median=9.91years, women: 9.00 years). Also, to account for effects of statin use on plaque composition, we further corrected for statin use frequency and duration, and in stratified analysis we excluded statin users. Also, we performed analysis excluding individuals with prevalent CVD at time of MRI scan. Additionally, we investigated whether

SHBG and FAI were associated with plaque composition and risk of stroke. Also, we restricted the analysis on sex steroids and stroke to the participants who had also information on plaque composition (n=1498). Among these subjects we further investigated the effect of carotid plaque characteristics and risk of stroke. We created additional models adjusting for carotid plaque thickness and for plaque composition (presence of calcification, lipid core and intraplaque hemorrhage in individual models, as well as the three variables in the model). We also restricted our analysis on sex hormones and risk of stroke to subtypes of stroke (ischemic only, and ischemic and unspecified types of stroke). Finally, we performed analysis including overall Rotterdam study population regardless presence of carotid atherosclerosis.

Results

Table 1 summarizes the baseline characteristics of the participants included in the main analysis (the association between sex steroids and carotid plaque composition). A total study population 1480, included 645 (43.58%) postmenopausal women and 835 (56.42%) men. The mean age of women was 65.54 years (SD 7.22), and men 63.79 (SD 6.72). Women were on average 17.29 years (SD 9.11) into menopause, and majority of the women (66.4%) experienced natural menopause. Calcified carotid plaques were present in 1220 (82.4%) participants, 532 women (82.5%) and 688 (82.4 %) men. Lipid core was detected in 651 (43.9%) participants, 238 (36.9%) women, and 413 (49.46%) men. Intraplaque hemorrhage was observed in 521 (35.2 %) individuals, 187 (29.0%) women and 334 (40.0 %) men. Selected characteristics of study participants for the analysis on sex steroids and risk of stroke were similar to the study population for plaque composition as outcome (**Supplemental Table 1**). As expected, considering that 36.9% of women and 25.7 % of men who did not perform MRI had contraindication or physical limitations, we found differences in age (women: mean age 68 vs. 65.5; men: 65.2 vs. 63.7) and incidence rate of stroke (women: 8.1/1000 person-years vs. 4.8/1000 person years; men: 9.3/1000 person-years vs. 4/1000 person-years) among participants who did not attend and who attended MRI visit (**Supplemental table 2**).

Table 1. Characteristics of the Study Population

	Women (n=645)	Men (n=835)
Age at baseline, mean (SD), y	65.54 (±7.22)	63.79 ±6.72
Age at time of MRI scan, mean (SD), y	73.44 (±9.07)	71.60±9.10
Carotid plaques		
Calcium plaques, yes	532 (82.5 %)	688 (82.4 %)
Lipid plaques, yes	238 (36.9 %)	413 (49.5 %)
Plaque hemorrhage, yes	187 (29.0 %)	334 (40.0 %)
BMI, kg/m²	26.85(±4.10)	26.96 (±3.13)
Smoking		
yes	153 (23.7%)	199 (23.8 %)
no	487 (75.5%)	633 (75.8%)
Alcohol intake g/day	8.40 (±13.39)	14.86 (±17.48)
Health indicators		
Systolic BP, mmHg	139.13 (±20.47)	141.90 (±19.84)
Antihypertensive therapy with indication, yes	231 (35.8%)	303 (32.2%)
Total cholesterol, mmol/l	6.0 (5.35-6.70)	5.63 (4.9-6.30)
LDL, mmol/l	3.975 (±0.97)	7.70 (±1.55)
HDL, mmol/l	1.51 (±0.39)	1.23 (±0.32)
Triglycerides, mmol/l	1.36(1.02-1.81)	1.45 (1.08-2.00)
Insulin, pmol/l	67.0 (48.0-98.0)	73.0 (51.0-107.0)
Glucose, mmol/l	5.40 (5.1-5.9)	5.60 (5.20-6.20)
CRP mg/l	1.70 (0.6-3.5)	1.40 (0.56-2.90)
Serum lipid lowering medication, yes	148 (22.9%)	181 (21.7%)
Prevalent diabetes mellitus	57 (8.8%)	117 (14.01%)
Prevalent CVD at baseline	36 (5.6%)	89 (10.7%)
Prevalent CVD at MRI	73 (11.3%)	166 (19.9%)
Hormones		
Estradiol, pmol/l	32.06 (18.35-56.85)	100.10 (76.38-129.30)
Testosterone, nmol/l	0.80 (0.60-1.08)	16.66 (13.09-20.88)
SHBG, nmol/l	59.09 (42.14-79.99)	43.21 (33.87-55.31)
DHEA, nmol/L	9.64 (6.26-14.13)	9.51 (6.31-13.99)
FAI	1.32 (0.92-2.03)	38.72 (31.84-46.61)
TSH mU/l	2.00 (1.28-3.17)	1.82 (1.27-2.60)
Women-specific variables		
Age at menopause, years[1]	48.16 ±5.85	NA
Years since menopause	17.29 (±9.11)	NA
Menopause type, natural menopause[1]	428 (66.4%)	NA
Age at menarche, years	13.56 (±1.69)	NA
Number of pregnancies	2 (1-3)	NA

Values are reported as number (percentage) for categorical variables, and mean ± SD or median (25th-75th quartile) for continuous variables; Age at menopause, age at menarche, type of menopause and years since menopause were not available for all women, the present values are based on 631, 637, 638 and 631 women respectively; NA: not available

Sex steroids and Plaque Composition

No associations were found between sex steroids and calcified carotid plaques in either sex (**Table 2**). After correcting for potential confounding factors, higher levels of TE were associated with higher prevalence of lipid core in carotid plaques in both postmenopausal women (TE levels > 18.35 comparing to ≤18.35 pmol/l , odds ratio (OR) and 95% CI 1.48 [1.02, 2.15]) and men (per 1SD TE increase, OR and 95% CI 1.23 [1.03,1.46]) (**Table 2**). No associations were found between TT and presence of lipid core in either sex (**Table 2**). Higher levels of TE (TE levels > 18.35 comparing to ≤18.35 pmol/l, OR and 95% CI 1.58 [1.03, 2.40]) and lower levels of TT (per 1SD TT increase OR and 95% CI 0.82 [0.68, 0.99] respectively) were also associated with higher prevalence of intraplaque hemorrhage in postmenopausal women irrespective of potential confounding factors but not in men (per 1SD TE increase, OR and 95% CI 0.94 [0.81, 1.09] and per 1SD TT increase OR and 95% CI 0.84 [0.69, 1.01]) (**Table 2**).

Association between sex steroids and risk of stroke

During a median follow-up of 10.0 years, we identified 57 incident cases of stroke in women and 56 new cases of stroke in men. In the multivariable model (Model 2), TE was associated with increased risk of stroke in women (TE levels > 18.35 comparing to ≤18.35 pmol/l, HR and 95% CI 1.98 [1.01, 3.88]), whereas no association was found between TE and risk of stroke in men (Table 3). Also, no associations were found between TT and risk of stroke in either sex (Table 3).

Table 2. Association between sex hormones and carotid plaque composition

WOMEN (n=645)	**Calcified plaques** OR (95% CI)	**Lipid Core** OR (95% CI)	**Intraplaque hemorrhage** OR (95% CI)	**MEN** (n=835)	**Calcified plaques** OR (95% CI)	**Lipid Core** OR (95% CI)	**Intraplaque hemorrhage** OR (95% CI)
Estradiol†				Estradiol			
Model 1	0.97 (0.77; 1.23)	1.40 (0.98; 2.00)	**1.49 (1.01; 2.23)**	Model 1	0.94(0.79;1.09)	**1.22(1.06;1.39)***	0.95(0.83;1.10)
Model 2	1.06 (0.66; 1.71)	**1.48 (1.02; 2.15)**	**1.58 (1.03; 2.40)**	Model 2	0.94(0.77;1.14)	**1.23(1.03;1.46)***	0.94(0.81;1.09)
Testosterone‡				Testosterone ‡			
Model 1	1.08 (0.96; 1.22)	0.99 (0.84; 1.17)	0.87 (0.73; 1.05)	Model 1	1.04 (0.93;1.17)	1.00(0.84;1.19)	0.86 (0.71;1.02)
Model 2	1.03 (0.81; 1.31)	0.99 (0.84; 1.18)	0.82 (0.68; 0.99)	Model 2	1.06(0.84;1.34)	1.00(0.84;1.19)	0.84(0.69;1.01)

Model 1: Age , time difference between hormone measurement and MRI scan, body mass index (BMI), sex hormones for each other (for Estradiol we adjusted for SHBG and TT, for TT we adjusted for SHBG)

Model2: Model 1+ total serum cholesterol (continuous), statin use (yes vs. no), prevalent diabetes mellitus (yes vs. no), systolic blood pressure (continuous), antihypertensive medication (yes vs. no), prevalent CVD at time of MRI, smoking status (yes vs. no) and alcohol consumption (continuous)

† TE levels 18.35 pmol/l comparing to ≤18.35 pmol/l as reference category; ‡Per 1SD increase of serum hormone; *Results are presented as Odds ratio (OR) and 95% Confidence interval (CI 95%); *Statistically significant results are bold (at level $p<0.05$); *Association remains significant at a Bonferroni corrected **P** < .025 for 2 tests.

Table 3. Association between sex steroids and risk of stroke

	WOMEN (n=1023) HR (95% CI) (57 stroke cases)	**P value**	**MEN** (n=1124) HR (95 % CI) (56 stroke cases)	**P value**
[a]Estradiol				
Model 1	**1.99 (1.42-2.79)**	0.04	0.96 (0.83-1.11)	0.77
Model 2	**1.98 (1.01-3.88)**	0.04	0.98 (0.75-1.29)	0.91
[b]Testosterone				
Model 1	1.06 (0.93-1.21)	0.67	1.13 (0.78-1.66)	0.52
Model 2	1.04 (0.79-1.37)	0.77	1.10 (0.75 -1.62)	0.61

Model 1: Age, body mass index (BMI), sex hormones for each other (for TT we adjusted for SHBG, for E we adjusted for SHBG and TT)

Model 2: Model 1+ total serum cholesterol (continuous), statin use (yes vs. no), prevalent diabetes mellitus (yes vs. no), systolic blood pressure (continuous), antihypertensive medication (yes vs. no), prevalent CVD before the date of hormone measurement, smoking status (yes vs. no) and alcohol consumption (continuous)

[a] In women TE levels > 18.35 comparing to ≤18.35 pmol/l, in men per 1SD estradiol increase; [b] per 1SD testosterone increase; Results are presented as hazard ratio (HR) and 95% confidence interval (CI 95%); Statistically significant results are bold; Analysis done in subjects diagnosed with carotid atherosclerosis using ultrasonography

Sensitivity analysis

The association between TE and lipid core presence in men remained significant after we applied the Bonferroni correction ($p<0.025$). In sensitivity analyses, using tertiles of sex hormones showed same results as the main analysis (**Supplemental figure 1a, 1b, 1c**). Also, substituting total cholesterol for other blood lipids, adjusting further for number of pregnancies, age of menarche and type of menopause, statin frequency use, as well as for glucose, insulin, TSH, CRP, DHEA, maximal carotid plaque thickness and exclusion of individuals with prevalent CVD at the time of MRI scan did not materially affect any of the associations (**Supplemental table 3a, 3b and 4**). Also, in the stratification analysis, no significant interactions were found for sex steroids with BMI, age, years since menopause or with carotid intima media thickness (**Supplemental table 3a, 3b and 4**). Furthermore, the

results on sex steroids and plaque composition did not materially changed when the analysis were stratified by median time difference in between sex steroids and MRI measurements or when we further corrected for statin use frequency and duration (**Supplemental table 3a, 3b**). After statin users were excluded from the analysis, the association between TE and lipid core presence and intraplaque hemorrhage was not anymore significant in women, but the magnitude of the effect did not materially change (**Supplemental table 3a,3b**). Also, no association was observed between SHBG and FAI with plaque composition and risk of stroke in either sex (**Supplemental table 5, 6**). The results on sex steroids and risk of stroke did not materially change when restricting the analysis to subjects with available data on plaque composition (**Supplemental table 4**). In these subjects carotid plaque thickness and characteristics of carotid plaques did not affect the direction and magnitude of the association between TE and TT and risk of stroke (**Supplemental table 7**). Exclusion of hemorrhagic stroke cases, and further of non-specified types, did not materially affect the associations of sex hormones with risk of stroke. (**Supplemental table 4**). Inclusion of all eligible subjects form the Rotterdam study regardless presence of carotid atherosclerosis did not affect the direction and magnitude of the association between TE and risk of stroke in women and men (**Supplemental table 8**).

Discussion

In this population-based study among postmenopausal women and elderly men, we show sex differences in the association between estradiol, carotid plaque composition and risk of stroke. While TE was associated with higher prevalence of lipid core in both men and women, TE was associated with higher odds of having intraplaque hemorrhage and risk of stroke in women but not in men. No consistent association was observed between testosterone and plaque composition and risk of stroke in either sex (**Illustration 1**).

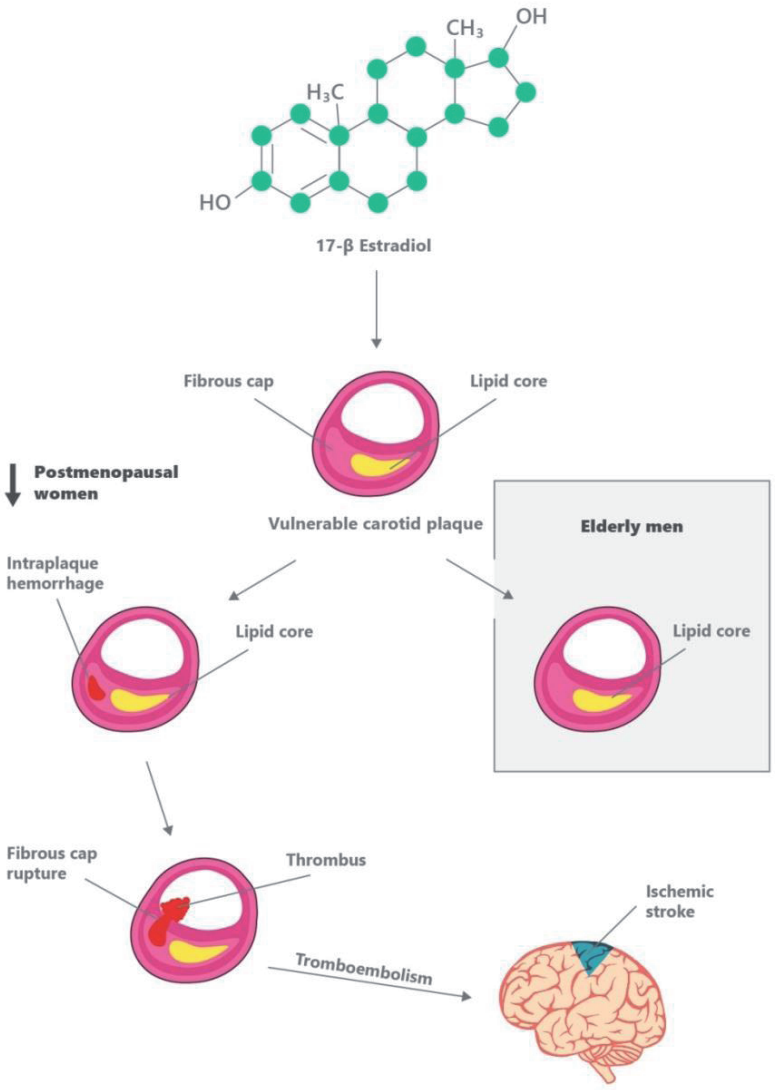

Illustration 1. Higher levels of endogenous estradiol are associated with vulnerable carotid plaque composition (lipid core presence and intraplaque hemorrhage) in postmenopausal women. Destabilization of atherosclerotic plaque may lead to plaque rupture, thrombus formation on the disrupted plaque surface and therefore to thrombus embolization into the distal vessels causing ischemic stroke. In our study we show that higher estradiol is associated with increased risk of stroke in postmenopausal women. In elderly men, higher estradiol levels was associated with presence of lipid core, however, we did not observe any association between estradiol and intraplaque hemorrhage and risk of stroke in men.

Estradiol, plaque composition and risk of stroke

Although is difficult to define normal hormonal range in elderly population, E levels <184 pmol/l are considered to be normal in male population [23], while in postmenopausal women, normal serum E range from 0 to 110.13 pmol/l [24]. In our sample only 2.8 % of men and 4.2 % of women had pathological values of serum E. Therefore, both, men and women had approximately the same percentage of pathological estradiol levels. These data indicate that the sex differences we found on TE, plaque composition and risk of stroke may not be due to differences in estradiol levels between men and women, but due to sex differences in the physiological actions of estradiol.

Our results on vulnerable plaque composition and increased risk of stroke with increasing levels of estradiol in women far from menopause (on average 26 years into menopause) are in line with the "timing hypothesis", which theorizes that estradiol has harmful vascular effects in elderly women in contrast to neutral or beneficial effects in younger women [25,26].

Findings from animal studies based on monkey models in premenopause show estradiol to prevent fatty streak deposition and progression of atherosclerotic plaque [27]. In contrast, in monkey models of female subjects 2 years into menopause (comparable to six postmenopausal years for women), no beneficial effect of estradiol was observed on the progression of coronary artery plaque [28]. Similarly, studies among premenopausal women or in women in menopausal transition show that women using hormone replacement therapy (HRT) have a lower incidence of carotid atherosclerotic lesions [29], and slower progression of CIMT [30], while studies in postmenopausal women report no or deleterious effects [31,32]. Also, TE has beneficial effects in the vascular system in premenopausal women, whereas in postmenopausal women, large clinical trials have reported use of exogenous estradiol, which increases circulating estrogen levels, to increase risk of stroke [33,34]. Women's Health Initiative (WHI) clinical trials reported harmful effect of HRT on ischemic stroke risk in women older than 50 years of age [35,36]. Observational data from the Nurses' Health Study confirmed those findings [37].

Although, emerging evidence supports the "timing hypothesis", the pathways still remain unclear. It is early to say if switch from protective to harmful estradiol effect is due to changes in ER signaling [11] or it is a consequence of age-related hyper-inflammatory state [38]. Some experimental studies suggest molecular mechanisms that may contribute to hyper-

inflammatory state and possibly promote pro-inflammatory effect of estrogens in the aging vasculature [39]. Also, the direct anti-\atherogenic effect of estrogen are present, absent, or reversed, depending on the state of the arterial endothelium. Coronary artery fatty streaks and small plaques are common in women at the time of perimenopausal transition, while advanced atherosclerotic plaques are common in aging women and in women 5–15 years after menopause. Endothelium changes related to atherosclerosis progression in elderly women might be another explanation why hormone replacement therapy initiated at the complicated plaque stage (beyond about 60 years of age) can have either no beneficial effects or deleterious effects [28].

In men, TE was associated with presence of lipid core, however, no association was observed with calcified carotid plaque, intraplaque hemorrhage or risk of stroke. Muller et al, demonstrated that higher E was associated with progression of CIMT of the common carotid artery [40]. Study done in men with DM II showed inverse association between E and carotid atherosclerosis [41], while another study did not find any correlation between E and atherosclerosis in men [42]. Evidences on association between E and risk of stroke in men are limited. In the Honolulu-Asia Aging Study, elevated serum E was cross-sectionally associated with frequency of lacunar infarcts found on MRI [43]. Study done in elderly men demonstrated positive correlation between E and stroke, however, they did not adjust for other sex hormones [44]. In line with our findings, two case-control studies did not find any correlation between E levels and stroke [45,46].

Total testosterone and plaque composition and risk of stroke

In the present study, no association was observed between TT and FAI, plaque composition and risk of stroke in either sex.

Animal models of atherosclerosis report contradictory results in both men and women. Few studies show that T has no effect [47] or beneficial effect on atherosclerosis in male animals [48], while in females exogenous androgen treatment may be atherogenic [49]. Similarly, while cross-sectional studies in men and women have demonstrated inverse relation between T, CIMT and carotid plaque [50], longitudinal studies and clinical trials show no impact of T to CIMT progression, coronary artery calcification and plaque area [51]. Limited and conflicting evidence exist also on T and stroke incidence. In a prospective observational study of 3443 elderly men with a median follow-up of 3.5 years, men with low-normal T levels had

increased risk of incident stroke and TIA combined [52]. In contrast, T therapy use in cohort of veterans with significant medical comorbidities was associated with increased risk ischemic stroke [53].

Strengths and limitations

To the best of our knowledge, this is the first and most comprehensive study to examine the associations of estradiol and testosterone and carotid plaque composition in sample of postmenopausal women and men. Our sample was drawn from middle-aged and elderly subjects from the general population, which is one of the major strengths of our study. Total testosterone was measured using chromatography-tandem mass spectrometry, at the moment considered to be the gold standard method. High-resolution protocol based MRI sequences are used to evaluate carotid plaque composition. Nevertheless, limitations of our study need to be discussed. First, a cross-sectional study design does not allow us to address the temporality of the observed associations, hence, we cannot draw any conclusions with regard to the causality of the observations. Second, in the RS there are no measures of bioavailable estradiol, which could have strengthened our results. Also, TE was measured using an immunoassay with a detection limit of 18.35 pmol/L, which is considered suboptimal particularly in postmenopausal women. Therefore, the analysis were done by categorizing the values into 0 if estradiol levels were ≤18.35 pmol/l and 1 if the values were >18.35 pmol/l. Categorization of a continuous variable introduces loss of information and power. Third, we found difference between participants who attend MRI visit and who did not attend. However, majority of subjects who did not perform MRI had contraindication or physical limitations (38.73 %), therefore they might have been sicker comparing to the participants who were eligible to attend MRI. Also, it has been shown that using a selected source population for a cohort study usually leads to bias towards the null [54]. Fourth, as our sample is population based we chose not to administer gadolinium contrast to the participants, although lipid core is more easily detected with a contrast-enhanced MRI [55]. However, in validation studies non-contrast-enhanced MRI sequences have shown a good accuracy and reproducibility [56]. Last, MRI assessment was measured only once and that measurement was taken after the exposure was measured, and therefore time difference between sex steroid measurements and MRI assessment exists. However, we did not find any difference in the results when main analysis was stratified by

the time difference between the two assessments. Also, this study was carried out in middle-aged and elderly patients, and in older age hormone levels are more stable over time [57]. Furthermore, the results on sex steroids and risk of stroke were on same direction as the results expected from sex steroids and plaque composition.

In summary, our findings suggest that endogenous estradiol may lead to the development of vulnerable carotid plaque composition and increase risk of stroke in middle-aged elderly women. Hence, HRT among postmenopausal women should be taken with caution, especially among those women who already have diagnosis of carotid atherosclerosis. Further, since the characteristics of carotid plaque did not explain the association between estradiol and risk of stroke, estradiol might help to predict ischemic stroke in women with subclinical atherosclerosis. However, further large-longitudinal studies are needed to confirm our findings and to investigate whether estradiol levels might be used in stroke prediction models.

Supplement available online at:

https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.117.311681

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CHAPTER 2.4

Serum testosterone and sex-specific risk of type 2 diabetes

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Manuscript under review

ABSTRACT

BACKGROUND: Sex differences are critical in the epidemiology and pathophysiology of metabolic disease, with an increased incidence of type 2 diabetes mellitus (T2DM) and cardiovascular disease in men

OBJECTIVE: Small scale evidence suggests that androgens have a sexually dimorphic impact on metabolic dysfunction. However, the sex-specific link between circulating androgens and risk of type 2 diabetes mellitus (T2DM) has not yet been examined in a large scale, longitudinal cohort, a task we undertook in this study.

METHODS: A retrospective cohort study in a UK primary care database. We included men and women with available measurement results for serum testosterone and sex hormone-binding globulin (SHBG). We categorized serum concentrations according to clinically relevant cut-off points and calculated crude and adjusted T2DM Incidence Rate Ratios (IRRs and aIRRs).

RESULTS: Serum testosterone concentrations were available in 70,723 men and 81,951 women; serum SHBG was available in 15,907 men and 42,042 women. In comparison to a reference cohort with serum testosterone ≥20nmol/l, men with lower serum testosterone had a significantly increased risk of T2DM, with the highest risk in those with serum testosterone <7nmol/l (aIRR 2.68, 95% CI 2.32-3.10, p<0.001). In women, the risk of T2DM started to increase significantly when serum testosterone concentrations exceeded 1.5nmol/l, with the highest risk in women with serum testosterone ≥3.5nmol/l (aIRR 2.0, 95% CI 1.58-2.55, p<0.001). The risk of T2DM increased in both men and women with serum SHBG <40nmol/L.

CONCLUSIONS: In this large longitudinal study, we found a sexually dimorphic association between serum testosterone and risk of incident T2DM. Androgen deficiency and excess should be considered important risk factors for diabetes in men and women, respectively.

INTRODUCTION

Sex differences are critical in the epidemiology and pathophysiology of metabolic disease, with an increased incidence of type 2 diabetes mellitus (T2DM) and cardiovascular disease in men [1]. Sex hormones such as androgens may mediate these differences, but the association between androgens and metabolic dysfunction is complex and sex-specific [2]. Androgen excess has recently been identified as an independent risk factor for non-alcoholic fatty liver disease (NAFLD) in women [3], and promotes lipid accumulation and lipotoxicity in female adipose tissue [4]. Female-to-male gender reassignment patients undergoing androgen therapy develop dyslipidemia and abnormal body composition [5,6]. Mirroring this, the adverse metabolic phenotype of male androgen deficiency bears a striking similarity to that of female androgen excess; lower testosterone levels in men are associated with impaired glucose homeostasis, hepatic steatosis and coronary artery disease [7-9]. A number of meta-analyses support a sex-specific relationship between androgens and the risk of metabolic dysfunction, and suggest that low circulating sex hormone-binding globulin (SHBG) concentrations may be metabolically harmful in both sexes [9,10].

Delineating an independent role for androgens in the pathogenesis of T2DM is confounded by changes in body composition, body mass index and lean mass observed in disorders of androgen excess and deficiency [11]. Against the background of a global epidemic of T2DM [12], there is an urgent health need to understand the sexually dimorphic role played by androgens in the pathogenesis of hyperglycemia. The shared constellation of risk factors observed in women with androgen excess and men with androgen deficiency suggests that circulating androgen concentrations common to both disorders may be metabolically disadvantageous [2]. To our knowledge, however, no large longitudinal studies have specifically examined the association between circulating androgen exposure *per se* and risk of T2DM in a sex-specific context.

The aim of this study was to investigate the independent sex-specific association between serum testosterone concentrations and the risk of hyperglycemia in men and women by undertaking a longitudinal retrospective cohort study in a large and diverse UK population base.

METHODS

Database

A large primary care database in the UK with contribution from over 700 general practices (14 million patients) was utilized for this study. Data from practices that use VISION Electronic Medical Record (EMR) are gathered, anonymized and released for research purpose [13]. The resulting database, The Health Improvement Network (THIN) database holds data on demographic characteristics, clinical diagnosis, physical measurement, laboratory results and prescriptions. The THIN database is broadly representative of the UK population structure [14] and has been utilized for numerous epidemiological studies, including studies on diabetes [15,16] and sex hormones [3,17,]

Testosterone and Sex Hormone Binding Globulin (SHBG) measurements

Men or women over the age of 16 who had a measurement of the serum concentration of testosterone or SHBG between 1st of Jan 2000 and 15th of May 2016 were eligible to take part in the study. Common clinical indications for these measurements include suspected polycystic ovary syndrome (PCOS) in women, infertility investigations in both sexes and erectile dysfunction in men [19,20] . Where multiple measurements were available in one individual, the first measurement was utilized. Patients with the outcome of interest (diabetes) preceding the index date were excluded from the study.

Exposure categories

Measurements were categorized by applying clinically relevant cut-off points for serum concentrations (nmol/L) to observe any gradient change in risk [3]. For women, testosterone was grouped as <1.0nmol/L (reference group), 1.0-1.49, 1.5- 1.99, 2.0-2.49, 2.5-2.99, 3.0-3.49 and >3.5 nmol/L. For men, the groups were as follows: <7, 7-9.9, 10.0-14.9, 15-19.9, >20.0nmol/L (reference group) nmol/L. For both sexes, SHBG was categorized as >60.0nmol/L (reference group), 50.0-59.9, 40.0-49.9, 30.0-39.9, 20.0-29.9 and < 20 nmol/L.

Follow-up period

The date of measurement of testosterone or SHBG served as the index date. Each participant was followed-up from the index date until the exit date. Exit date was defined as the earliest of the following dates: outcome (diagnosis of T2DM), study end, death or the

date they left the general practice or the general practice stopped contributing to the database.

Outcome and covariates

A clinical diagnosis of T2DM by the general practitioner was the outcome of interest. In the UK, the Quality Outcome Framework (QOF) in general practices ensures high quality data on important comorbidities such as cardiovascular disease, hypertension and diabetes [21] . Within the database, diagnostic codes for T2DM were identified based on Read codes, a hierarchical coding system to record signs, symptoms, procedures and diagnosis in primary care [3]. Covariates that are independent predictors of T2DM other than the exposure of interest were selected on the basis of biological plausibility and previous literature [22]. These included age, body mass index (BMI), Townsend deprivation score and smoking status.

Statistical analysis

Baseline data of each category in the serum testosterone and SHBG cohorts was reported separately for men and women as mean (standard deviation) or median (interquartile range) as appropriate for continuous variables and as proportions for categorical variables. Crude Incidence Rate Ratio (IRR) and adjusted Incidence Rate Ratio (aIRR) were calculated by applying Poisson regression offsetting for the person years of follow-up. Covariates adjusted for in the model were age, BMI, Townsend quintiles and smoking status. In women, an additional model included polycystic ovary syndrome (PCOS) as a covariate to explore if the risk of T2DM in women was independent of a diagnosis of PCOS.

Where missing data existed (BMI, Townsend or smoking), we created a separate category so that all available data is utilized in the analysis. BMI was categorized as per WHO recommendation into <25.0kg/m^2, 25-29.0kg/m^2 and >30kg/m^2. All analyses were performed in STATA 14.0.

Subgroup analysis

In women, we performed stratified analysis by age (<50 years and 50 years and above) to explore if the association was similar before and after the average age of menopause. A similar age-stratified analysis was also carried out in men.

Ethical Approval:

This study used routinely collected, anonymised primary care data. Patients were not involved in the study, and therefore no consent was required. Research using THIN data was approved by the NHS South-East Multicentre Research Ethics Committee in 2003, with the condition that studies undergo independent scientific review [23]. Approval for this analysis was obtained from the Scientific Review Committee for the use of THIN data in January 2018 (SRC reference number 17THIN106).

RESULTS

Characteristics of the cohorts with serum testosterone and SHBG measurements

A total of 152,674 participants in the cohort with available serum testosterone measurement results (testosterone cohort; 70,723 men and 81,951 women) and a total of 57,949 participants (15,907 men and 42,042 women) in the SHBG cohort, both derived from the THIN database, met the inclusion criteria and were included in the current study. The mean age for men was 51.6 (SD 14.8) years in the testosterone cohort and 51.7 (SD 16.0) years in the SHBG cohort. For women, mean age was 33.2 (SD 10.9) years in the testosterone cohort and 32.1 (SD 10.6) years in in the SHBG cohort. In total, 40,577 (57.4%) men in the testosterone cohort and 9,795 (61.6%) men in the SHBG cohort were overweight or obese (BMI ≥25kg/m^2). Among women, 36,663 (44.7 %) were obese or overweight in the testosterone cohort and 19,273 (45.8%) in the SHBG cohort. Approximately 21% of men and 22% of women were smokers across both testosterone and SHBG cohorts **(Table 1)**. Biochemical evidence of male androgen deficiency (serum testosterone <7nmol/L) was observed in 5,862 men (8.3%). Biochemical evidence of female androgen excess (serum testosterone >2nmol/L) was observed in 20,627 women (25.2%); of those, 2,543 women (3.1%) had severe androgen excess (serum testosterone ≥3.5 nmol/L). Serum SHBG concentrations <20nmol/L were observed in 2,517 (15.8%) men and 3,733 (8.9 %) women **(Suppl. Tables 1-4)**.

Table 1: Baseline characteristics of the testosterone and SHBG cohorts stratified by sex

Characteristics	Men		Women	
	Serum testosterone	Serum SHBG	Serum testosterone	Serum SHBG
Population n (%)	70,723 (46.32)	15,907 (27.45)	81,951 (53.68)	42,042 (72.55)
Age (years) mean (SD)	51.63(14.78)	51.73 (16.04)	33.23 (10.87)	32.11 (10.60)
Townsend index n (%)				
1 (least deprived)	20,048 (28.35)	3,997 (25.13)	18,478 (22.55)	8,754 (20.82)
2	15,515 (21.94)	3,427 (21.54)	15,695 (19.15)	7,688 (18.29)
3	13,729 (19.41)	3,033 (19.07)	17,063 (20.82)	8,684 (20.66)
4	11,033 (15.60)	2,565 (16.12)	15,308 (18.68)	8,157 (19.40)
5 (most deprived)	7,402 (10.47)	2,186 (13.74)	10,281 (12.55)	5,957 (14.17)
Missing or implausible data	2,996 (4.24)	699 (4.39)	5,126 (6.25)	2,802 (6.66)
BMI (kg/m^2) categorised n (%)				
<25	19,235 (27.20)	3,995 (25.11)	32,551 (39.72)	15,978 (38.00)
25-30	26,040 (36.82)	5,817 (36.57)	16,861 (20.57)	8,446 (20.09)
>30	14,537 (20.55)	3,978 (25.01)	19,802 (24.16)	10,827 (25.75)
Missing or implausible data	10,911 (15.43)	2,117 (13.31)	12,737 (15.54)	6,791 (16.15)
Smoking status n (%)				
Non-smokers	53,441 (75.56)	12,264 (77.10)	61,320 (74.83)	31,561 (75.07)
Smokers	15,376 (21.74)	3,377 (21.23)	18,049 (22.02)	9,315 (22.16)
Missing or implausible data	1,906 (2.70)	266 (1.67)	2,582 (3.15)	1,166 (2.77)
Follow-up in years median (IQR)	3.31 (1.46 – 6.07)	2.78 (1.25 – 4.91)	3.16 (1.34 – 6.15)	2.78 (1.20 – 5.39)

testosterone levels <7nmol/L, compared to the reference category of 20nmol/L (aIRR 2.68,

Association between sex hormones and T2D risk in men

Among 70,732 men with serum testosterone measurements, 3,163 developed diabetes during the follow-up period. After adjusting for age, BMI, Townsend index and smoking status, aIRR for T2DM in men increased with decreasing categories of serum testosterone concentrations, most notably a 168% higher risk of developing T2DM in those with testosterone levels <7nmol/L, compared to the reference category of 20nmol/L (aIRR 2.68,

95% CI 2.32-3.10, p<0.001) (Table 2). However, the risk of T2DM increased even within the normal male testosterone range (15-19.99 nmol/L, aIRR 1.28, 95% CI 1.12-1.46, p<0.001; 10-14.99 nmol/L, aIRR 1.88, 95% CI 1.67-2.13, p<0.001, **Table 2** & **Figure 1a**).

In the SHBG cohort, among 15,907 men studied, there were 708 cases of incident diabetes during the follow-up period. After adjusting for age, BMI, Townsend index and smoking status, the risk of T2DM increased in men with SHBG levels <40nmol/L; aIRR of incident T2DM increased across categories of decreasing SHBG concentrations as compared to the reference category (≥60nmol/L) and the risk was more than 5-fold higher in the group with SHBG <20nmol/L (aIRR 5.74, 95% CI 3.72-8.87, **Table 2 & Figure 1c**).

In addition to these findings, as expected, increasing age, overweight/obesity, smoking and higher social deprivation conferred a risk for T2DM **(Suppl. Table 5 and 6).**

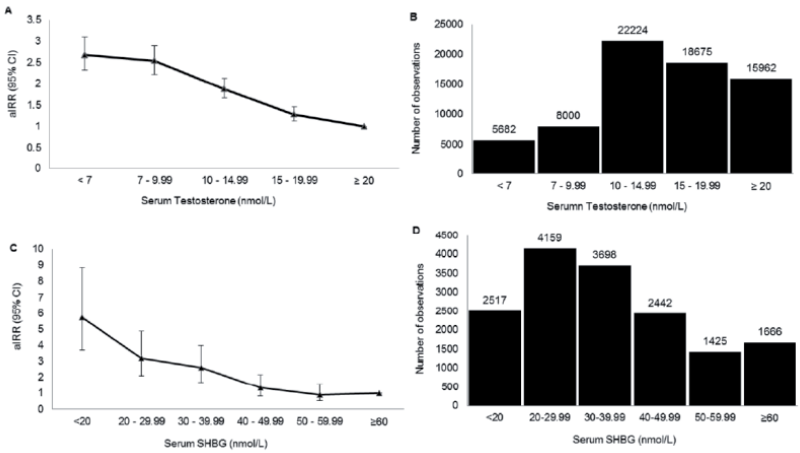

Figure 1: Risk of incident type 2 diabetes (T2DM) according to serum testosterone and sex hormone-binding globulin (SHBG) concentration categories in men. (a) Adjusted Incidence Rate Ratios (aIRRs) for diabetes in 70,723 men with serum testosterone measurements (b) Distribution of 70,723 men across each quintile of serum testosterone concentration. (c) aIRRs for serum SHBG concentrations for incident diabetes in 15,907 men. (d) Distribution of 15,907 men across each category of serum SHBG concentration.

Association between sex hormones and T2D risk in women

Among 81,951 women with serum testosterone measurements, 1,283 developed diabetes during the follow-up period. After adjusting for age, BMI, Townsend index and smoking status, T2DM aIRR tended to be higher with increasing serum testosterone levels. The risk increased significantly for serum testosterone levels >1.5 nmol/L, as compared to reference category (<1nmol/L), and continued to increase across each category of serum testosterone concentrations thereafter, with a two-fold increase in risk observed in women with serum testosterone ≥3.5 nmol/L (aIRR 2.0, 95% CI 1.58-2.55, p<0.001, **Table 2 & Figure 2a**). Further adjustment for a diagnosis of PCOS did not substantially change results (aIRR in subgroup of women with testosterone levels >3.5 nmol/L = 1.92, 95% CI 1.51-2.45, p<0.001).

In the SHBG cohort, among 42,024 women studied, there were 597 cases of incident diabetes during the follow-up period. The risk of incident T2DM increased with each category of decreasing SHBG concentration. Women with serum SHBG concentrations <20nmol/L had a 9-fold higher risk of developing T2DM compared to the reference category of ≥60nmol/L (aIRR 9.24, 95% CI 6.62-129, p<0.001), after adjustment for age, BMI, Townsend index and smoking status (**Table 2 & Figure 2c**). Additional adjustment for a diagnosis of PCOS did not alter the risk of T2DM (aIRR 9.14, 95% CI 6.54-12.77, p<0.001).

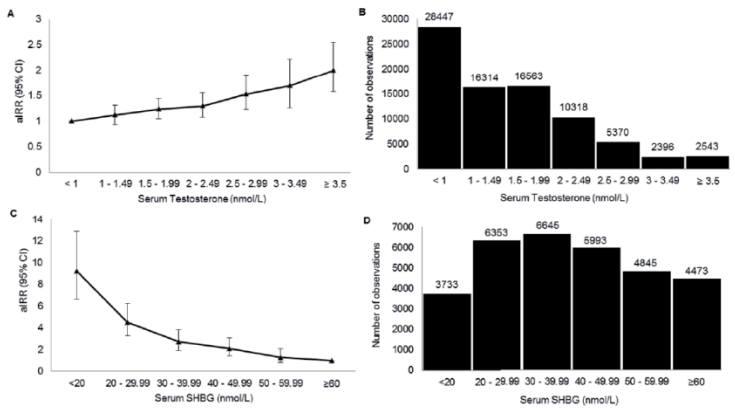

Figure 2: Risk of incident type 2 diabetes (T2DM) according to serum testosterone and sex hormone-binding globulin (SHBG) concentrations in women. (a) Adjusted Incidence Rate Ratios (aIRRs) for incident diabetes in 81,951 women with serum testosterone measurements. (b) Distribution of 81,951

women across each category of serum testosterone concentration. (c) aIRRs for serum SHBG concentrations for incident diabetes in 42,042 women with serum SHBG measurements. (d) Distribution of 42,042 women across each category of serum SHBG concentration.

Subgroup analyses

Subgroup analysis stratified by age (<50 and ≥50 years) did not alter the observed associations. In both age groups, a gradient increase in risk of T2DM was observed with increasing testosterone concentrations in women and decreasing testosterone concentrations in men **(Suppl. Fig 1 and Suppl. Tables 9-12).** Increased aIRRs for T2DM were noted with lower concentrations of SHBG in both age groups in men and women **(Suppl. Fig 2 and Suppl. table 13-16)**.

Table 2. Risk of incident T2DM according to the category of serum testosterone and SHBG at baseline

	Unadjusted IRR (95% CI)	P-value	Adjusted* IRR (95% CI)	P-value
MEN				
Serum testosterone concentration categories (nmol/L)				
< 7	4.43 (3.84 - 5.10)	< 0.001	2.68 (2.32 - 3.10)	< 0.001
7 - 9.99	4.03 (3.54 - 4.60)	< 0.001	2.54 (2.22 - 2.90)	< 0.001
10 - 14.99	2.58 (2.29 - 2.91)	< 0.001	1.88 (1.67 - 2.13)	< 0.001
15 - 19.99	1.51 (1.32 - 1.72)	< 0.001	1.28 (1.12 - 1.46)	< 0.001
≥ 20	1 (-)	-	1 (-)	-
Serum SHBG concentration categories (nmol/L)				
<20	4.00 (2.64 - 6.06)	< 0.001	5.74 (3.72 - 8.87)	< 0.001
20 - 29.99		< 0.001	3.20 (2.09 - 4.87)	< 0.001
30 - 39.99	2.45 (1.61 - 3.73)	< 0.001	2.61 (1.71 - 3.99)	< 0.001
40 - 49.99	1.27 (0.80 - 2.04)	0.309	1.36 (0.85 - 2.17)	0.207
50 - 59.99	0.97 (0.56 - 1.70)	0.925	0.91 (0.52 - 1.60)	0.748
≥60	1 (-)	-	1 (-)	-
WOMEN				
Serum testosterone concentration categories (nmol/L)				
< 1	1 (-)	-	1 (-)	-
1.0 - 1.49	1.00 (0.84 - 1.18)	0.980	1.12 (0.94 - 1.32)	0.203
1.5 - 1.99	1.07 (0.92 - 1.26)	0.384	1.23 (1.05 - 1.45)	0.011
2.0 - 2.49	1.14 (0.95 - 1.36)	0.156	1.30 (1.08 - 1.56)	0.005
2.5 - 2.99	1.26 (1.02 - 1.57)	0.032	1.53 (1.23 - 1.91)	0.000
3.0 - 3.49	1.50 (1.14 - 1.97)	0.004	1.69 (1.27 - 2.23)	0.000
≥ 3.5	2.03 (1.60 - 2.57)	0.000	2.00 (1.58 - 2.55)	0.000
Serum SHBG concentration categories (nmol/L)				
<20	13.54 (9.86 - 18.58)	< 0.001	9.24 (6.62 - 12.9)	< 0.001
20 - 29.99	7.21 (5.24 - 9.93)	< 0.001	4.49 (3.23 - 6.25)	< 0.001
30 - 39.99	4.12 (2.93 - 5.81)	< 0.001	2.71 (1.91 - 3.84)	< 0.001
40 - 49.99	2.65 (1.81 - 3.89)	< 0.001	2.08 (1.41 - 3.05)	< 0.001
50 - 59.99	1.54 (0.95 - 2.47)	0.078	1.29 (0.8 - 2.08)	0.301
≥60	1 (-)	-	1 (-)	-

* Adjusted for age, BMI, Townsend index, smoking status; Abbreviations: IRR, incidence rate ratio; SHBG, sex hormone-binding globulin; T2DM, type 2 diabetes mellitus

DISCUSSION

In this large longitudinal cohort study, we have demonstrated that androgens confer an independent sex-specific effect on the risk of incident T2DM. To our knowledge, this is the largest study, and the first longitudinal analysis, to address the impact of serum testosterone on risk of development of T2DM in both men and women. In the female cohort, aIRRs for T2DM increased significantly once serum testosterone concentrations increased above 1.5nmo/L; even those with circulating testosterone levels between 1.5 and 1.99nmol/L, conventionally considered within the normal physiological range for women, already had a 23% increased risk of T2DM compared to the reference group. Perhaps even more surprisingly, once male serum testosterone concentrations dropped below 20nmol/L, the risk of T2DM began to increase; men with circulating concentrations between 15 and 19.99nmol/L, i.e. within the normal physiological male range, had a 28% increased risk of T2DM over the study period. Reduced SHBG concentrations in both sexes, but particularly in women, also potently increased the risk of T2DM. Whilst the increased T2DM risk did not manifest in men until serum SHBG levels dropped below 30nmol/L, women with SHBG levels below 40nmol/L had a two-fold increased adjusted risk. This finding is in agreement with observations from previous studies, which demonstrated a stronger inverse association between SHBG levels and risk of T2DM in women compared to men [10,24].

A systematic review and meta-analysis, which included a total of 3825 men and 4795 women in 36 cross-sectional studies, as well as 368 cases from 7 prospective study populations, previously demonstrated that increased serum testosterone was associated with a 60% higher risk of T2DM in women; higher testosterone levels in men reduced the risk of T2DM by 42% [10]. In the same study, serum testosterone concentration were significantly higher in women with T2DM and significantly lower in men with T2DM; women in the highest quartile of bioavailable testosterone levels had a three-fold higher risk of T2DM compared to those in the other three quartiles. Goodman-Gruen *et al* also observed sex differences in the association between serum androgens and glucose tolerance status in an older community cohort of 775 men and 633 women above the age of 55 [25]. Men with impaired fasting glucose, impaired glucose tolerance and T2DM had significantly lower levels of serum testosterone, while women with T2DM had significantly higher levels of

bioavailable testosterone, independent of total body fat, fat distribution, physical activity and smoking. However, our study is the only longitudinal retrospective analysis to comprehensively evaluate these associations.

A number of key insights into the role of androgen excess in the development of metabolic dysfunction is provided by studies in women with polycystic ovary syndrome (PCOS), a disorder affecting up to 10% of the female population and primarily defined by the presence of hyperandrogenism and ovulatory dysfunction [26]. We have recently demonstrated that lean women with PCOS have an almost two-fold increased risk of NAFLD, a hepatic manifestation of metabolic dysfunction, and that androgen excess is an independent mediator of this increased risk [3]. In that study, we found that the hazard risk of NAFLD was 2.4-fold higher in women with serum testosterone >3nmol/L, independent of other risk factors, such as age, BMI, deprivation score and glycemic status, compared to the reference cohort of women with serum testosterone <1nmol/L. Androgen-mediated adipose tissue lipotoxicity may contribute to this increase in NAFLD risk [4,27]. PCOS women are at significantly increased risk of impaired glucose tolerance and T2DM at a young age, irrespective of body weight [28].A recent large Danish population register study concluded that the risk of T2DM was four-fold higher for women with PCOS, and diagnosed 4 years earlier, compared to women in the background population [29]. Androgen excess is likely to play a pathophysiological role in this process. Circulating androgen burden, with contributions from both the classic and the recently characterized 11-oxygenated pathway [30], is predictive of insulin resistance in PCOS women, and women with concurrent increases in both testosterone and the androgen precursor androstenedione appear to have the highest risk of dysglycemia [31]. The increased T2DM risk in the context of female androgen excess may also be mediated by adverse androgen effects on adipocyte lipid storage capacity; in addition, there is also convincing mechanistic evidence in both humans and rodents that androgen exposure in females induces direct deleterious effects on hepatic and adipose insulin sensitivity, as well as systemic glucose tolerance [32,33].

Male androgen deficiency occurs as a consequence of primary testicular pathology, hypothalamic-pituitary disorders, obesity or as part of the ageing process in older men [34,35]. Additionally, iatrogenic hypogonadism due to androgen deprivation therapy is observed in

men with prostate cancer [36]. Whilst the relationship between obesity and hypogonadism in men is complex and bidirectional [37], data from male cohorts treated with short term androgen deprivation therapy confirm that hypogonadism directly induces metabolically deleterious changes in body composition, with visceral fat mass expansion and loss of skeletal muscle bulk [38].

The results of this study are particularly surprising, given that an increased risk of T2DM was apparent at circulating testosterone concentrations considered physiologically normal, but below the reference group of 20nmol/L, independent of age, obesity and other potential confounding factors. However, our results do not imply endorsement of routine testosterone replacement to restore circulating testosterone levels above 20nmol/L in otherwise healthy men. Studies investigating a potential beneficial impact of androgen replacement therapy on cardiovascular and metabolic outcomes in men with testosterone concentrations in the low or low-normal range have shown at best conflicting results, and raise the possibility, in some instances, of overtly harmful effects [39-42]. The bulk of randomized clinical trials on the impact of androgen replacement therapy are insufficiently powered for rare cardiovascular outcomes, and large scale observational studies may be the only realistic avenue to improve our understanding of the relationship between hypogonadism and T2DM in men.

Potential mechanisms underlying the sexually dimorphic association between circulating androgens and risk of T2DM reported in this study merit some discussion. We and others have previously hypothesized the existence of a physiological window of circulating testosterone levels, outside of which disturbances in body composition and metabolic homeostasis are increased [2,43]. Serum testosterone levels found in female androgen excess frequently overlap with those in male androgen deficiency, and may predispose to visceral fat accumulation and an adverse metabolic phenotype; it is intriguing that circulating androgen concentrations in women correlate positively with fat mass in women but negatively in men [11]. Male androgen deficiency also adversely impacts on lean mass, particularly skeletal muscle bulk [44]; as skeletal muscle is the major site of glucose uptake, and thereby a key regulator of systemic glucose homeostasis [45], reduced muscle mass and function in the context of androgen deficiency is likely to predispose to hyperglycemia.

Disturbances in circulating concentrations are also likely to impact on tissue-specific androgen exposure. We have previously demonstrated that enhanced intra-adipose androgen activation in women with PCOS is responsible for local adipocyte hypertrophy and lipotoxicity, and may impact on mitochondrial oxidation of free fatty acids [4]. Indeed, effects on mitochondrial function may be a major driver of androgen-related metabolic disease, including T2DM [46]. Skeletal muscle from women with PCOS has distinct changes in genes responsible for mitochondrial oxidative phosphorylation compared to BMI-matched controls [47], and recent data suggest impaired fat oxidation in hyperandrogenic PCOS women *in vivo* [48]. Mirroring this, reduced testosterone levels are associated with abnormal mitochondrial respiration and impaired insulin sensitivity in skeletal muscle in men [49], with improvements in mitochondrial biogenesis in human and rodent studies after androgen replacement therapy [50,51].

Low circulating SHBG has been consistently identified as a risk factor for T2DM in both sexes in a number of smaller studies and meta-analyses [10,52,53], and our longitudinal study supports these observations. In a meta-analysis of 13 population-based studies with 1,912 incident cases of T2DM, low SHBG were associated with increased risk of T2DM in women, irrespective of menopausal status [52]. SHBG levels are typically higher in women, and our data confirm that reduced circulating concentrations are associated with a higher risk of T2DM than that observed in men. SHBG is a critical mediator of the association between sex steroids and metabolic dysfunction. Circulating testosterone and estradiol are both bound to SHBG, but the affinity of SHBG is more than two-fold higher for the former [54]; the majority of circulating testosterone is bound to SHBG, such that only the unbound or 'free' fraction is capable of exerting effects in target tissues [55]. Therefore, reduced SHBG levels in women are a surrogate marker of increased circulating active androgens. In men, low SHBG levels are not considered a marker of androgen excess, as unbound testosterone concentrations are physiologically much higher in men than women. Insulin is a potent regulator of hepatic SHBG output, which is suppressed in the context of hyperinsulinemia, leading to reduced SHBG, and therefore increased free androgens, in insulin resistant states such as PCOS in women [31]. It remains unclear whether SHBG plays a causal role in the pathophysiology of metabolic diseases such as T2DM, or whether it is simply a surrogate marker of insulin resistance and increased metabolic risk.

This study has a number of important limitations, not least its retrospective nature. Detailed clinical phenotyping in studies of this type are not possible. There are also no detailed data available on laboratory assays used to measure serum testosterone. This is not of particular concern in men, as physiologically higher testosterone concentrations do not represent a challenge for quantification by either radioimmunoassay (RIA) or tandem mass spectrometry techniques. In women, however, where low circulating concentrations pose significant analytical and quantification difficulties for standard RIAs, the consensus is that today measurements should be performed by liquid chromatography-tandem mass spectrometry to improve quantification and avoid cross reactivity [56]. Furthermore, we have no information on the time of day blood sampling for serum testosterone took place; in men, Endocrine Society guidelines emphasize that morning samples are crucial to accurately diagnose hypogonadism [57]. Lastly, we must assume that testosterone data were obtained from men and women with a clinical indication for serum measurement; this introduces a potential bias by indication. However, we believe that these limitations are ameliorated by the large patient numbers and the clear and potent gradient towards sex-specific diabetes risk in the study population.

In conclusion, in the largest retrospective longitudinal study of its kind, we have demonstrated evidence of a sexually dimorphic role for androgens in mediating the risk of T2DM. These observations persist after correction for typical confounding factors such as BMI. Serum testosterone concentrations above 1.5nmol/L in women, and below 20nmol/L in men, are associated with a linearly increased risk of hyperglycemia in the context of worsening female androgen excess and male androgen deficiency, respectively. Reduced SHBG levels in both sexes, but particularly in women, significantly increase the risk of T2DM. These data further define androgens as a novel metabolic risk factor in men and women, but potential mechanisms underpinning these observations remain to be clarified. We suggest that women with androgen excess and men with androgen deficiency should be systematically screened for T2DM. Future studies will be required to show if reducing androgens in women, and increasing androgens in men, will improve overall metabolic health and risk of progression to overt T2DM.

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CHAPTER 2.5

Prognostic value of dehydroepiandrosterone in type 2 diabetes: The Rotterdam Study

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Manuscript under review

ABSTRACT

BACKGROUND: Emerging evidence shows that high levels of dehydroepiandrosterone (DHEA) are associated with reduced risk of type 2 diabetes (T2D). It is not known whether DHEA is associated with prognosis of T2D.

OBJECTIVE: We examined whether higher levels of DHEA and its main derivate dehydroepiandrosterone sulphate (DHEAs) were associated with fewer complications of T2D.

METHODS: We included 586 men and 544 women with T2D from the Rotterdam Study, a prospective follow-up study. DHEA levels were measured at baseline. Complications of T2D included hypertension (HTN), chronic kidney disease (CKD), initiation of insulin therapy, stroke and death. HTN was defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or use of blood pressure lowering medication. CKD was defined as estimated glomerular filtration rate <60 ml/min/1.73 m^2. Incident stroke events and insulin therapy initiation were obtained on the basis of medical records. Mortality data were obtained by notification from the municipal administration. Logistic regression (for HTN, CKD and insulin therapy initiation) and Cox proportional hazards models (for stroke and mortality) with adjustment for relevant confounders were used to calculate odds ratios (ORs) and hazard ratios (HRs), and their 95% confidence intervals (CIs).

RESULTS: Cross-sectionally, higher levels of DHEA were associated with lower odds of having HTA (OR=0.67, 95%CI: 0.49-0.90) and CKD (OR=0.66, 95%CI: 0.47-0.92) which remained robust also in the longitudinal analysis. No consistent associations were found between DHEAs and HTA/CKD. During 19 years of follow-up, we identified 165 incident cases of insulin initiation, 145 cases of stroke and 559 deaths. Higher levels of DHEA were associated with later initiation of insulin therapy (OR=0.62, 95%CI: 0.41-0.93) and reduced risk of all-cause mortality (HR=0.84, 95%CI: 0.71-0.98) but no association was found between DHEA levels and risk of stroke (HR=0.83, 95%CI: 0.58-1.20). Further adjustment for glucose, insulin, downstream hormone levels such as total estradiol and testosterone and DHEAs hormone did not affected any of these associations. DHEAs was associated with reduced risk of mortality, but not with other diabetes complications.

CONCLUSIONS:

These findings suggest that high levels of DHEA are associated with fewer complications in diabetics. It is at present unclear whether medications or lifestyle factors that alter DHEA metabolism can be effectively used in prevention of diabetes complications.

INTRODUCTION

Type 2 diabetes mellitus (T2D) is a chronic metabolic disorder, that is becoming an epidemic due to increase of its prevalence. It is estimated that 439 million people would have T2D by the year 2030[1]. Diabetes, because of the poor glycemic control, is associated with macro- and microvascular complications, leading to hypertension, chronic kidney disease, blindness and stroke[2]. These long term complications contribute substantially to morbidity, mortality and economic burden of diabetes[3,4]. According to World Health Organization statistics, each diabetic patient spends about three times more money on his or her health than a person without diabetes[5].

Several factors have been associated with progression of diabetes and its complications, but they cannot fully explain the excess risk. Recently, dehydroepiandrosterone (DHEA) metabolism has been implicated in the etiology of diabetes. Randomized controlled trials have reported that DHEA replacement can reduce abdominal fat and improve insulin sensitivity[6]. Also, prospective population-based studies have reported serum DHEA levels to be associated with lower risk of type 2 diabetes in middle-age and elderly men and women [7,8]. Emerging evidence indicates that DHEA may be associated with progression of diabetes. Animal studies show that treatment with DHEA can increase insulin-induced glucose uptake in rat models of type 2 diabetes and moderate the severity of diabetes [9,10]. Further, DHEA may play a role on risk of stroke associated with diabetes since treatment with DHEA prevented both derangement of the oxidative state and neuronal damage induced by ischemia/reperfusion in experimental diabetes [11]. Along the same lines, a clinical trial showed that DHEA treatment ameliorates the oxidative imbalance induced by hyperglycemia, downregulates the tumor necrosis-alpha receptor system, and prevents advanced glycation end product formation, suggesting a beneficial effect on the onset and/or progression of chronic complications in T2D patients[12]. Despite the growing body of evidence on protective role of DHEA in T2D, information about the role of DHEA and T2D complications remains unclear. Therefore, we aimed to examine the association between DHEA and its main derivate dehydroepiandrosterone sulphate (DHEAs) and T2D complications. We hypothesized that higher levels of DHEA/DHEAs were associated with fewer complications of T2D, later initiation of insulin therapy and reduced risk of mortality.

METHODS

Study Population

The study used data from The Rotterdam Study (RS), a prospective ongoing population-based cohort study which commenced in 1990, in Rotterdam, the Netherlands. In the Rotterdam study, the first cohort of study participants (RSI), from the Ommoord district in Rotterdam, comprised 7983 persons aged 55 years and over. In 2000, the cohort (RSII) was extended to include an additional 3011 participants who moved into the study district or had become 55 years of age. A further extension of the cohort (RSIII) occurred in 2006 to include 3932 participants living in the research area and aged 45-54 years. For follow-up, examinations were scheduled every 3-5 years[13].The RS complies with the Declaration of Helsinki and has been approved by the Medical Ethics Committee of the Erasmus Medical Centre and also complies with the Dutch Ministry of Health, Welfare and Sport. All participants in the present analysis provided written inform consent to participate and to obtain information from their treating physicians [13].

DHEA assessment

DHEA and DHEAs were measured on a Waters XEVO-TQ-S system (Waters, Milford, MA, USA) using the CHS™ MSMS Steroids Kit (Perkin Elmer, Turku, Finland). The inter-assay coefficients of variation of androstenedione, DHEAs and DHEA were <6.5%.

Ascertainment of type 2 diabetes

T2D was defined, according to current WHO guidelines, as a fasting blood glucose ≥7.0mmol/l, a non-fasting blood glucose ≥11.1mmol/L (when fasting samples were absent) or the use of blood glucose-lowering medication[14]. Information regarding the use of blood glucose-lowering medication was derived from both structural home interviews and linkage to pharmacy records[15].

Ascertainment of type 2 diabetes complications:

For this study, diabetes complications were considered the presence and incidence of hypertension, chronic kidney disease (CKD), and initiation of insulin therapy, stroke incidence and mortality.

Blood pressure (BP) was measured in the sitting position on the right upper arm with a random-zero sphygmomanometer. Systolic BP was recorded at the appearance of sounds (first-phase Korotkoff) and diastolic BP at the disappearance of sounds (fifth-phase Korotkoff). Systolic and diastolic BP were calculated as the average of the 2 measurements. Hypertension was defines as a systolic BP ≥ 140mmHg, a diastolic BP ≥ 90mmHg, or the use of antihypertensive medication.

We defined prevalent and incidence CKD as an estimated glomerular filtration rate (eGFR) lower than 60mL/min/1.73m^2. Estimated glomerular filtration rate was calculated according to the CKD-EPI(CKD Epidemiology Collaboration) creatinine-cystatin C equation[16]. Similar with previous studies [17-19], to calculate the annual eGFR decline, we first subtracted the eGFR estimates of the follow up examination from the eGFR estimates at baseline and then divided by the time between the two visits. Incident cases of CKD were defined among the individuals free of CKD at baseline (eGFR>60ml/min per 1.73m^2), who had a decline in eGFR to less than 60 ml/min per 1.73m^2 between the two periodical examinations. To estimate the event date of the cases of chronic kidney disease we assumed a linear decrease in eGFR. Given this assumption, the date that each case had passed the eGFR threshold of 60ml/min per 1.73m^2 was taken as the event date and it was used to calculate the follow up time for incident cases.

Insulin therapy start until January 2012 was obtained on the basis of medical records and from the pharmacy data. Participants were continuously monitored for incident stroke, which were identified from medical records and confirmed by an experienced vascular neurologist[20]. Mortality data were obtained by notification from the municipal administration. Follow-up was complete until January 1, 2012, and April 20, 2017 for stroke incidence and mortality respectively.

Covariates

Information on current health status, medical history, medication use, smoking behavior, and other factors was obtained at baseline (RSI-3, RSII-1 and RSIII-1). Participants were asked whether they were currently smoking cigarettes, cigars or pipes. Alcohol intake was assessed in grams of ethanol per day. History of cardiovascular disease (CVD) was defined as having a history of coronary heart diseases (myocardial infarction, revascularization,

coronary artery bypass graft surgery or percutaneous coronary intervention), heart failure or stroke. This information was verified from the general practitioner's medical records. Information regarding the use of hormone replacement therapy was derived from structured home interviews.

Physical height (m) and body weight (kg) were measured at baseline with the participants standing without shoes and heavy outer garments. BMI was calculated as weight divided by height squared (kg/m^2). Waist circumference was measured at the level midway between the lower rib margin and the iliac crest with participants in standing position without heavy outer garments and with emptied pockets, breathing out gently. Physical activity was assessed using an adapted version of the Zutphen Physical Activity Questionnaire [15]. Every activity mentioned in the questionnaire was attributed a metabolic equivalent value according to the 2011 codes [21].

All biochemical variables were assessed in serum. Insulin and glucose were measured using a COBAS 8000 Modular Analyzer (Roche Diagnostics). The corresponding interassay CVs were insulin <8% and glucose <1.4%. Serum total cholesterol was determined by an automated enzymatic procedure in a non-fasting blood sample. High sensitivity C-reactive protein (CRP) was measured in non-fasting frozen serum of study participants using a rate near-infrared particle immunoassay (Immage Imunnochemistry System, Beckman Coulter, Fullerton, CA, USA). This system measures concentrations from 0.2 to 1.440 mg/l, with a within-run precision of 0.5%, a total precision <7.5% and a reliability coefficient of 0.995. TSH was measured by electrochemiluminescence immunoassay for thyroxine (Roche Diagnostics International Ltd, Rotkreuz, Switzerland) in serum samples stored at -80ºC). Serum cortisol was measured by luminescence immunoassay (IBL, Hamburg, Germany). Intra-assay and interassay coefficients of variation were below 6%. Sex hormone-binding globulin (SHBG) was measured using the Immulite platform (Diagnostics Products Corporation Brenda, the Netherlands,) while radioimmuniassay was used to measure the levels of total estradiol (TE2). The minimal corresponding amount of estradiol that can be measured was 18.35 pmol/liter. Undetectable estradiol was record as 18.35. Androstenedione and testosterone were measured on a Waters XEVO TQ-S system (Waters,

Milford, MA, USA) using the CHSMSMS steroids Kit(Perkin Elmer, Turku, Finland). The interassay CV of androstenedione and testosterone were <6.5% and <5%, respectively.

Population of analysis The present study used data from the third visit of the first cohort (RSI-3) and the baseline examinations of the second and third cohort (RSII-1 and RSIII-1). Overall, in these rounds of the RS, 11,732 subjects were invited for blood assessment, in which 1334 (11.4%) participants with diabetes were identified and eligible to be included in the analysis. Of these, for 134 participants there was no information on DHEA/DHEAs, so they were excluded from the analysis. Furthermore, 3 diabetic participants without information for systolic and diastolic blood pressure were excluded. Thus, 1130 participants were included in our final analysis for the association between DHEA/DHEAs, prevalent hypertension, prevalent CKD and mortality (**Figure 1**). Of 1130 participants, follow-up data on insulin therapy initiation were not available for 437 participants, and 149 participants were prevalent insulin users, and thus were excluded from the analysis on DHEA/DHEAs and initiation of insulin therapy, leaving 693 subjects for this analysis. Also, of 1130 participants, after excluding prevalent stroke cases (n=41) and participants whom did not give inform consent for stroke follow-up (n=47), 1042 participants were included in the analysis for incident stroke as outcome. Further, due to prevalent cases (hypertension, n=599; CKD, n=31) and no information on follow-up data on BP (n=378) and CKD (n=642), 153 and 457 diabetics patients were included for the analysis on DHEA/DHEAs and incidence hypertension and CKD respectively.

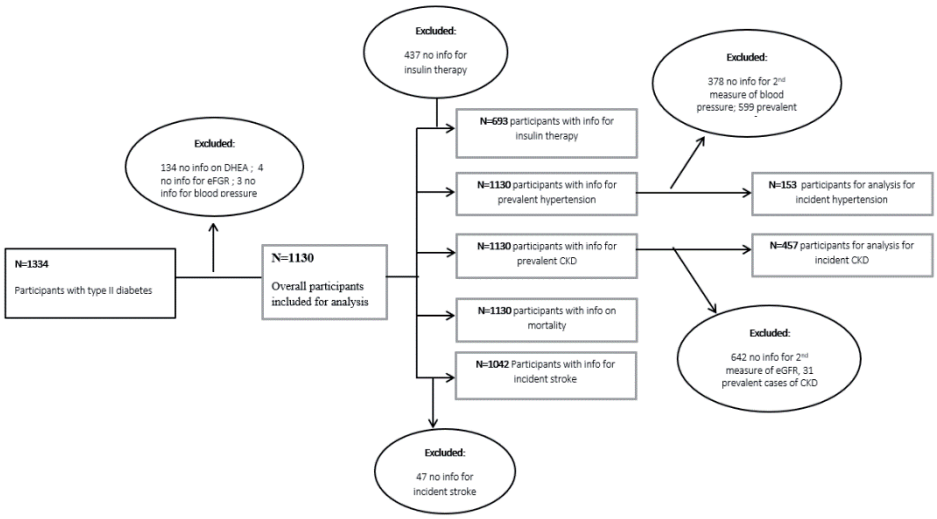

Figure 1. Flowchart of study participants

Statistical analyses

Continuous variables are reported as mean ± SD unless stated otherwise and categorical variables were presented as percentages. To achieve approximately normal distribution, skewed variables (DHEA, DHEAs, 17-hydroxy-progesterone, SHBG, insulin, androstendion, CRP, TSH) were natural log-transformed.

Multivariable logistic regression models were used to investigate whether high level of DHEA/DHEAs were associated with prevalent and incident hypertension, prevalent CKD, and initiation of insulin therapy. Cox proportional hazard models were used to examine the association between DHEA/DHEAs levels and incident chronic kidney disease, stroke and mortality. Odds ratios (OR) and hazard ratios (HR) and their 95% confidence intervals (95% CIs) were reported. In the basic model (Model 1) we adjusted for age, sex, RS cohort (RSI, RSII and RSIII), 17-hydroxy-progesterone(continuous), SHBG (continuous), cortisol (continuous), body mass index (BMI) (continuous), alcohol (continuous), smoking (current vs. former/never), physical activity, hormone replacement therapy (yes vs. no), prevalent cardiovascular disease (yes vs. no), serum total cholesterol (continuous), statin use (yes vs. no), prevalent oral medication for diabetes and prevalent insulin use(yes vs. no). In Model 2,

additionally to the variables in model 1, we adjusted for serum glucose (continuous) and insulin (continuous). In Model 3 we further adjusted for potential intermediator factors including in model 2 also the downstream hormones of DHEA such as androstenedione(continuous), total estradiol and total testosterone(continuous).

Sensitivity Analysis

We performed a series of sensitivity analyses using imputed data. To examine whether there were sex differences, an interaction term between DHEA/DHEAs and sex was tested. Additionally, stratified analysis by sex was performed. We also performed sensitivity analysis after excluding participants who had cardiovascular disease at baseline and were hormone replacement therapy users. Additional models were built by adding CRP, TSH or waist circumference to covariates in model 3, as well as adjusting DHEA/DHEAs for each other. There were missing values on one or more covariates. Because the missing values were likely to be missing at random and for avoidance of loss in efficiency, missing values were imputed using a multiple imputation technique (5 imputation sets) (**Supplemental Table 3**). Rubin's method was used for the pooled coefficients (odds ratio (OR) or HR) and 95% CIs. A p-value of less than 0.05 was considered as statistically significant. All analyses were done using SPSS statistical software (SPSS, version 21.0; SPSS Inc, Chicago, Illinois).

Results

Baseline characteristics

There were 585 (52%) men and 544 (48%) women with T2D. The mean age (SD) of the sample was 67.45 (9.60). The mean (SD) BMI was 29.38 ± 4.78 and 13.4% of the study population were prevalent insulin user. **Table 1** summarizes the baseline characteristics of 1,130 participants.

DHEA/DHEAs, hypertension and chronic kidney disease

Table 2 presents the associations between levels of DHEA/DHEAs and prevalent hypertension and CKD. Among 1130 diabetic subjects, 924 participants (81.8%) had hypertension while 197 participants (17.4%) had CKD. After adjustment for confounding factors (model 1), high levels of DHEA were associated with lower odds of having hypertension (OR=0.67, 95%CI: 0.49-0.90) and CKD (OR=0.66, 95%CI: 0.47-0.92). Similarly,

high levels of DHEAs were associated with lower odds of hypertension (OR=0.76, 95%CI: 0.57-0.99), but no association was found for prevalent CKD (**Table 2**). Further adjustment for levels of glucose and insulin or downstream sex hormones including androstenedione, total estradiol and total testosterone did not affect the association between DHEA/DHEAs, prevalent hypertension and CKD (**Table 2**).

During a median follow-up of 10 years, in 153 subjects free of hypertension at baseline and 457 subjects free of CKD, 81 cases of hypertension and 84 cases of CKD respectively were identified. Similarly to the cross-sectional findings, independent of confounding factors and potential intermediate risk factors, high levels of DHEA were associated with lower odds of developing hypertension (OR=0.39, 95%CI: 0.17-0.92) and lower risk of developing CKD (HR=0.63, 95%CI: 0.40-0.98) (**Table 2)**. No association was found between DHEAs and incident hypertension/CKD (**Table 2**).

Sensitivity analysis

The interaction with sex was not significant for any of the associations investigated (All *P*-values <0.05). Furthermore, stratification by sex did not show any sex-differences in the results (**Supplemental Table 1**). All associations that were statistically significant in the main analysis remained unchanged when the analyses were additionally adjusted for CRP, TSH or WC. Also, exclusion of participants with prevalent CVD or of participants who reported use of hormone replacement therapy did not materially change any of the associations. Further, adjustment for DHEAs, attenuated the association between DHEA, start of insulin therapy and mortality, but did not affect the association between DHEA and other diabetes complications (**Supplemental Table 2**). Also, adjustment for DHEA, did not affect any of the association between DHEAs and diabetes complications, but attenuated the association between DHEAs and mortality (**Supplemental Table 2**)

Table 1. Study participants characteristics (N=1,130)

Subjects characteristics		Subjects characteristics	
Age at measurements (y)	67.45 ± 9.60	Indication for hypertension ,n (%)	
Sex, n (%)		No indication HT	525(46.46%)
Male	586 (51.86 %)	Indication HT	605(53.54%)
Female	544 (48.14%)	Body mass index (kg/m^2)	29.38 ± 4.78
Smoking, n (%)		Waist circumference(cm)	100.8±11.95
Never smoker	908 (80.31%)	Statin users, n (%)	
Current smoker	222 (16.69%)	Users	316(27.95%)
Physical activity	69.86 ± 52.96	Non users	814(72.05%)
Alcohol Consumption	1.43(19.98)	Cholesterol (mmol/L)	5.42 ± 1.06
Hormone replacement therapy, n (%)		Glomerular filtration rate(creatinine) (ml/min/1.72m^2)	76.51 ± 17.95
Therapy users	18 (1.63%)	Glucose(mmol/L)	8.55 ± 2.75
Non users	1112(98.37%)	Insuline in serum (pmol/L)	111.50(105)
Oral hypoglycemic medication use, n (%)		C-Proteine reactive(mmol/L)	2.45(3.79)
Yes	514(45.47%)	TSH(Mu/l)	1.88(1.58)
No	616(54.53%)	Cortisol(nmol/L)	369,57 ± 119.35
Prevalent insulin use, n (%)		17-Hydroxy-Progesterone (mmol/L)	1.65(1.59)
Yes	151(13.35%)	Estradiol (pmol/L)	87.16 ± 86.38
No	979(86.65%)	Testosterone (nmol/L)	7.45 ± 8.22
CVD, n (%)		SHBG (nmol/L)	41.28(25.78)
No prevalent	892 (78.98%)	Androstendione (nmol/L)	2.60(1.65)
Prevalent	238 (21.01%)	DHEA (nmol/L)	7,97(7.32)
Systolic blood pressure	174.60 ± 30.73	DHEAS(nmol/L)	1884.02(1871.61)

Values are valid percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (interquartile range) for continuous variables with a skewed distribution.

Table 2 The association between dehydroepiandrosterone and dehydroepiandrosterone sulphate hormone levels with hypertension and chronic kidney disease

		Model 1 Relative risk (95%CI)*#	Model 2 Relative risk (95%CI)*#	Model 3 Relative risk(95%CI)*#
Prevalent Hypertension[†]	DHEA	**0.67 (0.49, 0.90)**	**0.67 (0.50, 0.92)**	**0.53 (0.36,0.78)**
	DHEAS	**0.76 (0.57,0.99)**	0.77 (0.58,1.02)	**0.70 (0.52,0.96)**
Incident Hypertension[†]	DHEA	**0.37 (0.16,0.83)**	**0.36 (0.17,0.83)**	**0.23 (0.08,0.71)**
	DHEAS	0.70(0.35,1.38)	0.68(0.40,1.36)	0.68(0.29,1.59)
Prevalent CKD[†]	DHEA	**0.66 (0.47, 0.92)**	**0.65 (0.47, 0.92)**	**0.52 (0.34,0.78)**
	DHEAS	0.82(0.63,1.12)	0.85(0.65,1.12)	0.80(0.60,1.09)
Incident CKD[‡]	DHEA	**0.64 (0.42,1.00)**	**0.64 (0.41,0.99)**	**0.57 (0.32,1.00)**
	DHEAS	0.89(0.59,1.32)	0.88(0.59,1.32)	0.91(0.57,1.44)

[†]Odds ratios estimated by using logistic regression models;[‡]Hazard ratios estimated by using Cox's proportional hazard models; *Result are reported per one unit increase in natural log transformed dehydroepiandrosterone or dehydroepiandrosterone sulphate; # Relative risks are odds ratio or hazard ratio

Model 1: adjusted for age, sex, cohort, progesterone, sex-hormone binding globulin, cortisol, body mass index, alcohol, smoking, physical activity, hormone replacement therapy, prevalent cardiovascular disease, serum total cholesterol, statin use, prevalent oral medication for diabetes, prevalent insulin use.
Model 2: additionally adjusted for glucose and insulin.
Model 3: additionally adjusted for estradiol, testosterone and androstenedion

DHEA/DHEAs, initiation of insulin therapy, stroke incidence and mortality

During the follow-up (median follow-up for insulin therapy 6 years, stroke 3 years and mortality 9years) ,165(16,8%) cases of new insulin users, and 145(13,4%) cases of incident stroke and 559(49,5%) deaths were identified.

As shown in Table 3, independent of confounding factors (model 1), higher level of DHEA hormone was associated with lower risk of starting insulin therapy (OR=0.61, 95%CI: 0.42-0.92), and mortality (HR=0.81, 95%CI: 0.69-0.95), whereas no significant association was found between DHEA levels and stroke (HR=0.83, 95%CI: 0.58-1.18). High levels of DHEAs were associated with lower risk of mortality (HR=0.78, 95%CI: 0.68-0.92), whereas no association was found between DHEAs, start of insulin therapy and stroke incidence (**Table 3**). Additional adjustment for glucose, insulin and downstream sex hormones did not affect the association between DHEA and initiation of insulin therapy, as well as the association of DHEA/DHEAs with mortality (**Table 3**).

Table 3. The association between DHEA and DHEAs hormone levels and initiation of insulin therapy, stroke and risk of mortality

		Model 1 Relative risk (95%CI)*#	Model 2 Relative risk (95%CI)*#	Model 3 Relative risk (95%CI)*#
Insulin therapy[†]	DHEA	**0.62(0.41, 0.93)**	**0.60(0.40, 0.91)**	**0.57(0.34,0.97)**
	DHEAS	0.77(0.54,1.09)	0.79(0.54,1.12)	0.83(0.55,1.24)
Stroke[‡]	DHEA	0.83(0.58, 1.18)	0.83(0.58, 1.19)	0.83(0.57, 1.20)
	DHEAS	0.78(0.58, 1.05)	0.78(0.58,1.06)	0.82(0.60,1.12)
Mortality[‡]	DHEA	**0.84(0.71,0.98)**	**0.84(0.72,0.98)**	**0.80(0.66,0.97)**
	DHEAS	**0.85(0.74,0.97)**	**0.85(0.74,0.98)**	**0.84(0.72,0.98)**

[†]Odds ratios estimated by using logistic regression models ;[‡]Hazard ratios estimated by using Cox's proportional hazard models *Result are reported per one unit increase in natural log transformed dehydroepiandrosterone or dehydroepiandrosterone sulphate
Relative risks are odds ratio (initiation of insulin therapy) or hazard ratios (for stroke and mortality)
Model 1: adjusted for age, sex, cohort, progesterone, sex-hormone binding globulin, cortisol, body mass index, alcohol, smoking, physical activity, hormone replacement therapy, prevalent cardiovascular disease, serum total cholesterol, statin use, prevalent oral medication for diabetes, prevalent insulin use. **Model 2**: additionally adjusted for glucose and insulin. **Model 3**: additionally adjusted for estradiol, testosterone and androstendion.

DISCUSSION

In this prospective study of diabetes individuals, we found that, high levels of DHEA were associated with lower risk of developing diabetes complications, including chronic kidney disease, hypertension, initiation of insulin therapy and death; whereas no association was found between level of DHEA hormone and stroke. No consistent associations were found between DHEAs and diabetes complications, however our results show DHEAs levels can predict mortality in patients with diabetes.

Most of the evidence on role of DHEA in prognosis of diabetes comes from animal studies. Animal studies have reported therapeutic effect of DHEA in diabetic mice. Treatment of genetically diabetic mice of both sexes with DHEA was very efficacious in moderating the severity of the ensuing diabetes by reducing hyperglycemia [9,22]. Also, treatment with DHEA and/or DHEAs in genetically diabetic or obese mice increased the sensitivity to insulin and prevented the development of severe diabetes[23]. Further, treatment of diabetic rats with

DHEA improved vascular reactivity, which may contribute to the clinical manifestations of cardiovascular disease, the pathogenesis of nephropathy and diastolic dysfunction in subjects with diabetes mellitus. Indeed, DHEAS has been shown to cause dilation of preconstricted vascular rings and to prevent dexamethasone-induced hypertension[24]. However, a study in diabetic mice, despite showed DHEA treatment to decrease blood glucose, increased plasma creatinine and decreased glomerular filtration rate, indicating that DHEA treatment may be harmful to renal tissue[25], which is not in line with our findings on a potential protective effect of DHEA on CKD.

Considering the lower amounts of DHEA in laboratory animals, and that DHEA may have a distinct role in humans since its biosynthesis pattern is specific to higher primates, caution is required in extrapolating results obtained in animal systems to humans[26]. Findings in humans on prognostic value of DHEA/DHEAs for patient with diabetes is scarce. Similarly to findings on DHEA/DHEAs as a prognostic marker in diabetic patients, a meta-analysis showed that patients with cardiovascular disease who have lower DHEAs levels may have poorer prognosis than those with higher DHEAs, since lower levels of DHEAs were associated with significant increased risk of mortality[27]. In the same line, low levels of DHEAs were associated with worst renal function and increased risk of mortality in CKD hemodialysis men[28]. Further, clinical trials in healthy postmenopausal women report that DHEA/DHEAs replacement therapy significantly improves metabolic syndrome parameters that are linked to an increase risk of diabetes, cardiovascular and mortality, but also influence prognosis of diabetes[29-31]. However, cross-sectional observational data in healthy individuals have raised concerns that DHEA/DHEAs[32,33] may increase blood pressure levels[34], which are not supported by clinical trials in healthy subjects[29,35]. Contrary to the cross sectional study findings the trials showed blood pressure-lowering effects of DHEAs, a finding noted in our cross sectional and longitudinal analysis of diabetic subjects.

Cerebrovascular complications make diabetic patients 2–6 times more susceptible to a stroke event[36]. Animal studies show that DHEA may play a role on risk of stroke associated with diabetes[11], which is not supported by our study. However, the null effect found between DHEA/DHEAs and stroke in our study can be also due to few incident cases of stroke (n=145) we observed during the follow-up. An observational cohort study, using a

nested-case control design with 461 ischemic stroke events concluded that lower DHEAS levels were associated with a greater risk of ischemic stroke[37].

Initiation of insulin therapy indicates greater severity of diabetes and difficulty in maintaining glycemic control, also because of reduced insulin sensitivity[38]. Data on DHEA and insulin sensitivity in humans are contradictory, with some showing that DHEA administration increases[6,39], has no effect[40,41] or decrease[42]insulin sensitivity. However, in our study we found that higher levels of DHEA were associated with later initiation of insulin therapy. Some clinical trials but not all, report DHEA/DHEAs supplementation to reduce weight and body fat[6], which on the other hand determine initiation of insulin therapy in diabetes patients[43].

Both DHEA and DHEAs can be converted in peripheral tissues to androstenedione, testosterone and dihydrotestosterone, and both are aromatized to estrogens[44]. Therefore, we would expect that DHEA/DHEAs can exert their functions via downstream hormones. However, androstenedioine, testosterone and estrogen could not explain the association between DHEA/DHEAs and diabetes complications in our study. Therefore, other pathways might be involved. In addition to adrenal synthesis, evidence also point out that DHEA and DHEAS are synthesized in the brain and there is a need to further investigate the role of these hormones in brain function[45]. The most biological action of DHEA/DHEAs involve neuroprotection, catecholamine synthesis and secretion, antioxidant and anti-inflammatory effects[46]. The mechanism action of antioxidant effect of DHEA hormone has been explained by inhibiting the nuclear factor-kappaB(NF-Kβ)activation in hippocampus of diabetic rats[47]. The anti-inflammatory effect is reached by decreasing pro-inflammatory cytokine production both *in vivo* and *in vitro*[48,49]. DHEA and DHEAs inhibited both basal and TNFα-stimulated NF-Kβ-dependent luciferase transcription in a time- and dose-dependent manner[49]. Oxidative stress and inflammation are involved in the pathogenesis of diabetes and its complications. Therefore, DHEA/DHEAs, due to their antioxidant and anti-inflammatory effect may reduce oxidative stress and alleviates diabetic complications.

DHEA and DHEAs circulate in the blood mostly bound to albumin but with a small amount not bound to a protein. These steroid hormones inter convert and about 6% of DHEA will re-enter the blood as DHEAs, while 60-7-% of DHEAs will re-enter as DHEA. Concentrations

of DHEAs are between 250 and 500 times higher than concentrations of DHEA in women and men, respectively. This difference in concentrations between DHEA and DHEAs depends mainly on the fact that DHEA is cleared rapidly from the blood, with a metabolic clearance rate (MCR) in the range of 2000l/day, but the clearance of DHEAs is much slower and its MCR is in the range of 13 l/day. Therefore, DHEAs has a half-life of 10-20h, while the half-life of DHEA is 1-3h. (27)

There are several key strengths of our study: the population-based design, prospective setting, the long follow-up and availability of broad data about risk factors, which allowed us to control for several potential confounders. Moreover, our study is unique because the association between DHEA/DHEAs and complications of T2D have not been investigated before.

Different limitations of this study should also be acknowledged. First, we did not have data on HbA1c, microvascular complication of retinopathy and on neuropathy, which would have strengthened our findings. Although the definition of CKD based on KDIGO (Kindey Disease: Improving Global Outcomes) criteria requires 2 eGFR values <60 mL/min/1.73m^2 at least 90 days apart, we only had a single measurement of eGFR measured in two different period of times. However, CKD definition based on eGFR<60mL/min/1.73m^2 has been used frequently in the population-based research setting. Finally, our findings cannot be generalized to other geographic regions or ethnicities.

Conclusion

We have demonstrated that DHEA metabolism may play a role in diabetes prognosis. Future studies are needed to understand the underlying mechanism and to test whether medications or lifestyle factors that alter DHEA metabolism can be effectively used in prevention of diabetes complications.

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SUPPLEMENTAL MATERIAL

Supplemental Table 1. The association between DHEA/DHEAS and complications of T2D, initiation of insulin therapy and risk of mortality in men and women

		Model A Relative risk(95%CI)	Model B** Relative risk(95%CI)
Prevalent Hypertension†	DHEA	0.77(0.52,1.14)	M 0.81(0.46,1.41); **F 0.36(0.20,0.65)**
	DHEAS	0.80(0.54,1.20)	M 0.82(0.51,1.27); F 0.62(0.41,0.95)
Prevalent Chronic Kidney disease†	DHEA	**0.57(0.35,0.89)**	**M 0.48(0.26,0.88); F 0.53(0.29,0.99)**
	DHEAS	**0.85(0.56,1.29)**	M 0.81(0.50,1.32); F 0.66(0.42,1.05)
Incident hypertension	DHEA	0.40(0.15,1.06)	M **0.05(0.003,0.62);** F 0.23(0.03,1.83)
	DHEAS	0.65(0.25,1.71)	M 0.31(0.05,1.77); F 0.62(0.13,3.01)
Incident chronic kidney disease	DHEA	**0.57(0.33,0.98)**	M **0.44(0.20,0.97);** F 0.91(0.33,2.53)
	DHEAS	0.83(0.48,1.43)	M 0.79(0.41,1.51); F 1.15(0.53,2.48)
Initiation of insulin therapy†	DHEA	**0.52(0.30,0.90)**	M 0.72(0.31,1.65) **F 0.49(0.24,0.99)**
	DHEAS	0.64(0.38,1.10)	M 1.01(0.50,2.06); F 0.76(0.46,1.30)
Stroke‡	DHEA	0.73(0.43,1.25)	M 1.04(0.41,2.65); F 0.89(0.29,2.75)
	DHEAS	0.55(0.87,0.54)	M 1.01(0.51,2.00); F 0.86(0.43,1.74)
Mortality‡	DHEA	0.85(0.69,1.04)	**M 0.76(0.58,0.99);** F 0.87(0.65,1.15)
	DHEAS	0.85(0.70,1.03)	M 0.81(0.65,1.02)**;** F 0.87(0.69,1.08)

†Odds ratios estimated by using logistic regression models ; ‡Hazard ratios estimated by using Cox's proportional hazard models; *Result are reported per one unit increase in natural log transformed dehydroepiandrosterone or dehydroepiandrosterone sulphate; **Stratified by sex; # Relative risks are odds ratio (initiation of insulin therapy) or hazard ratios (for stroke and mortality); Model A: Model 2 and interaction term between DHEA/DHEAS

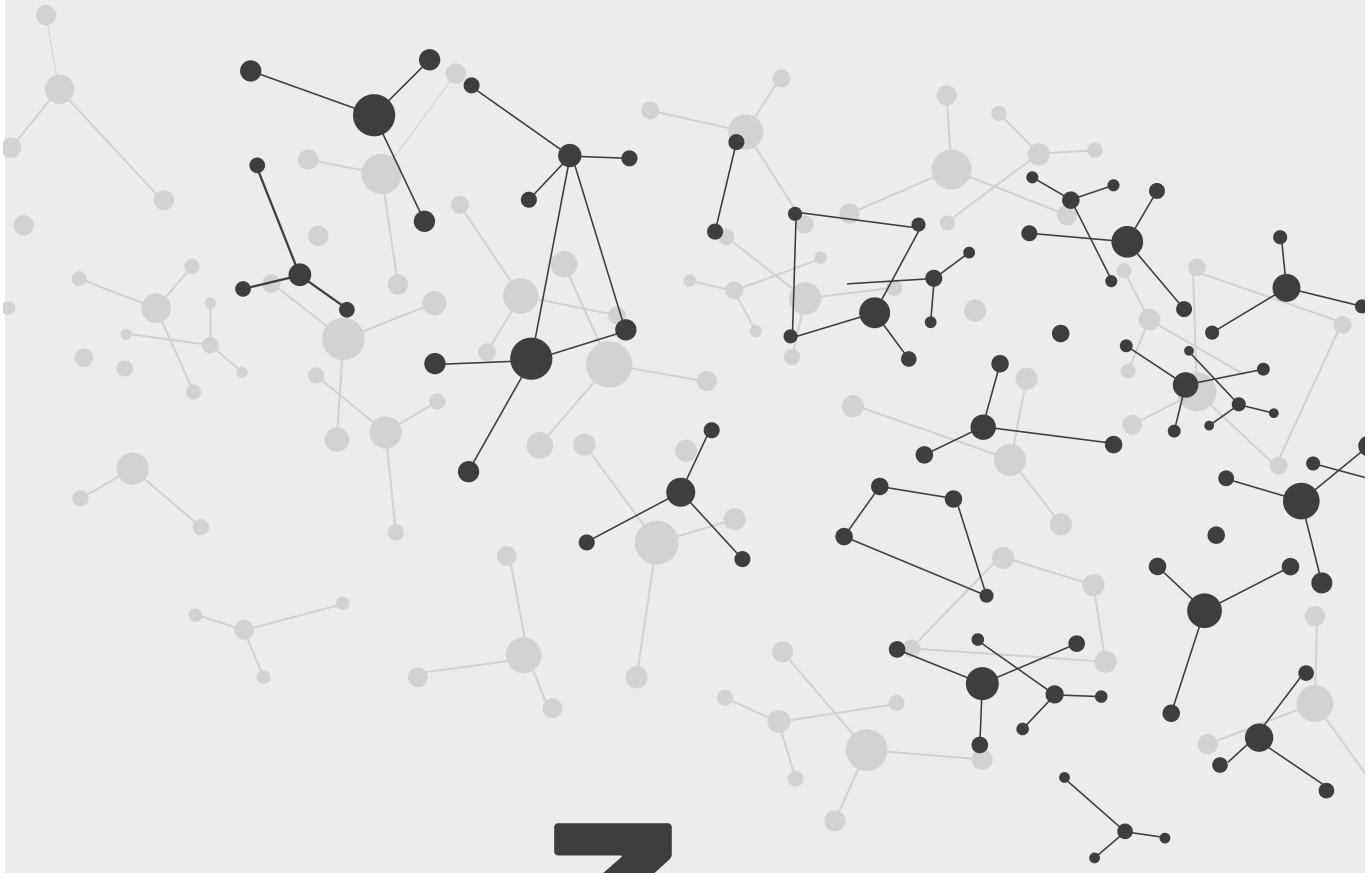
Supplemental Table 2 Sensitivity analysis on the association between dehydroepiandrosterone and dehydroepiandrosterone levels, diabetes complications, initiation of insulin therapy and risk of mortality

		Multivariable model + CRP Relative risk(95%CI)	Multivariable model + TSH Relative risk(95%CI)	Multivariable model + Waist circumference Relative risk(95%CI)	Multivariable model + DHEA/DHEAS Relative risk(95%CI)	After excluding CVD prevalent Relative risk(95%CI)	After excluding hormone therapy users Relative risk(95%CI)
Prevalent Hypertension†	DHEA	**0.53(0.36,0.78)**	**0.53(0.36,0.78)**	**0.53(0.36,0.78)**	**0.55(0.35,0.88)**	**0.55(0.37,0.84)**	**0.52(0.35,0.77)**
	DHEAS	**0.70(0.52,0.96)**	**0.69(0.51,0.95)**	**0.70(0.52,0.96)**	0.89(0.62,1.28)	**0.66(0.47,0.92)**	**0.70(0.52,0.96)**
Prevalent CKD†	DHEA	**0.53(0.35,0.80)**	**0.52(0.34,0.78)**	**0.51(0.34,0.77)**	**0.48(0.30,0.79)**	0.57(0.34,0.95)	**0.52(0.34,0.78)**
	DHEAS	0.80(0.59,1.09)	0.81(0.59,1.11)	0.80(0.59,1.09)	1.07(0.73,1.55)	0.83(0.57,1.21)	0.78(0.57,1.07)
Incident Hypertension	DHEA	**0.22(0.07,0.72)**	**0.16(0.05,0.56)**	**0.20(0.06,0.65)**	**0.17(0.05,0.63)**	**0.23(0.07,0.70)**	**0.19(0.06,0.66)**
	DHEAS	0.68(0.27,1.71)	0.63(0.25,1.58)	0.66(0.27,1.62)	1.31(0.44,3.91)	0.63(0.26,1.53)	0.63(0.25,1.59)
Incident CKD	DHEA	0.64(0.35,1.15)	0.57(0.32,1.01)	**0.56(0.32,1.00)**	**0.45(0.22,0.93)**	**0.45(0.24,0.85)**	0.58(0.33,1.03)
	DHEAS	1.08(0.61,1.91)	0.97(0.51,1.84)	1.08(0.61,1.91)	1.21(0.58,2.52)	0.88(0.53,1.47)	0.91(0.58,1.45)
Initation of insulin therapy†	DHEA	**0.57(0.36,0.96)**	**0.57(0.34,0.97)**	0.60(0.36,1.01)	0.55(0.28,1.05)	0.57(0.31,1.04)	0.63(0.37,1.07)
	DHEAS	0.82(0055,1.23)	0.81(0.54,1.21)	0.86(0.57,1.28)	1.12(0.67,1.87)	0.77(0.49,1.22)	0.82(0.54,1.25)
Stroke‡	DHEA	1.11(0.67,1.85)	1.10(0.66,1.82)	1.20(0.72,1.99)	0.99(0.48,2.07)	1.45(0.81,2.62)	1.08(0.64,1.77)
	DHEAS	1.04(0.70,1.55)	0.98(0.66,1.46)	1.20(0.81,1.05)	1.15(0.66,2.01)	1.22(0.79,1.87)	1.07(0.72,1.60)
Mortality‡	DHEA	**0.82(0.68,0.99)**	**0.80(0.66,0.97)**	**0.81(0.67,0.98)**	0.86(0.68,1.10)	0.84(0.66,1.07)	**0.80(0.66,0.97)**
	DHEAS	**0.85(0.73,0.99)**	**0.84(0.56,1.29)**	**0.85(0.73,0.99)**	0.92(0.76,1.12)	0.92(0.77,1.10)	**0.84(0.72,0.98)**

†Odds ratios estimated by using logistic regression models; ‡ Hazard ratios estimated by using Cox's proportional hazard models; *Result are reported per one unit increase in natural log transformed dehydroepiandrosterone or dehydroepiandrosterone sulphate levels# Relative risks are odds ratio (for chronic kidney disease, hypertension and insulin therapy) or hazard ratios (for stroke and mortality); Multivariable model: adjusted for age, sex, cohort, progesterone, sex-hormone binding globulin, cortisol, body mass index, alcohol, smoking, physical activity, hormone replacement therapy, prevalent cardiovascular disease, serum total cholesterol, statin use, prevalent oral medication for diabetes, glucose, insulin, testosterone, estradiol and androstendion

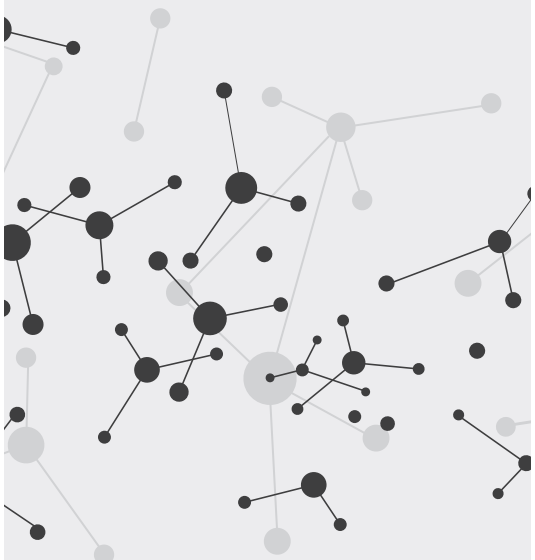
Supplemental Table 3 Details of the Multiple Imputation Modeling

	Multiple imputation procedure
Software used	SPSS 21.0 for windows
Imputation method and key settings	Fully conditional specification(MCMC) Maximum iterations 10
No of imputed data sets created	60
Variables included in the imputation procedure: • imputed and used as predictors of missing data	• smoking status, waist circumference, body mass index, prevalent cardiovascular disease, physical activity, natural log transformed sex-hormone binding globulin, estradiol, natural log transformed dehydroepiandrosterone sulfate, natural log transformed androstenedione, natural log transformed thyroid-stimulating hormone, hormone replacement therapy, natural log transformed insulin, glucose, natural log transformed c-reactive protein, total cholesterol, statin use, prevalent oral medication for diabetes, prevalent insulin intake and alcohol intake
• Used as predictor only	• Age, sex, cohort, natural log transformed progesterone, cortisol, testosterone, egfr baseline, egfr second measure, incident insulin therapy, incident stroke, indication for hypertension, mortality
Treatment of non-normally distributed variables	Predictive mean matching
Treatment of binary/categorical variables	Logistic regression models



Chapter 3

Exogenous Sex Hormones and Cardiometabolic Risk in Women



3.1

Association between progestin-only contraceptive use and cardiometabolic outcomes: A systematic review and meta-analysis

3.2

The route of administration, timing, duration and dose of postmenopausal hormone therapy and cardiovascular outcomes in women: a systematic review

CHAPTER 3.1

Progestin-only contraceptive use and cardio-metabolic outcomes

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Eur J Prev Cardiolog 25, 1042-1052

ABSTRACT

AIMS: The association between progestin-only contraceptive use (POC) and risk of various cardio-metabolic outcomes has been rarely studied. We performed a systematic review and meta-analysis to determine the impact of POC use on cardio-metabolic outcomes including venous thromboembolism, myocardial infarction, stroke, hypertension and diabetes.

METHODS AND RESULTS: Nineteen observational studies (7 cohort, and 12 case-control) were included in this systematic review. Of those, nine studies reported the risk of venous thromboembolism, six reported risk of myocardial infarction, six reported risk of stroke, three reported risk of hypertension and two studies reported the risk of developing diabetes with POC use. The pooled adjusted relative risks for venous thromboembolism, myocardial infarction and stroke for oral POC users versus non-users based on the random effects model were 1.06 (95% CI 0.70 to 1.62), 0.98 (95% CI 0.66 to 1.47) and 1.02 (95% CI 0.72 to 1.44) respectively. Stratified analysis by route of administration showed that injectable POC with a RR of 2.62 (95% CI 1.74 to 3.94), but not oral POC [RR 1.06 (95% CI 0.7 to 1.62)], was associated with increased risk of venous thromboembolism. A decreased risk for venous thromboembolism in a subgroup of women using intrauterine Levonorgestrel device was observed with RR 0.53 [95% CI 0.32 to 0.89]. No effect of POC use on blood pressure was found, but there was an indication for an increased risk of diabetes with injectable POC, albeit non-significant.

CONCLUSIONS: This systematic review and meta-analysis suggests that oral POC use is not associated with increased risk of developing various cardio-metabolic outcomes, whereas injectable POC use might increase the risk of venous thromboembolism.

INTRODUCTION

A number of studies have debated the association between combined oral contraceptive use and risk of cardio-metabolic outcomes [1-3], with some studies reporting an increased risk of venous thromboembolism (VTE), stroke and myocardial infarction (MI) for users of combined oral contraceptives (COC) [3,4]. COC can affect lipid profiles, carbohydrate metabolism, hemostatic factors and thrombolysis and this may be the pathway by which they affect the risk of developing various cardio-metabolic outcomes [5-8]. It has been postulated that the increased risk of various cardio-metabolic outcomes is mainly attributed to the estrogen content of these contraceptives [9]. Therefore, over the years the estrogen content of combined oral contraceptive pills has decreased and new oral contraceptives with progestin-only content have been developed, which are considered to be safer [9]. Type of progestin as well as route of administration are important factors in predicting risk of various cardio-metabolic outcomes. Progestins such as gestodene, norgestimate and desogestrel, have been associated with a greater VTE risk than the older progestins (levonorgestrel, lynestrenol, norethisterone) [10]. Also, studies have reported an elevated risk of VTE with the use of depot medroxyprogesterone (DMPA) which has relatively higher dose of progestin [11,12]. The weight gain is cited as a common side effect and major reason for discontinuation of DMPA [13]. Previous studies have generally found no association between use of injectable or implantable POC and the development of glucose intolerance [14], still, epidemiological evidence suggested possible increased risk of diabetes among DMPA users [14]. A rise in blood pressure as side effects of combined oral contraceptives have been theorized to be the critical mechanism for increased cardiovascular risk in women on COC, still the evidence on POC effect on blood pressure remains limited [15]. To date, there is scarce evidence on how POCs affect the various cardio-metabolic outcomes, which might be because of low chronic diseases occurrence among women of reproductive age and therefore low statistical power to estimate the reliable risk due to usage of POCs [15]. Although few reviews have evaluated the role of POCs and risk of VTE, stroke and MI [10,15-18], these reviews have some limitations. They are focused on specific outcomes (MI or VTE or stroke), include only specific study designs (case-control only), search available literature only within few databases and are non-quantitative or largely nonsystematic in nature. Therefore, an updated and comprehensive quantitative review is important, given the

different types of cardio-metabolic outcomes that may be affected by POC use by women of childbearing age.

This systematic review and meta-analysis aims to investigate the impact of POC use on the risk of developing various cardio-metabolic outcomes such as MI, stroke, VTE, diabetes and hypertension.

METHODS

Data Sources and Search Strategy

The Cochrane Handbook for Systematic Reviews of Interventions and PRISMA and MOOSE guidelines were used to guide the conduct and reporting of this review [19,20]. We conducted a literature search of articles from the following electronic databases from the earliest record to January 16, 2017: PubMed, Web of Science and EMBASE. The search strategy was built based on the PICOS and followed the recommendations of the Cochrane review for progestin only pill [21]. The following key words were searched: "progesterone only pill", "progesterone", "progestin only", "progestogen only", "cardiovascular disease", "heart disease", "cerebrovascular disease", "stroke", "myocardial infarction", "coronary artery disease", "venous thromboembolism", "diabetes" and "hypertension". Additionally, reference lists of the included studies and relevant reviews, as well as studies that have cited these articles, were searched with Elsevier's Scopus, the largest abstract and citation database. The detailed master search strategy is shown in Supplemental Figure 1.

During the first phase of screening, two reviewers evaluated the titles and abstracts against the inclusion and exclusion criteria. For each potentially eligible study, two reviewers independently assessed the full-text. In cases of disagreement, a decision was made by consensus or, if necessary, a third reviewer was consulted.

Study Selection and Eligibility Criteria

Studies were included if they met all of the following inclusion criteria: (i) used a randomized trial, case-control, cohort (prospective or retrospective), or cross sectional study design; (ii) reported presence of a treatment arm featuring use of POCs; (iii) reported use of progestin for the purpose of contraception only; (iv) collected data on incidence of cardiovascular disease (MI, stroke, heart disease, VTE events), diabetes and hypertension;

and (v) were based on human data only and reported odds ratio or relative risk comparing use of POCs with non-users of contraceptives.

Data Extraction

Two reviewers independently extracted data and consensus was reached in case of any inconsistency with involvement of a third reviewer. A piloted data extraction form was used. This included data on study size; study design; baseline population; location; age at baseline; duration of follow-up; reported degree of adjustment; type of POC use, type and numbers of outcomes; how outcomes were ascertained; and reported risk ratios. In instances of multiple publications, the most up-to-date information was extracted.

Assessing the Risk of Bias

Bias within each individual study was evaluated by two independent reviewers using the validated Newcastle-Ottawa Scale (NOS), a semi-quantitative scale designed to evaluate the quality of non-randomized studies [22]. The assessment of the study quality was based on the selection criteria of participants, comparability of cases and controls, and exposure and outcome assessment. Studies that received a score of nine stars were judged to be of at low risk of bias; studies that scored seven or eight stars were considered at medium risk; those that scored six or less were considered at high risk of bias. Detailed information on the assessment of study quality and risk of bias is provided in **Supplemental Table 1 and 2**.

Patient involvement

Patients were not involved in our study.

Statistical Analysis

We estimated the risk ratio of cardiovascular diseases (VTE, MI and stroke) for users of POCs versus non-users in subgroups according to route of administration (oral, injectable and intrauterine). Based on previous reports estimating the yearly incidence of those events to about 0.06% per year [23], we considered that cardiovascular events had a low incidence (<10% a year) in women aged <50 years taking oral contraceptives. For infrequent events, the relative risk and odds ratio are considered equivalent measures of relative risk [24,25]. For initial disease risks of 10% or less, even odds ratios of up to eight can reasonably be interpreted as relative risks [26]. For each study, we used the most adjusted relative risk with

its 95% confidence interval (CI) and we used the inverse variance weighted method to combine relative risks to produce a pooled relative risk using random-effects meta-analysis models, to allow for between study heterogeneity. We also conducted sensitivity analyses using fixed effects models and we present the results in the forest plots. Furthermore, where a study reported more than one risk estimate, the pooled relative risk was obtained using fixed-effects model. A narrative synthesis and construction of descriptive summary tables were performed for those study outcomes that could not be quantitatively pooled. Heterogeneity was quantified using the I^2 statistic, classified as low (I^2 ≤25%), moderate (I^2 >25% and <75%), or high (I^2 ≥75%) [27]. Additionally Q statistic was used to assess the presence of heterogeneity. $P_{Q\ statistic}$ ≥0.05 was considered to indicate no significant heterogeneity among the included studies. Publication bias was assessed through a funnel plot and asymmetry was assessed using the Egger's test. It was not feasible to perform sensitivity analyses due to small number of included studies. All tests were two-tailed and p-values of 0.05 or less were considered significant. STATA 14 (Stata Corp, College Station, Texas) was used for all statistical analyses.

RESULTS

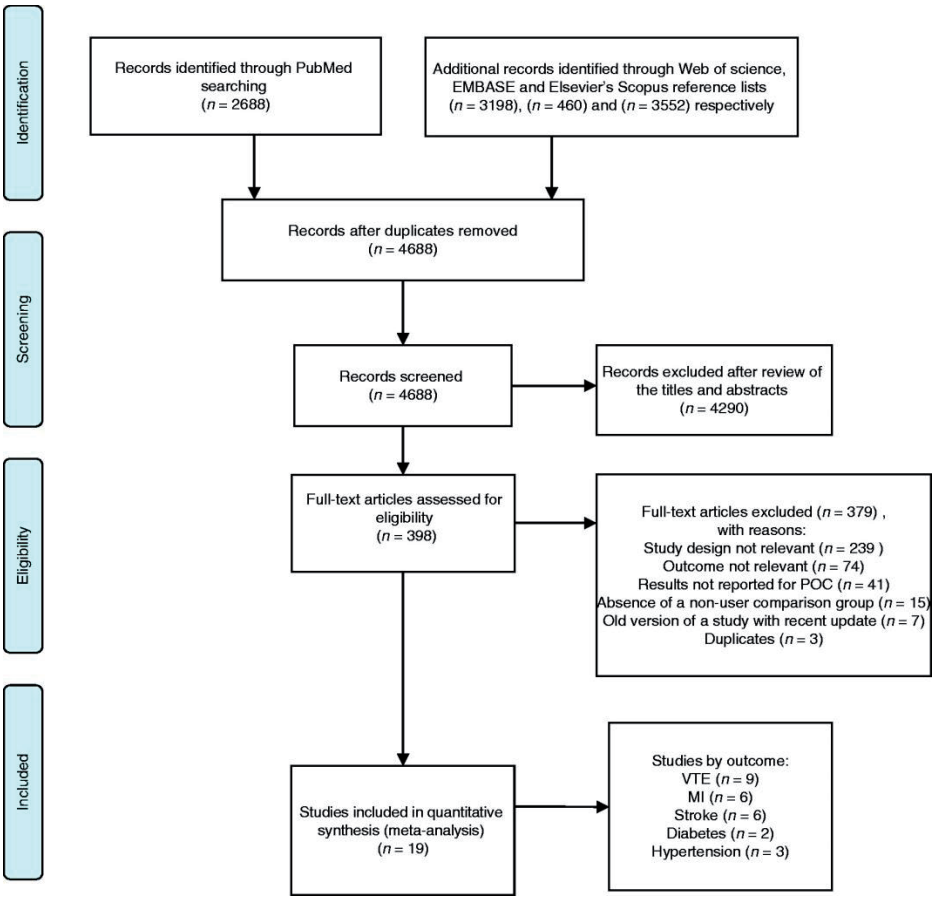
Study Identification and Selection

A total of 9898 references were identified: 2688 from PubMed, 3198 from Web of Science, 460 from EMBASE and 3552 from the search in Elsevier's Scopus **(Figure 1)**. Of these, 5210 duplicates were removed, and 4290 were excluded after review of the titles and abstracts, leaving 398 articles for full-text screening. After full-text assessment 19 articles were included in this review. Of these studies, 2 were nested case-control studies, 10 case-control studies, and 7 cohort studies. No randomized clinical trial was found. Nine studies reported the risk of VTE, 6 studies reported risk of MI, 6 reported risk of stroke, 3 reported risk of hypertension and 2 studies reported risk of developing diabetes. *Characteristics of Included Studies*

In total, 19 studies were included in this review, including data from 62 088 women of which 11 930 women reported using POC. The majority of the included studies were conducted in Europe (n=12) followed by USA (n=5). In addition, there were two multi-country studies. The age of participants ranged from 15 to about 66 years. Fifteen studies

reported on POC administered orally, five studies by injection, implant or intrauterine device.

Figure 1: Flow diagram of studies included in the review



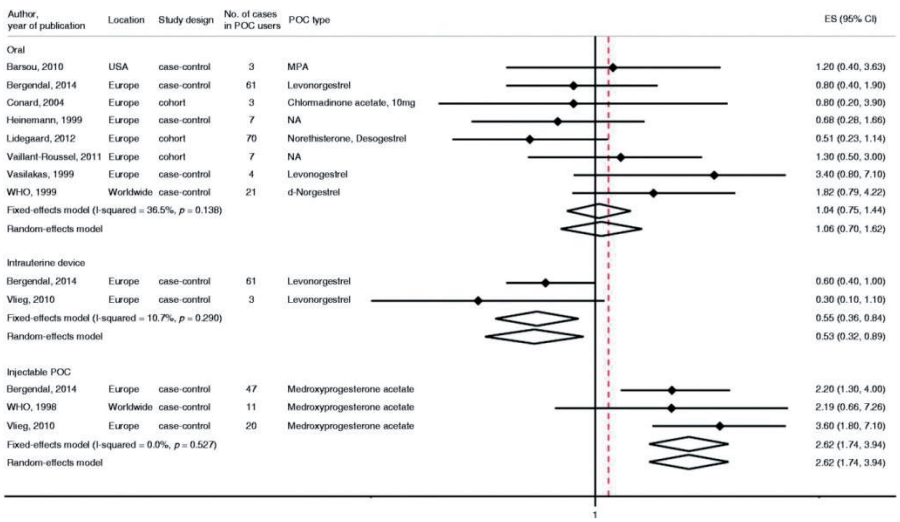
Progestin-only contraceptive use and risk of Venous Thromboembolism

POC use and the risk of VTE were reported in nine articles [11,12,28-34], four of which were retrospective case-control studies, two were nested case-control studies and three were cohort studies. The details on study participants can be found in **Supplemental table 3**. Eight studies investigated the risk of VTE with oral, two studies with intrauterine and three with

injectable POC. Therefore, we have estimated the fully adjusted (as reported in studies) risk ratio of VTE for POC users versus non-users in each subgroup according to route of administration (oral, injectable and intrauterine).

Pooled fully adjusted risk ratios, based on more than 500 women using POC and 176 VTE events, showed no significant association of oral POC use with the risk of VTE when comparing users with non-users [pooled risk ratio, 1.06 (95% CI, 0.7 to 1.62)]. There was no evidence of high between-study heterogeneity for POC use and risk of VTE in these studies (I^2 = 36.5% and $P_{Q\ statistic}$ 0.14). Only three case-control studies reported risk of VTE with injectable (72 controls 78 cases) and two studies reported on intrauterine (125 controls and 64 cases) progestin administration. The pooled risk ratio of VTE for users of intrauterine POC formulation (Levonorgestrel) was 0.53 (95%CI 0.32 to 0.89), I^2 = 10.7% and $P_{Q\ statistic}$ 0.29. On the other hand, the RR of VTE for injectable progestin formulation (DMPA) was 2.62 (1.74 to 3.94), I^2 = 0% and $P_{Q\ statistic}$ 0.53 **(Figure 2)**.

Figure 2: The association between POC use and risk of venous thromboembolism by route of administration

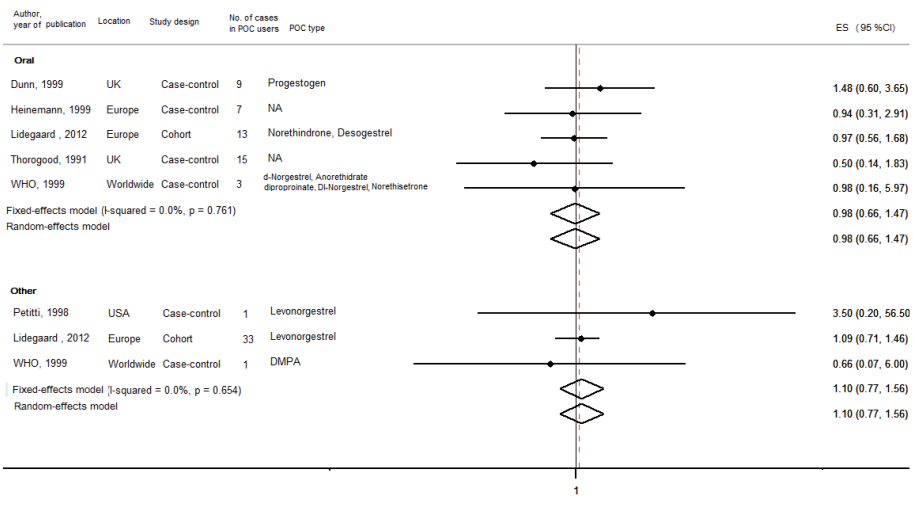


The summary estimates presented were calculated using random effects and fixed effects models; Size of data markers are proportional to the inverse of the variance of the odds ratio; CI confidence interval (bars). P comes from Q statistics

Progestin-only contraceptive use and risk of Myocardial Infarction

Six studies reported the risk of MI with POC use [29,35-39] **(Supplemental table 4)**. Of those, five were case-control studies and one was a cohort study. Five studies reported RR after oral POC administration, two studies reported RR in women using progestin implants, and one study reported RR of MI after injectable and intrauterine POC administration. The adjusted relative risk of MI for users of POC versus non-users varied from 0.5 to 3.5, none of the studies reporting a statistically significant association. Pooled results for the fully adjusted models, based on more than 150 women using POC and 47 MI cases, showed that there was no significant association of MI risk with those who used POC orally versus those who did not use hormone therapy [pooled risk ratio, 0.98 (95% CI, 0.66 to 1.47)] (**Figure 3)**. In addition, there was no evidence of between-study heterogeneity for POC use and risk of MI in these studies (I^2= 0% and $P_{Q\ statistic}$ 0.72). The pooled RR for MI in the subgroup of women using progestin otherwise than orally was 1.10 (95 %CI 0.77 to 1.56), I^2= 0% and $P_{Q\ statistic}$ 0.65.

Figure 3: The association between oral POC use and risk myocardial infarction by route of administration

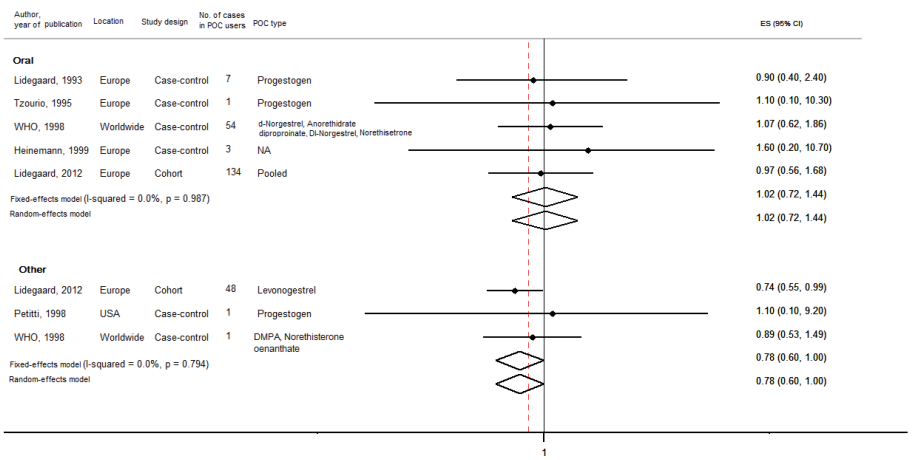


The summary estimates presented were calculated using random effects and fixed effects models; Size of data markers are proportional to the inverse of the variance of the odds ratio; CI confidence interval (bars). P comes from Q statistics

Progestin-only contraceptive use and risk of Stroke

Six studies examined the association between POC use and risk of stroke [29,36,38-41] **(Supplemental table 5)**. Of those, five were case-control studies and one was a cohort study. The adjusted relative risk of stroke for users of POC versus non- users varied from 0.89 to 1.6, none of the studies reporting a statistically significant association. The summary measure from pooled analysis including 350 women using progestin contraceptives orally and 199 stroke events showed no significant evidence to suggest that the use of POC is associated with the risk of stroke [pooled risk ratio, 1.02 (95 %CI 0.72 to 1.44)] for the fully adjusted model (**Figure 4**). There was no evidence of between-study heterogeneity for stroke risk and POP use (I^2=0% and $P_{Q\ statistic}$ 0.99). The pooled RR of stroke in women applying POC other than orally was 0.78 (95CI% 0.6 to 1), I^2=0% and $P_{Q\ statistic}$ 0.79.

Figure 4: The association between oral POC use and risk of stroke by route of administration



The summary estimates presented were calculated using random effects and fixed effects models; Size of data markers are proportional to the inverse of the variance of the odds ratio; CI confidence interval (bars). P comes from Q statistics

Progestin-only contraceptive use and risk of Hypertension

Only three cohort studies were found to report the impact of POC use with the risk of developing hypertension [42-44] **(Supplemental table 6)**. A study by Spellacy and Birk et al. [42] followed 415 predominantly American black women for two years and reported that those who used POC had a significant drop in diastolic blood pressure ($p<0.05$). Still, most of women in this study were 4 weeks postpartum, and subsequently using mini pills as contraceptive method, which might present a bias in case selection. However, two studies [43,44] of 119 and 593 participants respectively, reported that POC use had no significant effect on blood pressure. These studies were limited by small sample size, inadequate adjustment for confounders and lost to follow up bias.

Progestin-only contraceptive use and risk of Diabetes

We found two epidemiological studies that investigated the association between POC and risk of developing T2D. A case-control study by Kim et al. (2001) [45] reported the association of POC use and risk of developing type II diabetes (DM II) in a health centre in USA **(Supplemental table 7)**. Diabetic cases (n = 284) and non-diabetic controls (n = 570) were matched by age. It was found that users of POC (DMPA) were at an increased risk of developing diabetes compared to those who used combined pill (estrogen-progestogen), odds ratio: 3.6 [95% CI, 1.6 to 7.9], after adjusting for age and BMI. When compared with no history of hormonal contraceptive use, POC was still associated with the risk of developing diabetes, odds ratio 2.1 [95% CI 1.03-4.2], when adjusted for age, BMI and parity. However, further adjustment for gestational diabetes diagnosed after contraceptive given attenuated and abolished the association, odds ratio 1.6 [95%CI 0.77- 3.5]. In cohort study [46], Norplant users (n=7 977) were prospectively compared with age-matched, non-hormonal intrauterine device users (n=6 625) and women who underwent sterilization (n=1419). Twelve T2D cases were identified- nine in Norplant initiators (eight current users), two in IUD initiators (three current IUD users) and one in a sterilized woman. The crude incidence rate was higher in current Norplant users compared with controls, but the crude and adjusted rate ratios for Norplant users compared with controls were not significantly different. After adjusting for clinic, age, and body weight, the implant current users didn't have significantly higher incidence of T2D as compared with the group using IUD or sterilization, RR, 2.4 (95 % CI 0.7-8.1).

Study Quality and Publication Bias

Three studies were classified to have low risk of bias, five to have medium risk of bias and the rest were classified to have high risk of bias. We did not find evidence for publication bias from the funnel plots of VTE, MI and stroke, as shown in **Supplemental figure 2.**

DISCUSSION

Overall, the available body of literature suggests that use of oral POCs is not associated with excess risk of VTE, MI, stroke and hypertension. We found limited evidence that DMPA is associated with increased risk of VTE while intrauterine application of Levonorgestrel was associated with decreased risk of VTE. There was, also, an indication for increased risk of diabetes with injectable POC, albeit non-significant.

Our findings suggest no effect on VTE risk after oral POC and a decreased risk of VTE in subgroup of women using intrauterine Levonorgestrel device with RR 0.53 (95% CI 0.32 to 0.89). However, the subgroup analysis based on three studies [12,33,39], including 78 VTE events, showed 2.6-fold increased risk of VTE for injectable progestin users comparing to non-users. A study that contributed most to the summary statistic for DMPA and risk of VTE, excluded women in highest risk of VTE (personal history of venous thromboembolism) (12), therefore, it is less likely that the effect observed on injectable progestin is due to confounding by indication. POC intake causes a decrease in sex hormone–binding globulin, which is a marker of venous thrombosis risk, and this effect varies with the dose and type of progestogen used [47,48]. Indeed, the plasma concentration of Levonorgestrel with intrauterine device range between 74 and 166 pg/mL[49], while after intramuscular injection of 150 mg of DMPA the peak plasma concentration is 2500–7000 pg/mL and remains greater than 430 pg/mL at three months [50,51]. Also, progestins may express prothrombotic properties via modulation of protein C resistance [52], by affecting cellular expression of tissue factor and circulating tissue factor pathway inhibitor [53,54]. The third generation progestins (eg. desogestrel) are suggested to be more prothrombotic than earlier formulations such as levonorgestrel or norethisteron [10]. Levonorgestrel does not increase the activated protein C resistance, suggesting that this contraceptive does not have a prothrombotic effect [52]. While, in a mouse model of vascular injury MPA increased thrombin formation and changes

in vascular gene expression resulting in altered plaque matrix either alone and in combination with estradiol [55].

A previous meta-analysis of six case-control studies reported that there was no increase in the MI risk with POC use [16]. In our meta-analysis we excluded one of the studies included in previous estimates, as it was investigating the effect of COC (contained up to 50 μg of estrogen combined with a fixed dose of progestin) [56], and not progestin-only pill, still our results are in line with previous findings. The result was similar according to the route of administration, including implant, injectable, and oral POC. Furthermore, our findings are in line with a previous meta-analysis of six case-control studies [17] showing that POC use had no significant effect on the risk of developing stroke. Similarly, a systematic review looking at the association of POC use with high blood pressure also concluded that POC use does not affect diastolic and systolic blood pressures [15]. However, all of the included studies were written in 70s and 80s, therefore, they have investigated the 1st and 2nd generation of POC, while the information on 3rd generation of progestins are lacking. Contraceptive Use advocates the use of POC for women at high risk of cardiovascular disease (70), which may be a safe recommendation as also supported. Furthermore, an important limitation of these studies is the fact that they investigated POC use in normotensive women, yet, future studies should investigate the effects of POC on blood pressure in women with history of hypertension.

A case-control study done among Navajho women showed that use of injectable POC significantly increased the risk of developing T2D when adjusted for age, BMI and parity, however, after further adjustment for gestational diabetes diagnosed after contraceptives given the association was attenuated and not any more significant [45]. Women with gestational diabetes are at higher risk for developing T2D, and women who used DMPA were significantly more likely to have a history of gestational diabetes [45]. Therefore, it might be that the gestational diabetes is on the pathway between POC use and T2D development, which needs further investigation. Nevertheless, a study conducted in breast-feeding Latina women with prior gestational diabetes mellitus, demonstrated that oral POC were associated with increased risk of diabetes as compared with equal use of low dose combination OCs, indicating that, if an association between POC use and diabetes exists,

other pathways other than gestational diabetes may be present [57]. In this study, however, low-dose progestin and COCs were not associated with risk of diabetes [57].

The mechanism linking POC use with potential increased risk of diabetes is unknown. A possible mechanism might be the adverse effect that POC use has on obesity, which is an important risk factor for diabetes [45]. The 2016 Cochrane review investigated the association between progestin-only contraception use and weight changes. They report little evidence of weight gain when using progestin-only contraceptives. Actual mean weight gain was generally low (<2 kg for most studies) for 6 to 12 months of follow-up. [58]. However, the effect on weight varied with different formulations and routes of POC administration [13,59-61]. Furthermore, using contraception reduces numbers of pregnancies, that is also considered to be a risk factor of developing diabetes [45]. Also, decrease in sex-hormone binding globulin is associated with injectable DMPA [62] and a low circulating levels of sex hormone–binding globulin are a strong predictor of the risk of type 2 diabetes in women and men [63]. The other possibility is that women taking COC comparing to one receiving DMPA are healthier and have lower risk of developing T2D [45]. Indeed, a systematic review on studies in nondiabetic women based on 6 studies investigating DMPA use reported elevation of insulin concentration (compared with baseline before DMPA) at 2–3 h after the glucose challenge [14], however, most of the studies included in review did not find any effect of injectable contraceptives on glucose concentrations in lean glucose tolerant women. Studies that reported increased glucose were done in subjects who had higher baseline body weight or had longer duration of POC use [14].

Strengths and Limitations

Our results are consistent with previously published reviews [10,15-18], however, this is the first systematic review and meta-analysis that looks at the association of POC use with multiple cardio-metabolic outcomes such as VTE, MI, stroke, hypertension and diabetes. Nevertheless, there are number of limitations of this review. The studies included in this review were limited by study design and methodology: (i) all studies were observational in nature and thus prone to bias and confounding, (ii) had small number of participants using POC, which explains the wide confidence intervals of some of the studies and (iii) studies did not specify the type and dosage of POC or the type and dosage of POC varied

considerably. Although, the prevalence of intermediate risk factors for cardiovascular is low among women of reproductive age, still, 10% of women ages 18–44 years have high blood pressure, while, 15% of women ages 20–45 years have high cholesterol, also, 3% of women in reproductive age have T2D [64,65]. Overall, the studies included in this review adjusted for only a limited number of potential confounders and mediators mainly age and BMI, however, the outcomes under study are complex and thus other potential confounders should have been taken into account to make valid conclusions. Eg. increasing evidence show the association between use and risk of T2D [66], therefore, future studies should take this into account when investigating the risk of diabetes with POC use.

Implications for policy and future research

European Guidelines on cardiovascular disease prevention in clinical practice emphasized the role of cardiologist in assessing the bassline cardiovascular risk before advising the type of contraceptives to be used [67]. U.S. Medical Eligibility Criteria for Contraceptive Use advocates the use of POC for women at high risk of cardiovascular disease [68], which may be a safe recommendation as also supported by our findings of no association between oral POC use and VTE, MI and stroke in women in general. Although, the U.S. Medical Eligibility Criteria for Contraceptive Use [68] recognize previous history of MI and stroke, as well as hypertension as contraindications for injectable POCs use, previous history of VTE is not recognized as contraindication for DMPA. Therefore, based on our findings of increased risk of developing VTE and present indication of increased risk of type 2 diabetes further investigation is required in order to rule out potential harmful effect of DMPA in these women.

CONCLUSIONS

In conclusion, studies included in this meta-analysis suggest that progestin-only contraceptive use is not associated with increased risk of developing various cardio-metabolic outcomes. However, our findings, based on limited evidence, suggest that increased risk of VTE, might be present for injectable POC, as well as some indication for increased diabetes risk. Also, there is some indication that intrauterine Levonorgestrel device might be safe choice in regard of VTE risk. Nevertheless, this systematic review must be interpreted with caution as the studies included in the review were observational in

nature and meta-analyses results were based on studies with small sample size. Rigorous studies with better quality design and lower risk of bias are needed to determine the true impact of POC use on various cardio-metabolic outcomes.

Supplement available online at:

http://journals.sagepub.com/doi/suppl/10.1177/2047487318774847/suppl_file/Supplemental_material.pdf

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CHAPTER 3.2

The route of administration, timing, duration and dose of postmenopausal hormone therapy and cardiovascular outcomes in women: a systematic review

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Manuscript under review

ABSTRACT

BACKGROUND: The effect of postmenopausal hormone therapy (HT) on cardiovascular disease (CVD) risk remains controversial.

OBJECTIVE AND RATIONALE: We aimed to systematically review the evidence regarding the role of dose, route of hormone administration, timing of initiation and duration of HT on cardiovascular risk among postmenopausal women.

SEARCH METHODS: The electronic databases Medline Ovid, Web of Science, and Cochrane Central were systematically searched to identify studies published before 30th January 2018. Reference lists, using Elsevier's Scopus, of the included studies were searched for further identification of relevant studies. Clinical trials and observational studies that assessed clinical and subclinical cardiovascular outcomes in relation to dose, route of administration, duration of use, or timing of HT initiation among postmenopausal women were included. Data were extracted by independent reviewers using a pre-designed data collection form.

OUTCOMES: In total, 33 unique studies (6 trials and 27 prospective observational studies) were identified, including a total of 2 588 327 women. Overall, the evidence did not support the concerns that oral nor transdermal HT increases heart disease risk. Contrary, observational data showed that beneficial cardioprotective effect can be observed even with use of low doses of oral HT (effect of 0.3mg/d of oral CEE was similar to that seen with the standard dose of 0.625mg/d), but clinical trials to support cardioprotective benefit of HT in primary prevention have not been identified. Furthermore, the current data suggested that oral and transdermal HT, in dose-dependent manner and irrespective of HT formulation, may increase thromboembolic risk, as well as risk of stroke. However, transdermal estrogen with <50 μg/d of estrogen combined with micronized progesterone appears to be safer choice with respect to thrombotic and stroke risk. Also, vaginal HT administration may play a role in myocardial infarction and stroke risk prevention, but this is based on limited evidence and requires further investigation. The timing of HT initiation and duration may be important factors to consider when prescribing HT especially in women with adverse cardiometabolic profile and pre-existing conditions such as coronary/carotid atherosclerosis, which are at risk of developing, and thus progressing to cardiovascular disease.

CONCLUSIONS: Use of low dose oral and transdermal HT appears to be safe with regard of CVD risk in women in menopausal transition and within the first years (e.g. 10 years) after menopause onset. In women with increased baseline thromboembolic risk, transdermal estradiol alone or with micronized progesterone shall be suggested as first-line treatment. In case that HT shall be initiated after 10 years since the menopause onset (>60years old), due to greater absolute risks of coronary heart disease, stroke and venous thromboembolism, HT shall be used for the shortest time possible and in lowest possible dose, and preferably transdermal administration should be recommended. However, individualized treatment approach including baseline CVD risk assessment shall be applied when prescribing HT.

Introduction

Menopause, Climacteric Symptoms and Hormone Therapy

Menopause is considered the end of a woman's reproductive life and is generally defined by cessation of menstrual periods for 12 consecutive months [1,2]. Menopausal transition may start several years before and is characterised by irregular menstrual cycles and the presence of menopausal symptoms [3]. The most challenging climacteric symptoms are vaginal dryness and vasomotor symptoms with 50.3% to 82.1% of menopausal women reporting hot flashes or night sweats [4,5]. The duration and intensity of menopausal symptoms varies considerably among women, although most women report that they last between six months to two years [6]. Also, symptoms could be of different severity, with up to 42% women aged 60 to 65 years experiencing moderate to severe vasomotor symptoms [7]. Certainly, vasomotor symptoms are the main indication for hormone therapy (HT) use. Estrogen products are proven to be efficient in the reduction of hot flashes and are superior to other non-hormonal therapies [8]. However, the effectiveness of HT greatly varies with HT characteristics and currently there are no arbitrary limits regarding the dose and duration of use of HT. While most women will no longer have symptoms after 5 years of treatment, some women may experience long-term hot flashes, in extreme cases even lifelong [9]. Also, women with premature ovarian failure might need a higher dose of estrogen to control vasomotor symptoms than their older counterparts [9].

HT formulations can include either estrogen alone (estrogen-only HT) - mainly indicated for women who have hysterectomy (surgical removal of uterus), or estrogen combined with progestogen (combined HT) - which is mainly indicated for women with uterus [6]. HT is used in a variety of formulations and doses and can be taken orally, and as an implant, skin patch or cream (trans-dermally, vaginally). The clinical effects vary according to the type of HT and the duration of its use [6]. The most commonly prescribed is oral HT, and the most common estrogens used are conjugated equine estrogen (CEE), synthetic conjugated estrogens, micronized 17b-estradiol and ethinyl estradiol, while commonly used progestins are medroxyprogesterone acetate (MPA), norethindrone acetate, and native progesterone [10]. Though MPA is mainly given orally, levonorgestrel and norethisterone are available in transdermal patches combined with estradiol; and levonorgestrel can be delivered directly to the uterus with an intrauterine device [9].

For decades HT has been crucial for achieving menopausal symptom relief and improving the quality of women's lives. However, HT has been accompanied by specific cardiovascular health concerns, which could depend on HT preparations and dosages [11].

Endogenous vs. exogenous estrogen and CVD Risk: The conundrum

Premenopausal women have a lower CVD risk compared to age-matched men, however, this sex-advantage for women gradually disappears after menopause[12]. This increase in CVD risk after menopause has been attributed to the sharp decline of estrogen levels, suggesting a potential cardioprotective effect of endogenous estrogen in women before the menopause [12]. Various potential cardioprotective effects of endogenous estrogen have been suggested. Estradiol has beneficial effects on key elements in the pathogenesis of CVD: inflammation [13], endothelial function [14] and lipid profile [15]. When HT was introduced it was hypothesized to reduce CVD risk. Although observational data have suggested that HT decreases the risk of CVD and reduces mortality in postmenopausal women with heart disease [16-19], large-scale clinical trials (Women's Health Initiative-WHI and the Heart and Estrogen/Progestin Replacement Study (HERS) I and II) indicated an unfavourable HT effect on CVD risk [13]. WHI reported 30–40% elevated risk of stroke for women given estrogen combined with progestin or estrogen alone [20,21]. In line with this, the Nurses' Health Study (NHS) reported 35% increased risk of stroke with current use of hormone therapy [16]. Yet, the latest update from WHI showed that HT with CEE + MPA or with CEE alone was not associated with risk of all-cause, cardiovascular, or cancer mortality during a cumulative follow-up of 18 years [22]. Although consistent evidence suggests estrogen therapy may be cardioprotective if started around the menopause onset and harmful if started in later stages of menopause (> 10 years), the evidence was inconsistent with combined HT, suggesting a potential attenuation of the coronary benefit with using a continuous progestogen [23]. The current evidence on HT and CVD risk is conflicting, with HT being reported to cause both beneficial and detrimental effects. Many potential factors have been suggested to contribute to the adverse outcomes: the dose, route, the type of HT given (conjugated equine estrogen with progestin), the timing of HT initiation/the age of women, a history of CVD/increased CVD risk and the thromboembolic properties of estrogen and progestin. To date, despite the widespread use of HT, there is no a comprehensive review on how CVD risk differ by dose, duration, route and timing of initiation of HT treatment.

We aimed to systematically review and summarize the available evidence on the association between HT and CVD risk in post-menopausal women and whether these effects differed by timing of initiation, route of administration, duration and dose of HT.

Methods

Data Sources and Search Strategy

This review was conducted using a predefined protocol and in accordance with PRISMA and MOOSE guidelines. Three electronic databases (Medline Ovid, Web of Science and Cochrane Central) were searched until 30th January 2018 without any language restriction. The computer-based searches combined terms related to (1) the menopause (e.g., *menopausal*) in humans, (2) HT and the factors relevant to this review (e.g., *timing, duration, dose, administration*) and (3) cardiovascular outcomes (atherosclerosis, peripheral arterial disease, *carotid intima-media thickness, stroke, transient ischemic attack, heart failure, coronary heart disease, angina, chest pain, venous thromboembolism*). Details of the search strategy can be found in Supplemental table 1.

Two independent reviewers screened the titles and abstracts of all studies initially identified, according to the selection criteria, and any disagreement was resolved through consensus or consultation with a third independent reviewer. Full texts were retrieved from studies that satisfied all selection criteria. Reference lists of the included studies and relevant reviews, as well as studies that have cited these articles, were hand-searched and searched with Elsevier's Scopus, the largest abstract and citation database.

Study Selection and Eligibility Criteria

Intervention studies were eligible if they: (i) were randomized controlled trials (RCTs), non-randomized controlled trials, or prospective observational studies; (ii) assessed the effects of the timing, duration, dose or route of administration of HT in menopausal, or postmenopausal women compared to a placebo or no treatment and (iii) collected subclinical or clinical cardiovascular endpoints. To maintain consistency and due to difficulty in interpreting results, head-to-head trials that compared non-hormonal therapies with estrogen or with other medications were excluded. No restrictions on length of follow up were applied.

Data Extraction

Two authors independently extracted data and a consensus was reached in case of any inconsistency with involvement of an additional author. A predesigned electronic data

abstraction form was used to extract relevant information. This included questions on baseline population; location; age at baseline; study design; number of participants; type and dose of intervention; duration of treatment or follow-up; timing of intervention; route of administration; comparisons; outcome measures, and results for each outcome (odds ratios, risk ratios, hazard ratios or mm/year for subclinical measurements). Additionally, for intervention studies, allocation concealment and blinding were also recorded. In case of multiple publications, the most up-to-date or comprehensive information was extracted.

Assessing the Risk of Bias

The Cochrane Collaboration's tool [24] and the Newcastle-Ottawa Scale [25] were used by two independent investigators to assess the risk of bias in RCTs and in prospective observational studies, respectively. The Cochrane Collaboration's tool evaluates seven possible sources of bias: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting and other bias. The Newcastle-Ottawa Scale uses a star system (with maximum of nine stars) to evaluate three domains: selection of participants; comparability of study groups; and the ascertainment of outcomes and exposures of interest. Studies that received a score of nine stars were judged to be of at low risk of bias; a score of seven or eight stars was medium risk; those that scored six or less were considered at high risk of bias. Furthermore, we applied the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach to score the quality of evidence and therefore improve the communication with the users and make sure they are aware how much confidence they can place in the recommendation provided by this review. The GRADE judgment about the quality of evidence is based on two key concepts: magnitude of effect and quality of evidence. The evidence is graded from high, moderate and low to very low. RCT start as high quality and observational studies start as low quality. Limitations in study quality, important inconsistency of results, or uncertainty about the directness of the evidence can lower the grade of evidence. Also, certain factors such as evidence of a dose response gradient or strong evidence of association based on consistent evidence from two or more observational studies with no plausible confounders may increase the grade [26].

Results

Study Identification and Selection

In total, we identified 11,591 relevant citations, of which 3,982 were duplicates. After screening based on titles and abstracts 7, 480 studies were excluded, and 129 articles were selected for detailed evaluation of their full texts. Of those, 54 articles, based on 33 unique studies, met the inclusion criteria and were included in the review: 16 studies examined the dose of HT, 12 studies examined the route of administration, 8 studies examined the role of timing of HT initiation and 30 examined the duration of HT use (**Figure 1**).

Characteristics of Included Studies

Among the 33 included studies, 6 were clinical trials and 27 were prospective observational studies. In aggregate, the studies reported results for 2 588 327 women (2 541 092 from observational studies and 47 235 from RCTs). Seventeen studies were based in Europe; 16 in North America; and none in South America, Australia, Asia and Africa. The baseline age of participants ranged from 30 to 94 years. For trials, the duration of the interventions ranged from 0.5 years to 7.2 years, while for prospective observational studies ranged from 1 to 28 years.

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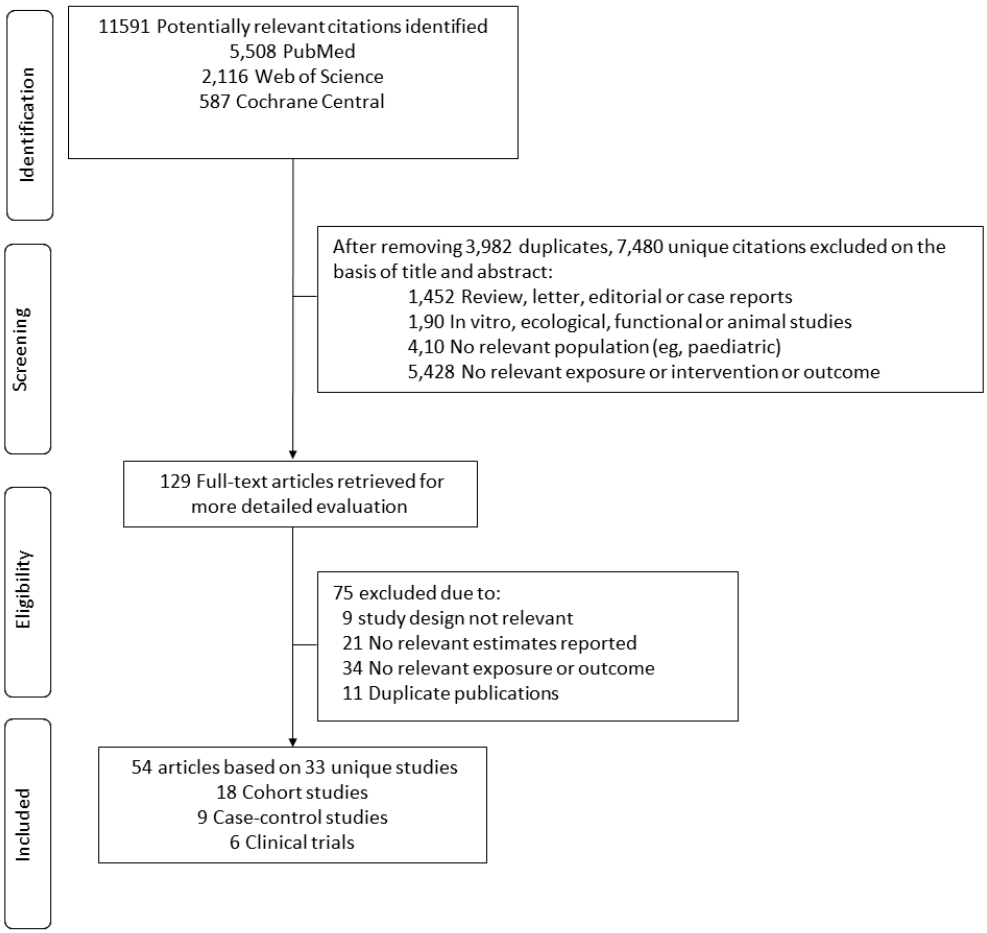
Dose of HT and CVD risk

Sixteen studies, one RCT and 15 observational studies [16,17,27-40] examined the association between HT dose and various CVD outcomes. Findings are summarized in **Key box 1** and detailed study characteristics are provided in **Supplemental table 2.**

Figure 1. Flow diagram of the study selection process



Key box 1. Hormone therapy dose and cardiovascular risk	Evidence Quality
Low HT dose:	
• In observational studies, beneficial cardioprotective effect has been observed even with low HT doses (effect of 0.3mg/d of oral CEE was similar to that seen with the standard dose of 0.625mg/d).	**B**
• The RCTs to support cardioprotective benefit of HT in primary prevention have not been identified.	**D**
High HT dose:	
• VTE and stroke risk increases in dose-dependent manner with higher estrogen dose in estrogen alone or combined HT formulations; caution is needed with >0.625mg/d of estrogen in oral formulations and >50 μg/d in transdermal formulations.	**B**
• Thrombotic risk was significantly higher with preparations containing medroxyprogesterone acetate (MPA).	**B**
General conclusion:	
• HT should be used in the lowest effective dose to avoid adverse cardiovascular effects, and with advancing age HT dose should be reduced.	**B**

Findings are based on sixteen studies, one RCT and 15 observational studies examined the effect of HT dose on various CVD outcomes; Low dose: 0.3mg- 0.625mg; Medium dose: 0.625-1.25mg; High dose: ≥1.25mg
Abbreviations: HT: hormone therapy; VTE: venous thromboembolism; B: moderate quality of evidence (We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different); C: low quality of evidence (Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect)

Nine observational studies reported the association between HT dose and heart disease risk, reporting in general no association or cardioprotective effect. In Nurses' Health Study (NHS), among women with no history of heart disease (during 488 801 person-years of follow-up), the risk for coronary events was similarly reduced in those currently taking 0.625 mg of oral CEE daily (RR 0.54, 95%CI, 0.44-0.67) and those taking 0.3 mg of oral CEE daily (RR 0.58, 95%CI 0. 37- 0.92) compared with never-users [16]. The latest publication from the same study, investigating estrogen only and combined HT in women with history of heart disease, reported 30% lower risk of CHD for women using estrogen alone or combined HT compared with postmenopausal women who never used hormones. Although, findings were similar across various doses of oral conjugated estrogen, only the medium estrogen dose (0.625mg/d combined with progestin) was significantly associated with reduced CHD risk, RR 0.70 (95% CI 0.59–0.83)[28]. In WHI, in a subset of 1 246 women and during a median

of 10.4 years of follow-up, women who used oral low dose CEE (<0.625mg/d) had non-significantly lower rates of CHD, total CVD and CVD mortality comparing to women who used oral conventional-dose CEE (0.625mg/d) [29]. A study of 635 women reported a decreased MI risk with a medium dose of oral/transdermal HT; the corresponding ORs for low, medium, and high doses were 0.96 (95% CI 0.55 to 1.65), 0.59 (95% CI 0.42-0.82), and 0.75 (95% CI 0.48-1.19) respectively [31]. Similarly, a larger study of 24 420 women showed a decreased risk of MI with oral low and medium dose of estrogen, but there was no evidence of decreased MI risk with high estrogen dose [33]. A cohort study among 9 236 Swedish women reported reduced risk of developing MI with medium estrogen dose (0.625mg/d of CEE, or 2mg/d of estradiol) as compared to low dose HT, (RR 0.75, 95% CI 0.56-0.99) [39]. In addition, we found three observational studies that showed no evidence that MI risk varied with HT dose [30,32,41]. In the single RCT we included in our review, in 321 healthy postmenopausal women at increased CVD, neither of combined HT regimens (with standard and low progestin) slowed intima-media thickness (IMT) progression within 1 year of follow-up [27].

Two observational studies reported an increased risk of particular vascular events such as VTE and TIA with increasing HT dose – one study showed an association of VTE with estrogen only and combined oral and transdermal HT [34] and another demonstrated increased risk of TIA associated with oral and transdermal estrogen only HT [42].

We identified seven studies reporting the association between HT dose and stroke risk, with conflicting results. A matched case-control study including more than 70 000 women reported a dose-dependent relationship between transdermal estrogen and stroke risk, with no increased stroke risk with ≤50 μg of transdermal estrogen, and an 1.89-fold increased stroke risk with > 50 μg of transdermal estrogen. However, among women using oral estrogen only and combine HT regimes the stroke risk was increased 1.25 to 1.48-fold in both HT regimes (≤0.625mg/d or ≤2mg/d of estradiol and >0.625mg/d of estrogen or >2mg of estradiol) as compared to non-users [38]. Canonico et al. reported increasing-dose dependent ischemic stroke risk with oral estrogen- the risk was borderline significant with low to medium estrogen dose (<1mg/d) (OR 1.39, 95%CI 1.00 to 1.99), and the greatest in those using high (>1mg/d) estrogen doses (OR 2.41, 95%CI 1.43 to 4.07), however, in contrast to the findings of Renoux et al, stroke risk was not increased with increasing doses of transdermal estrogens [40]. Another study (in more than 15 000 women) reported dose-

dependent stroke risk with no increased stroke risk with low oral estrogens, and increased risk with medium and high dose (≥0.625mg/d) [16]. Lemaitre et al found no evidence of an increased ischaemic stroke risk in users of medium (0.625mg) compared to low (0.3mg) estrogen dose (among 864 women), however, when comparing high (>0.625mg) with low estrogen use, a 2.41-fold increased ischemic stroke risk was observed [35]. In contrast to this, findings from Women's Health Initiative did not find significant difference in stroke risk when comparing low (≤0.625mg) and medium oral CEE dose (>0.625mg), RR 1.07, 95%CI 0.76-1.49 [29]. Two observational studies investigated the risk of hemorrhagic stroke, and there was no significant association observed [16,34]. A study that investigated all routes of estrogen HT administration reported protective effect of HT against death due to stroke, yet there was no difference in regard to HT dose [17]. The inconsistent findings on stroke risk may be the consequence of different HT regimes investigated across different studies. Indeed, increased stroke risk was observed with oral estrogen irrespectively of the dosage [38,40] and with high dosages (>0.625mg/d) or with combined oral HT [16,43]. Transdermal estrogens either did not increase stroke risk [40] or increased the risk in regimes with high dosage of estrogen (>50 μg/d)[38]. No association was found between ischaemic stroke and use of progesterone, pregnanes and nortestosterones, however, ischemic stroke risk was increased with norpregnanes (OR, 2.25; 95% CI, 1.05-4.81)[40]. Also, the greatest VTE risk was observed with HT formulations containing medroxyprogesterone acetate (RR 2.67, 95% CI 2.25- 3.17) [44].

Route of HT Administration and CVD risk

Twelve studies, one RCT and eleven observational studies [4,29,32,38,40,44-50], investigated the association between route of HT administration and CVD risk. Findings are summarized in **Key box 2** and detailed study characteristics are provided in **Supplemental table 3.**

Key box 2. Route of hormone therapy administration and cardiovascular risk	Evidence Quality
Oral HT administration:	
• Does not increase heart disease risk and may be cardioprotective.	**B**
• Increases thromboembolic risk and may increase risk of stroke.	**B**
Transdermal HT administration:	
• Is safe with regard to coronary heart disease risk.	**B**
• Is safer with regard to thrombotic risk as compared to oral HT administration.	**B**
General conclusion:	
• Transdermal estrogen preparation may be safe with regard to CHD and thrombotic risk, and limited evidence indicates no increased risk of stroke associated with use of transdermal estrogen in formulations with < 50 μg of estradiol per day.	**C**
• Besides the route of administration, in combined HT the risk from HT may vary with progestin type used.	**B**
• Vaginal HT administration may play a role in myocardial infarction and stroke risk prevention, but data are limited.	**C**

*Findings are based on twelve studies, one RCT and eleven observational studies; Abbreviations: CHD: coronary heart disease; HT: hormone therapy; A: high quality of evidence (We are very confident that the true effect lies close to that of the estimate of the effect); B:moderate quality of evidence (We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different)

Findings on the association between route of HT administration and heart disease were reported in five studies and in general indicated protective or no effect. In a matched-cohort study involving 5 102 women, transdermal estrogen therapy was associated with a 19% lower incidence of CVD events compared with oral estrogen therapy use (IRR 0.81, 95%CI 0.67-0.99), and the observed association was driven mainly by lower incidence of congestive heart failure and VTE [50]. Similarly, in a larger study of 93 676 women, transdermal estrogen was associated with a lower risk of CHD compared to oral CEE (HR, 0.63; 95% CI, 0.37-1.06), albeit non-significantly [29]. Three studies investigated the association between route of HT administration and risk of MI in comparison to never users, and reported beneficial or no effect with oral and transdermal HT on MI risk. De Vries and colleagues, in a study of 9 390 women showed decreased age adjusted risk of MI with both use of oral and transdermal estrogen only and combined HT [47]. Similarly, in a case-control study of 1 533 women, Chilvers reported a reduced risk of MI with oral HT, but not with transdermal HT [48]. In the large study that followed more than 400 000 women (during more

than 2 million women-years), Lokkeggard et. al reported no associations of oral estrogen only HT with MI, neither of oral nor transdermal combined HT with risk of MI, but showed a decreased MI risk with estrogen only transdermal HT (RR 0.62, 95% CI 0.42-0.93). Additionally, vaginal rote of HT administration was associated with decreased MI risk (RR 0.56, 95% CI 0.44-0.71)[32].

Four studies reported on stroke risk and one trial investigated carotid artery media thickness (CIMT) in regard to route of HT administration. In a 4-year double blind RCT (including 727 recently postmenopausal women at low risk of CVD), low-dose oral or transdermal estrogen, with cyclic oral progesterone favourably altered certain CVD risk factors (lipid levels with oral CEE and insulin resistance with transdermal estrogen), however the effect of HT on carotid atherosclerosis was neutral regardless of the route of HT administration [45]. Findings from WHI, in a subset of 314 women, reported lower (but not statistically significant) stroke risk with transdermal compared to oral conventional-dose CEE (RR 0.87, 95% CI 0.55-1.38) [29]. In a French medical database (including 15 305 women), route of estrogen administration and type of progestogens were shown to be important determinants of ischemic stroke risk. While oral estrogens significantly increased the risk of ischemic stroke (OR 1.58, 95 % CI 1.01-2.49) in a dose-dependent manner, transdermal estrogens showed no association (OR 0.83, 95 % CI 0.56-1.24). Although there was no significant association of ischemic stroke with progesterone, pregnane derivatives, and nortestosterone derivatives, norpregnane derivatives were found to increase ischemic stroke risk [40]. In a large cohort study, including 980 003 women, oral unopposed estrogen or estrogen/progestin treatment was associated with an increased risk of ischemic stroke, whereas there was no increased stroke risk with transdermal application, while vaginal route of administration was associated with decreased stroke risk (RR 0.65; 95% CI, 0.59–0.70) [49]. Four observational studies reported the risk of venous thromboembolism (VTE) in regard to route of HT administration. Three studies reported 1.52 to 4.2-fold increased risk of VTE with oral HT, and no association between transdermal HT and VTE risk [4,38,44]. A retrospective matched-cohort study (among 5 102 women) reported lower VTE risk with transdermal estrogen only as compared to oral estrogen only HT, IRR 0.42 (95% CI 0.19-0.96) [50]. A large population-based study among more than a million women reported variations in RR of VTE with regard to HT formulation and time since initiation. The risk of VTE varied considerably by HT formulation, greater VTE risk was observed with oral

estrogen-progestin HT (RR 2.07, 95%CI, 1.86-2.31) than with oral estrogen-only therapy (RR 1.42, 95% CI 1.21-1.66), with no increased risk with transdermal estrogen-only therapy. The greatest risk increase was observed with HT formulations containing medroxyprogesterone acetate (RR 2.67, 95% CI 2.5-3.17). Also, current users of oral HT had twice the risk of VTE in the first 2 years after starting HT compared to subsequent years [44]. In line with this, Renoux et al. reported increased VTE risk with oral estrogen and estrogen-progestogen therapy that increased with estrogen dose, and no increased VTE risk with transdermal estrogen alone or combined with progestogen. The risk of VTE with oral HT formulations was particularly elevated during the first year of use but disappeared 4 months after discontinuation [5].

One study evaluated the effects of route of administration on atrial fibrillation risk- while overall HT was associated with 9-37 % decrease in risk of atrial fibrillation in the first year after MI, the lowest risk of atrial fibrillation was observed in women ≥80 years old for use of overall HT and vaginal estrogen compared to non-users (HR 0.63, CI 0.42-0.94, and HR 0.58, CI 0.34 -0.99, respectively [46].

The Timing of HT initiation and CVD risk

Eight studies, two RCTs and six observational [11,28,43,51-55] examined the role of the timing of HT initiation on CVD risk. Different studies looked at different lengths of time between menopause onset and HT initiation: two studies reported on HT initiation in the first four years 4 after menopause, 3 studies at 5 years since menopause, 2 studies at 10 years since menopause and one study reported on HT initiation 6 years since menopause. Findings are summarized in **Key box 3**, detailed study characteristics can be found in **Supplemental table 4.**

Key box 3. The timing of hormone therapy initiation and Cardiovascular risk	Evidence Quality
Early HT initiation:	
• In healthy recently postmenopausal women (<60 years old or who are within 10 years of menopause), the current evidence suggests that use of HT is associated with reduced CHD risk and mortality and no increase stroke risk.	**B**
• There is indication of increased VTE risk even when HT starts near menopause onset, yet, the risk might be minimized using low dose estrogen-only transdermal/vaginal therapy or combined HT with proper choice of progesterone (e.g. micronized progesterone).	**B**
• HT initiation 0–5 years after menopause onset was associated with reduced or null risk of future stroke.	**B**
Late HT initiation:	
• Observational studies reported no evidence of increased risk CHD/MI risk with later HT initiation (10+ years after the menopause onset).	**C**
• Observational studies reported increased thromboembolic and stroke risk albeit non-significant.	**C**
General conclusion:	
• Individual CVD risk factors evaluation before HT initiation is strongly advised.	**C**
• The age-related pre-existing conditions (coronary/carotid atherosclerosis, even subclinical) at the time of HT initiation may have a profound impact on the effect of HT on CVD outcomes.	**C**

*Findings are based on eight studies, six observational and two RCTs; Early HT initiation: within 10 years since menopause onset; Late HT initiation: 10+ years since menopause onset; Abbreviations: CHD: coronary heart disease; CVD: cardiovascular; MI: myocardial infarction; HT: hormone therapy; B:moderate quality of evidence (We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different); C: low quality of evidence (Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect)

An intervention trial among 643 women that evaluated subclinical atherosclerotic measures in relation to timing of HT onset reported that oral estradiol therapy, with or without progesterone, was associated with less progression of subclinical atherosclerosis (measured as CIMT) than was placebo when therapy was initiated within 6 years after menopause but not when it was initiated 10 or more years after menopause [51].

Five studies investigated the risk of VTE and stroke with regard to timing of HT initiation. The stroke risk during intervention phase in WHI was increased by 37% with conjugated equine estrogen and medroxyprogesterone acetate (CEE/MPA) and by 35% with CEE reflecting increased ischemic, but not haemorrhagic, stroke risk. However, in stratified analysis by 10-year age groups, risk of stroke was elevated but non-significantly in both intervention groups. CEE/MPA was observed to significantly increase risk of MI among women more than 20 years past menopause onset [11]. Prentice et al. combined both WHI

clinical trial data and observational study data, to investigate HT initiation <5 and ≥5 years after menopause. Findings indicated increasing VTE risk with CEE with increasing years from menopause to first use of hormone therapy, and strong early VTE risk elevations with CEE/MPA among recently postmenopausal women without prior hormone therapy [54]. However, the risk of stroke did not depend significantly on a gap time from menopause to first use of CEE and CEE/MPA hormone therapy. However it is important to note that it was not possible to calculate RR within first 5 years since menopause due to small number of events with CEE, therefore, results should be taken with caution. Findings from the Nurses' Health Study indicated 30–40% increased risk of stroke for women currently taking HT, either estrogen alone or combined with progestin and no difference in the relation of HT to stroke for women initiating therapy near to menopause (<4 years) versus 10+ years after menopause [43]. The latest findings based on pooled individual participant data from more than 88,000 postmenopausal women from 5 population-based Swedish cohort studies, showed that HT initiated early, in relation to menopause onset, was not associated with increased risk of incident stroke, regardless of the route of administration, type of HT, active ingredient, and duration. Also, while HT initiation 0–5 years after menopause onset, as compared to never use, was associated with a decreased risk of stroke (and haemorrhagic stroke), late HT initiation was associated with elevated risks of stroke and haemorrhagic stroke when conjugated equine estrogen was used as single therapy, furthermore, late initiation of combined HT was associated with increased haemorrhagic stroke risk [55].

Additionally, we identified four observational studies that evaluated the risk of CHD or MI, and none of studies reported an increased risk with later HT initiation regardless of HT formulation [28,52-54].

Duration of HT use and CVD risk

Thirty studies, three RCTs and 27 observational studies [5,16,17,30,32,34-36,38,47,48,52-54,56-71], examined the effect of duration of HT use on CVD risk. The findings are summarized in **Key box 4**, detailed study characteristics can be found in **Supplemental table 5.**

Key box 4. The duration of hormone therapy and cardiovascular risk	Evidence Quality
Short duration:	
• HT duration is important predictor of future VTE events irrespective of HT formulation and route of administration indicating high risk even with short HT duration (<1year).	A
• HT was associated with reduced or null risk of future stroke if initiated relatively soon after the onset of menopause, yet, optimal duration of HT from the perspective of stroke risk remains to be determined.	B
Long duration:	
• Evidence from observational studies on HT and MI/CHD and CHD mortality with long term use (5+) years is conflicting.	C
• Long HT duration (5+ years) is associated with increased thromboembolic and stroke risk.	B
General conclusion:	
• Late HT initiation (10+ years after menopause onset) shall be followed with the HT duration for the shortest time possible.	B

*Findings are based on thirty studies, three RCTs and 27 observational studies; Short duration <5 years; Long duration 5+ years; Abbreviations: CHD: coronary heart disease; CVD: cardiovascular; MI: myocardial infarction; HT: hormone therapy, VTE: venous thromboembolism; A: high quality of evidence (We are very confident that the true effect lies close to that of the estimate of the effect.); B:moderate quality of evidence (We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different); C: low quality of evidence (Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect)

We found four studies that investigated atherosclerotic changes in regard to HT duration. A population-based study among 3 784 postmenopausal women showed decreased CIMT in the common carotid artery in women who had used HT for ≥1 year compared with never users, while the use of HT for <1 year was not associated with a change in CIMT [60]. Yet, a small 6-month RCT with 2.5 mg/d of tibolone showed no significant effects of tibolone on either intima-media thickness or blood flow resistance in the carotid arteries in postmenopausal women [58]. In a longitudinal study among 815 women, oral and transdermal (estrogen only and combined) HT had protective effect on carotid atherosclerotic plaque occurrence after 4+ years of use, but not in the group that used HT for less than 4 years. When stratified by HT regime, this relationship was observed only in oral combined HT and not in the estrogen only group [59]. However, analysis of 10 739 women from WHI, showed

increased risk of peripheral arterial disease with unopposed oral CEE after 6 years of follow-up [57].

We found fourteen studies that investigated VTE and stroke risk with regard to HT duration, and the results were inconsistent. In a population-based cohort study, HT with estradiol was associated with a 3-fold increased risk of VTE, but this increased risk was restricted to the first year of use, crude OR (<1 year of HT use) was 3.54 (95% CI 1.54-8.2) while crude OR after first year of use was 0.66 (95% CI 0.39-1.10)[71]. The findings from HERS trial are in line with those, oral estrogen-progestin HT was associated with 3.29-fold increased VTE risk within first year of HT use, while the risk was not observed with longer HT duration [56]. In a large case-control study, 23 505 cases of VTE were matched with 231 562 controls, and the risk of VTE was increased up to 2-fold in users of oral estrogen only and combined HT compared to non-users, irrespective of HT duration (≤1 year and > 1year) [5]. Similarly, in small case-control study of 210 women, estrogen-only therapy was associated with increased VTE risk, and there was also as suggestion of a duration effect[36]. In a case control study of 15 710 stroke cases matched with 59 958 controls, oral estrogen only HT was associated with 1.35-fold increased stroke risk with >1 year of HT duration and not with shorter duration (≤1 year). There was no association between estrogen-only transdermal therapy and stroke risk irrespective of HT duration [38]. Pretnice et al. combined observational study data and clinical trial WHI data and reported no increased VTE (HR 0.84, 95% CI 0.47-1.51) risk but increased stroke risk with longer HT duration (5+ years) in intervention arm with oral CEE, HR 1.68, 95% CI 1.06-2.66, as compared to never users, while there was no association between oral CEE and stroke risk with 0-4 years of HT use. Yet, in CEE/MPA intervention arm, there was no significant association between oral CEE/MPA and stroke risk, while increased risk of VTE was observed across all subgroups by HT duration , with highest risk within first two years of HT use, HR 5.30 (95% CI2.58-10.89)[72]. In a case-control study among 864 women, the risk of both types of stroke was transiently increased after initiation of oral estrogen HT. Compared with current HT use initiated earlier (≥0.5 years), a 2-fold increase in the risk of ischemic stroke (OR, 2.16; 95% CI, 1.04-4.49), and hemorrhagic stroke (OR, 2.20; 95% CI, 0.83-5.81), was seen [35]. In a large population based study among 76 875 women, 35% reduction in incidence of hospitalisation for CVD among women who took oral and transdermal HT for more than 3 years as compared to those treated for less than 6 months was observed. When comparing

route of HT administration, the reduction in hospitalization was observed in those treated long term with transdermal HT, but not for those who took oral HT [65]. One study investigated stroke mortality in pre (1995-2001) and post-WHI (2002-2009), and found no measurable changes in mortality in estrogen HT users, however, longer HT duration (1-8 years) was associated with stronger decrease in stroke morality than with shorter duration of HT use (<1 year) [62]. In contrast, Paganini-Hill at al., and Cauley et al. did not report a beneficial effect of estrogen only HT on stroke mortality, irrespective of HT duration (< 8 years and 8+ years and 1- and >10 years) [17,63]. In a nested case-control study of relatively healthy 9 429 postmenopausal women current use of unopposed and opposed estrogen was associated with a 34% increase in the risk of cerebrovascular events. No clear treatment duration (≤1 year and > 1year) pattern was observed for ischemic and hemorrhagic stroke, subgroup analysis by HT duration was not statistically significant, yet, the OR of TIA was significantly increased in HT users, and was higher in first year of HT as compared to longer duration [34]. Similarly, in NHS, during more than half million of years of follow-up, subgroup analysis by oral HT duration did not show variation in stroke risk with increased HT duration, and results remained non-significant across the strata [16]. Findings from a nested case-control study including 30 048 women showed no variation in stroke risk with duration of HT (all regimes except estradiol and dydrogesterone), while current short-term (1 year), mid-term (2-4 years) and long-term users (5+ years) had an increased relative risk of developing a VTE as compared to non-users [70].

Sixteen studies reported on heart disease risk in relation to HT duration. In general, existing literature suggested no association between HT duration and CHD risk or suggested a protective effect on MI and CHD with longer duration. The HERS was the first large scale RCT designed to test the efficacy of HT in prevention of CHD in women with history of CHD, coronary revascularisation or MI. After an average of 4.1 years of follow-up, there was no difference in nonfatal MI and coronary death between the hormone (CEE 0.625 mg/d plus MPA 2.5 mg/d) and placebo arms. A post hoc time-trend analysis revealed a significant 52% increase in cardiovascular events (42.5/1000 person-years versus 28.0/1000 person-years) in the first year in the HT group compared with placebo, with a nonsignificant trend toward fewer events in the treatment arm compared with placebo in later years (23.0/1000 person-years versus 34.4/1000 person-years)[56]. Findings from WHI trial suggest no association between CEE and CEE/MPA oral HT and CHD risk irrespective of HT duration [72].

In line with this, findings from observational studies suggest no association between oral and combined HT duration and risk of MI [30,32,53,67,69]

In a population-based study with more than half million of person years of follow-up, MI risk was decreased across all HT duration subgroups, with beneficial effect observed even with short term HT use (<1 year)[16]. In another population based study with 4537 cases of MI during 2.62 million person years of follow up, decreased MI risk was observed with longer overall HT duration (>1year and 5+ years) while there was no association with <1 year of HT, yet, due to small number of MI cases this might be the consequence of underpowered analysis[47]. In a case-control study among 864 women there was a trend for decreased risk of MI with an increased duration of overall HT use, with significant a decrease observed with 5+ years of HT use (trend Chi^2=28.6, P<0.001)[48]. Similarly, two observational studies reported decreased MI risk with a long (8+ years) estrogen HT [68], and CHD/CVD risk with long (3+years) HT use in general (all regimes)[65].

Additionally, five observational studies investigated CVD related mortality in regard to HT duration, and one study reported HT duration-independent decreased MI mortality [66] Two studies observed decrease in CHD risk after 10 years of HT [63] and 5 years of HT use [62], while two studies reported no association between HT duration and CHD mortality [52,64].

Study quality and between-study heterogeneity

Four of the six included trials demonstrated a medium or high risk of bias within one or more areas of study quality, as evaluated using the Cochrane Collaboration tool (**Supplemental table 6**). Only one observational study was considered to be at low risk of bias, with 16 of 48 studies considered to be at high risk of bias (**Supplemental table 7 & 8**). The variety of available studies (baseline characteristics of study populations, heterogeneity in exposure, outcome and follow-up duration) precluded our ability to quantitatively estimate risk for all cardiovascular outcomes. After assessing the study quality we applied the GRADE approach to determine the quality of the evidence taking into account the risk of bias, study design, consistency and directness of findings. The grading of each statement based on the current review was indicated in **Key boxes 1-4.**

Discussion

Summary of the Findings

This review, based on data from more than 2,5 million menopausal women gives an important overview of the current knowledge on the cardiovascular risk related to HT use. In general, oral HT was not associated with increased risk of heart disease, but contrary, beneficial cardioprotective effects may be observed with low doses of oral and transdermal HT. Also, there were some indications that vaginal HT may decrease MI and stroke risk, but the evidence is limited and requires further investigation. However, oral HT might be associated with increased risk of VTE and stroke. VTE and stroke risk increased in dose-dependent manner with estrogen dose in oral and transdermal HT composed of estrogen alone or in combination formulation. In women with increased baseline thromboembolic risk, transdermal estradiol alone or with micronized progesterone appears to be safer with regards to CVD risk. Also, due to greater absolute risks of coronary heart disease, stroke and VTE, late HT initiation (10 years since the menopause onset or >60years old) shall be recommended for the shortest time possible and in lowest possible dose, and preferably transdermal low dose HT (<50 µg/d of estrogen) should be advised.

Biological mechanisms underlying the controversial findings on HT and CVD risk

In the first ten years after menopause onset, the estrogen levels decrease by 60 to 80% as compared to premenopausal women [73]. However, in both cases, higher levels of estrogen are reported to be associated with adverse cardiometabolic outcomes. Early exposure to estrogen (i.e. an early age at menarche)[74,75] and pregnancy (which is characterized by high endogenous estrogen levels) in premenopausal women [76,77], and a high endogenous estradiol in postmenopausal women [78] have been linked with insulin resistance and an increased risk of T2D, as well as adverse cardiovascular health and increase risk of cardiovascular disease[79]. While on contrary, in a population based study, women with premature ovarian insufficiency, compared to premenopausal women, showed lower estrogen levels, but also a lower mean carotid intima media thickness and decreased odds of plaque presence [80]. Therefore, exogenous factors such as HT that alter serum levels of estradiol could play a role in cardiometabolic risk, and this role may depend on the magnitude the HT alters estradiol levels, and thus may vary by the dose of HT. Indeed, the

conventional estrogen HT doses (0.625mg/d) may increase plasma estradiol concentrations in postmenopausal women, affecting the CVD risk [81]. Contrary, a lower dose of estrogen replacement, which alters estradiol levels to a lower magnitude, has been found to improve cardiac function and remodelling in murine models of myocardial infarction, while at increased doses that raised plasma estrogen far beyond the physiological level, estrogen was detrimental to the heart[12,82]. Also, low doses of conjugated equine estrogen in monkeys were associated with reduction in coronary atherosclerotic plaque extent [83]. These observed beneficial effects may be due to improved endothelial function, lipid profile and restoration of plasma estradiol to biological levels that is found when low-dose estrogen is administered. In contrast, greater increases in plasma estradiol of two-to three-fold might lead to endometrial hyperplasia [68,82,83]. This finding could support the increased ischemic stroke risk with greater HT doses noted in this review, and also the greater impact of orally administered HT than transdermal patches. Oral estrogen therapy undergoes the first pass metabolism in the liver, which is associated with a number of adverse haemostatic effects (decreased low-density lipoprotein particle size, increased triglycerides/C-reactive protein, increased production of certain coagulation factors), whereas transdermal administration of estrogen therapy largely avoids these effects [84]. Also, the formulation of HT, especially, the type of progestogens in combined HT, could be an important determinant of thrombotic risk. Progestins downregulate estrogen receptors and, via progestin receptor activation, they may oppose the actions of estrogen, and MPA may cause this effects in greater extent than other progestins [56]. Findings from RCTs showed that norpregnane derivatives increased markers of blood coagulation activation and induced activated protein C resistance, an established risk factor for venous thromboembolism [85], and that combined transdermal HT with MPA increased prothrombin fragment 1+2 concentration [86]. Yet, nortestosterone derivatives used in transdermal estrogen therapy did not cause changes in matrix metalloproteinase (MMP)-2, or in LDL particle size [87] or had beneficial effects on haemostatic parameters [88]. Also, there is evidence that oral (not transdermal) estrogens activated blood coagulation and induced activated protein C resistance [89,90].

Recently, a large population-based study has indicated that in women with carotid atherosclerosis, endogenous estradiol may play a role in the development of vulnerable carotid plaque composition and increase the risk of stroke [91]. Similarly, endogenous estradiol in postmenopausal women was associated with increased risk of developing type 2

diabetes, a major risk factor for coronary artery atherosclerosis, stroke and overall CVD risk [78]. Findings from monkey models support the hypothesis that estrogen therapy may have a cardiovascular benefit when initiated early after the onset of menopause. Based on monkey models in premenopause, estradiol may prevent fatty streak deposition and progression of atherosclerotic plaque [92], Also, monkeys starting HT in early menopause reduced coronary artery atherosclerosis by about 50–70%. In contrast, delaying initiation of HT in these monkeys for about 6 years in human terms diminished this protection [93]. Coronary artery fatty streaks and small plaques are common in women at the time of perimenopausal transition, whereas advanced atherosclerotic plaques are common in aging women and in women 5 to 15 years after menopause [94]. Endothelium changes related to atherosclerosis progression in elderly women might be another explanation why HT initiated at the complicated plaque stage might have deleterious effects (beyond ≈60 years of age)[93]. The underlying mechanisms are not fully understood, but the changes in estrogen receptor signalling [13,95] or age-related hyper-inflammatory state[96] might be important factors.

The duration of HT cannot be observed as a single factor affecting CVD risk. Longer duration occurs simultaneously with the natural aging process, and other important factors are time of HT initiation and underlying endothelium characteristics/presence of other CVD risk factors. Long term estrogen use may have favourable effects on lipid profile and slow down the atherosclerotic process if administered in women with healthy vasculature [68]. Although the majority of observational studies evaluating stroke risk [16,35,70] reported null findings, there was some indication of increased stroke risk after ≥5 years of HT use [72], and increased risk of TIA irrespective of HT duration [34]. However, this may be a consequence of HT characteristics, and also characteristics of underlying population investigated.

The Criticism of the Current Evidence and Directions for Future Research

The synthesis of the existing knowledge on this topic was challenging due to inconsistent findings between some studies, caused by substantial diversity in scientific rigor and quality across the available evidence. The majority of studies included in the current review are from North American and European populations which might limit the generalizability of the findings of this review to the other populations. Furthermore, the HT formulation used within studies also differed, i.e., whether they included progestin or the form of estrogen, for example, 17β-estradiol or conjugated equine estrogens, which may make the interpretation challenging. Other important factors such differences in underlying CVD risk

factors in study populations, differences in age ranges, and variability in adjustment levels made the synthesis of the knowledge challenging. The importance of the age is clearly seen in the example of WHI and HERS trials. The first results from WHI [97] and HERS trials [98], changed the clinical practice and lead to conduction of multiple trials and studies to delineate the elements that explain the conflicting findings on HT risks and benefits. However, women included in those trials were considerably older than the age at which most women enter the menopause, mean age in WHI was 63 and in HERS 66.7 years, while the mean age of menopause onset is around the age of 50 [99]. Therefore, the results of those trials although very important might be driven by age-related changes that occur simultaneously with HT use. However, those two trials were extremely important and from them arose the so called "timing hypothesis" that suggests different clinical effects depending on whether HT is initiated close to the onset of menopause (<6 years) or several years later [8].

Our review emphasizes the gaps in the literature and should stimulate future research to investigate: (i) the risk of VTE and stroke with transdermal/vaginal and oral HT containing different types of progestogens and assess the association with coagulation factors and (ii) the role of underlying diseases and genetic traits in CVD risk, among which genetic variance in estrogen receptor, dyslipidaemia, history of gestational diabetes and preeclampsia and carotid atherosclerosis might be the most important. To properly investigate the role of timing of HT initiation, it may be more feasible to conduct large population-based studies rather than RCT. The trials should recruit women that recently entered the menopause or generally those in their 50s and follow them for sufficient amount of time (> 5 years). However, CVD rates are considerably low during this period of life, therefore, the sample size needed to detect a potential adverse effect would most probably make this kind of study design costly and non-feasible. Therefore, retrospective large-population based studies using general practitioner registries may be a better approach to address this research question. A good example is a national historical cohort of women established by linking five Danish registries and including 980 003 women, and 20 199 stroke cases [49]. It is of high importance to focus the future research on better understanding the endothelial dysfunction during the perimenopausal transition and in the first 10 years after the menopause onset. The progression of atherosclerosis may lead to a substantial reduction in

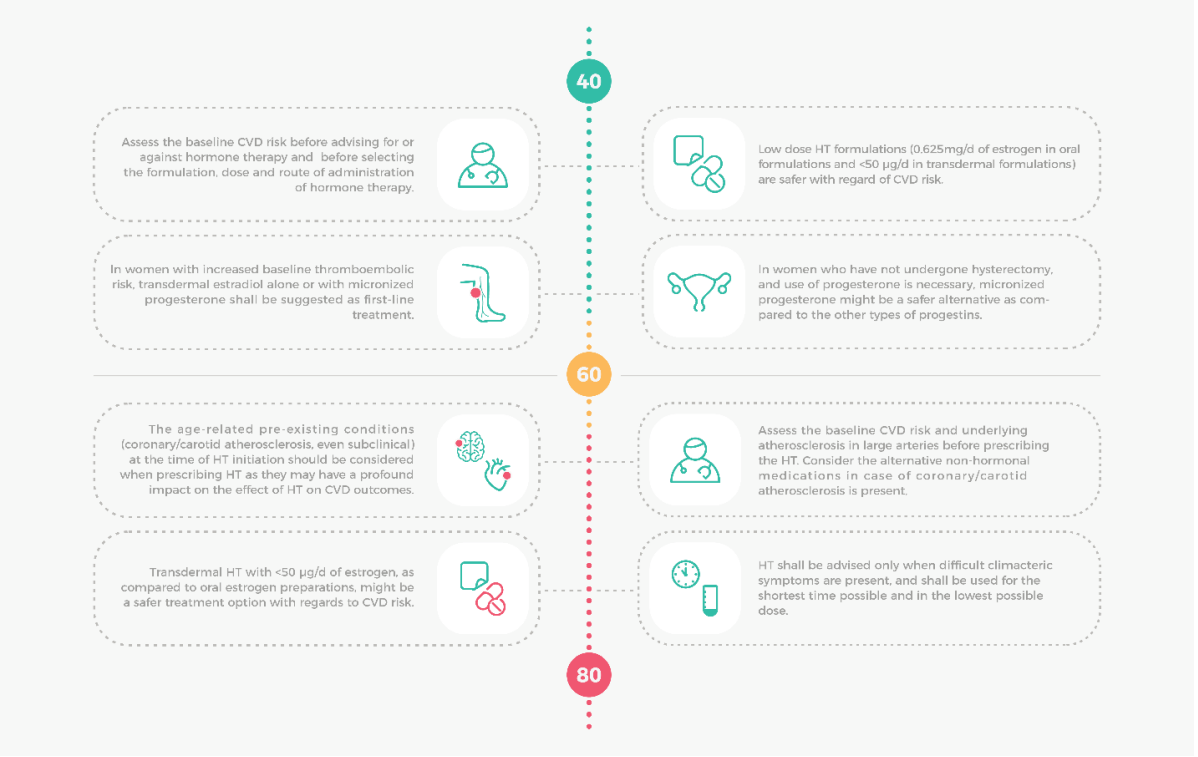
estrogen receptors and have a profound impact on observed increased CVD risk with later HT initiation.

Conclusions and Clinical Implications

The current review presents a cutting edge summary of HT and CVD risk, however, the recommendations from this paper shall be interpreted with caution. The quality of evidence included in this review was in general low or moderate with only few statements being supported with high quality evidence. The most important clinical recommendations based on this review are summarized in **Figure 2**. Use of HT should be individualized and not initiated nor discontinued solely based on a woman's age. Before advising HT use, it is necessary to evaluate baseline CVD risk, age and time since menopause onset. For example, women further from the menopause (e.g. more than 10 years from the menopause) have a more adverse CVD risk profile and are more prone to CVD as compared to women who are in first years of the menopause, therefore the use of HT should be recommended to the lowest dose and for the shortest time period possible. In particular, it is crucial to assess age-related pre-existing conditions (clinical and subclinical coronary/carotid atherosclerosis) at the time of HT initiation as they may have a profound impact on the CVD outcomes. Also, it is recommended that medical professionals discuss with their patients which route of administration might be safer for them, as well as the formulation of HT. The evidence so far shows that the use of transdermal estrogen, as compared to oral estrogen preparations, is less likely to lead to thrombotic events, and perhaps also to stroke and coronary artery disease, and therefore might be a better treatment option for women. While different formulations of HT exist, the use of HT should be based also on women's medical history, and particularly on the type of menopause women experienced. For instance, in women who have not had a hysterectomy, when the use of progesterone is necessary, micronized progesterone is considered the safer alternative as compared to the other types of progestins.

Overall, the evidence on HT and CVD risk in women is not robust, but supports the role of different factors such as route of administration, formulation, age and duration since the menopause as important determinants of CVD risk related to HT.

Figure 2. Clinical implications of findings



Vertical dotted line refers to women's age; Age from 40 to 60 years refers to menopausal transition and early menopause, while age above 60 years old refers to late menopause; CVD: cardiovascular disease; HT: hormone therapy

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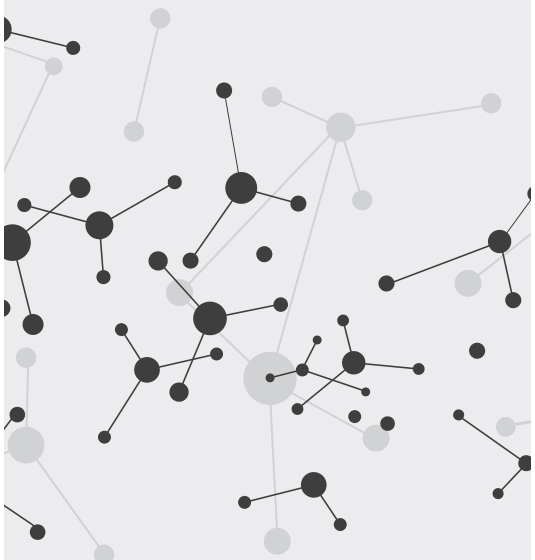
Chapter 4

Estrogen-like compounds and Metabolic Risk in Women



4.1

Associations between phytoestrogens, glucose homeostasis and risk of diabetes in women: a systematic review and meta-analysis

4.2

Phytoestrogen supplementation and body composition in postmenopausal women: A systematic review and meta-analysis of randomized controlled trials

CHAPTER 4.1

Associations between phytoestrogens, glucose homeostasis and risk of diabetes in women: a systematic review and meta-analysis

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Accepted for publication in Advances in Nutrition

ABSTRACT

Phytoestrogens might have advantageous effects on diabetes in women. We performed a systematic review and meta-analysis to determine the effect of phytoestrogens on glucose homeostasis and risk of type 2 diabetes (T2D) among women. Randomized clinical trials (RCT) and prospective observational studies that assessed associations of phytoestrogens (supplementation, dietary intake or biomarkers) with fasting glucose or insulin, homeostatic model assessment of insulin resistance (HOMA-IR), or with risk of T2D were included. We identified 18 RCTs (n=1,687 individuals) investigating the effect of phytoestrogen supplementation on glucose homeostasis, and 9 prospective population-based studies (n=212,796 individuals) examining the association between phytoestrogen intake and risk of T2D. As compared to placebo, phytoestrogen supplementation resulted in improvements in fasting glucose and HOMA-IR, the pooled mean differences of changes were –0.12 mmol/L (95% CI -0.20 to -0.03) and -0.24 (95%CI: -0.45 to -0.03) respectively. While there was no significant decrease in insulin levels with overall phytoestrogen supplementation, pooled mean difference of changes was -0.99 pmol/L (95%CI: -4.65 to 2.68). However, the results of RCTs vary by type of phytoestrogens, soy derived isoflavones and genistein improved glucose homeostasis, isoflavone mix and daidzein had no or were associated with adverse glycemic profile. Higher dietary phytoestrogen intake was associated with a 10% lower risk of developing T2D in observational studies (pooled relative risk; 0.90 [95% CI, 0.85 to 0.96] for highest versus the lowest quantiles). Results were similar when the analyses were restricted only to medium and high-quality studies. Overall phytoestrogens may have a positive influence on glycaemia and could be used for diabetes prevention in women. However, for some individual types of phytoestrogens, such as mixed isoflavones, caution is needed in recommending their use in women, as their use could lead to adverse glycemic profile in women.

INTRODUCTION

Phytoestrogens, nonsteroidal plant compounds with estrogen-like biological activity, have been suggested to improve women's health[1]. Many women in Western countries choose to use phytoestrogens as complementary therapy for treatment of menopausal symptoms [1,2]. Recently, a meta-analysis of clinical trials showed that specific phytoestrogen supplementation led to relief of menopausal symptoms [3], which, also due to the potentially negative health consequences of hormone replacement therapy [4,5], may motivate women to use these herbal medications. Furthermore, phytoestrogens are becoming progressively common constituents of human diets due to the latest dietary guidelines on substituting animal protein with soy-based foods[6]. Therefore, there is an increased interest in the potential health effects of phytoestrogens beyond menopausal symptoms. Due to the structural similarity to estrogen, phytoestrogens bind weakly to estrogen receptor-α and more strongly to estrogen receptor-β. They may possess organ-specific estrogenic and antiestrogenic effects depending on the circulating estrogen level (if the circulating estrogen level is high they exert an antiestrogenic effect, when the estrogen level is low, their effect becomes more estrogenic) [7,8]. Emerging evidence is showing that estradiol signaling can increase the risk of diabetes in postmenopausal women [9], also it has been suggested that phytoestrogens can avoid the estradiol-induced effects on type 2 diabetes (T2D) because of the ability of these compounds to compete with estradiol to bind its receptors, as well as via estrogen-independent pathways[7]. In vitro studies have shown that isoflavones, phytoestrogen compounds commonly found in soy, have antidiabetic properties [10,11], animal studies have indicated that phytoestrogens improve glycemic control and insulin sensitivity [12,13]. However, evidence from studies in humans on effects of phytoestrogens on glucose homeostasis and T2D risk are inconsistent, some studies showed adverse effects [14], some no association[15], while others showed a beneficial effect [16]. Previous quantitative reviews are limited by (i) a focus on specific populations (e.g. only Asian women); (ii) evaluation of only glycemic traits not risk of T2D, and (iii) including heterogeneous interventions (e.g. phytoestrogen supplementation plus dietary restrictions) making interpretation of results challenging [17-19].

Thus, we performed a systematic review and meta-analysis of intervention studies and prospective population- based studies evaluating the association between phytoestrogens use, glucose homeostasis and risk of T2D among women.

METHODS

Data Sources and Search Strategy

The Cochrane Handbook for Systematic Reviews of Interventions and PRISMA Statement were used to guide the conduct and reporting of this review[20,21]. A literature search was done using 5 electronic databases (Medline via Ovid, EMBASE via embase.com, Web of Science Core Collection, Cochrane CENTRAL via Wiley and Google Scholar) from inception to June 30, 2017 (date last searched). Additionally, reference lists of the included studies and relevant reviews, and studies that have cited these articles, were searched with Elsevier's **Scopus**, the largest abstract and **citation database.** Details on the search strategy are provided in **Supplemental table 1**.

Study Selection and Eligibility Criteria

Studies were included if they met the following inclusion criteria: (i) were randomized controlled trials (RCTs) or prospective observational studies; (ii) reported longitudinal associations of phytoestrogen supplementation/dietary phytoestrogens/phytoestrogens in serum and urine with serum glucose, insulin, homeostatic model assessment of insulin resistance (HOMA-IR) and risk of incident T2D; (iii) were performed among adult women or, when conducted in both men and women, showed results stratified by sex and/or reported that the interaction with sex was not significant (P-value > 0.05) and (iv) among participants who did not use glucose lowering medications. Only RCTs comparing intervention with a placebo were included. Thus, RCTs that compared intervention group with estrogen, other medications containing phytoestrogens, or intervention with phytoestrogens in combination with specific diets were excluded. Two reviewers (MG and NK) independently evaluated the titles and abstracts according to the inclusion and exclusion criteria. For each potentially eligible study, two reviewers assessed the full-text. In cases of disagreement, a decision was made by consensus or, if necessary, a third reviewer was consulted.

Data Extraction

A predesigned data extraction form was used to collect relevant information. In case of multiple publications of the same study, the most recent information was extracted. For each study, the most adjusted estimates were extracted. When studies included both men and women, did not report estimates separately for women, and reported no significant interaction with sex ($p > 0.05$), we extracted the estimates of the overall population.

Assessing the Risk of Bias

Bias within each individual study was evaluated by two reviewers. To assess the quality of RCTs we used The Cochrane Collaboration's tool for assessing risk of bias[22]. Studies are judged to be at low or high risk of bias based on criteria evaluating random sequence generation, allocation concealment, blinding of participants/personnel and outcome assessment, and lastly, incomplete outcome data and selective reporting[22] . Studies are considered to have low risk of bias if allocation concealment, blinding of participants and outcome assessors were all coded *yes*, if a compliance assessment was done, and the number of dropouts and reasons for dropout were reported. In case that ≥3 quality criteria were not met the study was classified as having high risk of bias; others were classified as having moderate (meeting 2 quality criteria) and low (<2 quality criteria) risk of bias (**Supplemental table 2)**. The validated Newcastle-Ottawa Scale (NOS), a semi-quantitative scale designed to evaluate the quality of non-randomized studies was used to evaluate bias within each observational study [23]. The assessment of the study quality was based on the selection criteria of participants, comparability of cases and controls, and exposure and outcome assessment. Studies that received a score of nine stars were judged to be of at low risk of bias; studies that scored seven or eight stars were considered at medium risk; those that scored six or less were considered at high risk of bias (**Supplemental table 3**).

Data Synthesis and Analysis

Intervention effects were defined as the differences in outcomes (glucose, insulin, HOMA-IR) between the intervention and placebo groups at the end of the trial. For continuous outcomes (e.g. glucose), summary measures were presented as mean differences, and for dichotomized outcomes (incident diabetes, yes or no), we presented relative risks (RR). In case of cross-over trials, the data from the first period only were used. To enable a

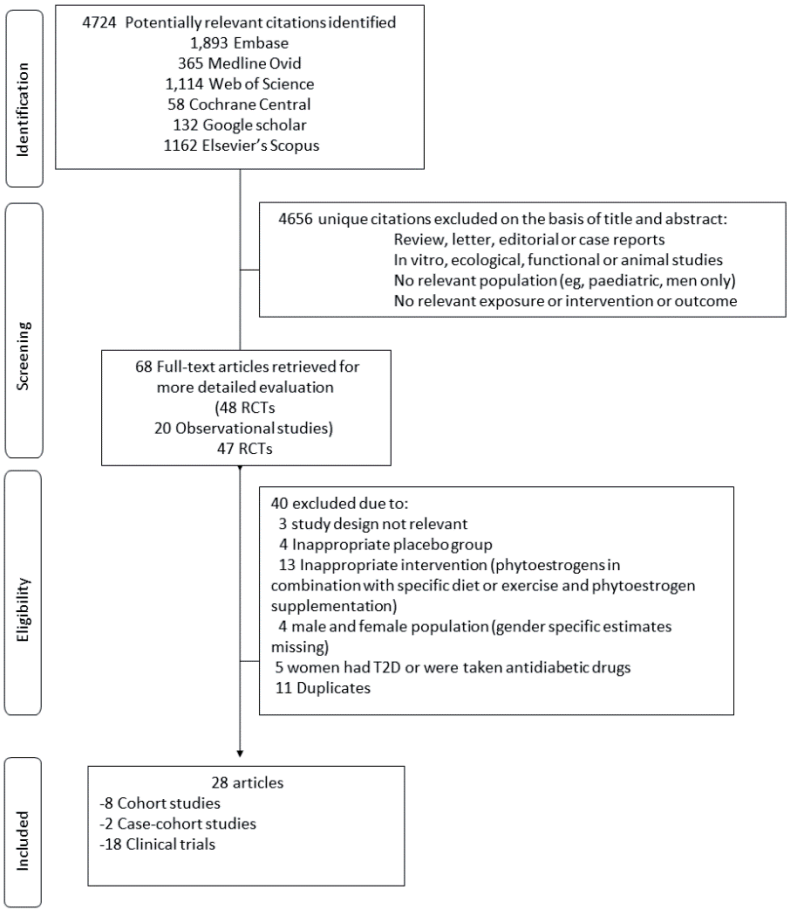
consistent approach to the meta-analysis and improve interpretation of the findings, units of measurement were converted to common units. For observational studies, we used previously described methods [24] to transform estimates, which were often differentially reported by each study (for example comparing quarters or thirds), to estimates corresponding to comparison of the top versus the bottom quintile of the baseline phytoestrogen intake distribution in each study. Briefly, we transformed the log RR by assuming a normal distribution, with the comparison between extreme quintiles being equivalent to 2.80 times the log risk ratio for one standard deviation increases (or equivalently as 2.54/2.80 time the log RR for a comparison of extreme quarters). We calculated standard error of the log RR by using published CIs and standardized them in the same way. Furthermore, where a study reported more than one risk estimate (e.g., for different types phytoestrogens), the pooled RR (e.g., for any type of phytoestrogens) from the study to be used in meta-analysis was obtained using fixed-effects models. The inverse variance weighted method was used to combine relative risks to produce a pooled relative risk using random effects meta-analysis models to allow for between study heterogeneity. Also, as a sensitivity analysis, we reported the estimates using fixed effect models as shown in the forest plots. Heterogeneity was quantified using the I^2 statistic, classified as low (I^2 ≤25%), moderate (I^2 >25% and <75%), or high (I^2 ≥75%) [25]. Additionally, Q-statistic was used to assess the presence of heterogeneity. $P_{Q\ statistic}$ ≥0.05 was considered to indicate no significant heterogeneity among the included studies. Study characteristics including geographic location, study population, median number of participants, median duration of intervention, median dosage of intervention, difference threshold between lowest and highest phytoestrogen quintiles intake, route of administration, menopausal status, median baseline age and body mass index (BMI) of participants, risk of bias and RCT/observational design were pre-specified as characteristics for assessment of heterogeneity and were evaluated using stratified analyses and random-effects meta-regression if 10 or more studies were included in the meta-analysis [26]. To assess the influence of each individual study to the overall estimates of the rest of the studies, leave-one-out sensitivity analysis was performed iteratively removing 1 study at a time to confirm that our findings were not driven by any single study. Publication bias was evaluated through a funnel plot and asymmetry was assessed using the Egger's test. All tests were two-tailed and p-values of

0.05 or less were considered statistically significant. STATA release 14 (Stata Corp, College Station, Texas) was used for all statistical analyses.

Results

The search strategy identified 4,724 references. Following initial screening based on titles and abstracts, the full texts of 68 articles were retrieved and evaluated further. As shown in **Figure 1** after full text assessment, 40 studies were excluded. The remaining 28 articles (based on 27 unique studies) were included in the review and meta-analysis. Of those, 18 were RCTs and 9 were observational prospective studies.

Figure 1. Flow diagram of studies of included in the current review



(i) Clinical Trials

Characteristics of the 18 trials included in this review can be found in **Supplemental table 4** [27-45]. In total 1,687 women (1,006 in intervention group and 681 in the control group) with baseline age ranging from 18 to 75 years were included. Fifteen RCTs were conducted in postmenopausal women, two included adult women regardless of the menopausal status, and one RCT was done in women and men but reported no sex interaction. Five RCTs were performed in North America, 5 in Europe, 5 in Asia, 2 in South America, and 1 in Australia. The Included RCTs reported data on different types of isoflavones (isoflavones mixture supplementation 9 RCTs; soy derived isoflavones 6; isolated genistein supplementation 4 RCTs; isolated daidzein 2 RCTs, flaxseed 1), and glucose homeostasis (glucose 17 RCTs; insulin 15 RCTs; and HOMA-IR 11 RCTs), but none reported data on risk of T2D. Thirteen of the RCTs included healthy women, two RCTs included women with metabolic syndrome, two included women with pre-diabetes, and one clinical trial included women six months after treatment for breast cancer. None of the women included in our meta-analysis used glucose lowering medications. Details on average changes between baseline and intervention on serum glucose, insulin and HOMA-IR can be found in **Supplemental table 5**.

(ii) Observational prospective studies

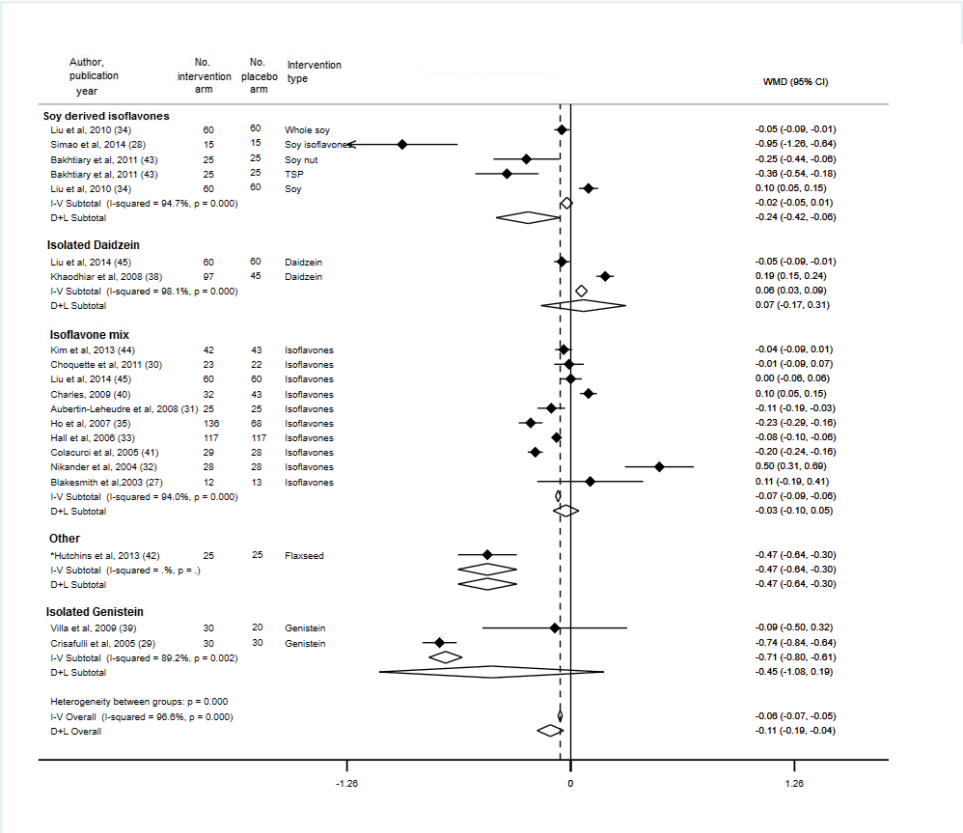
Detailed study characteristics of the 10 articles based on prospective observational studies, which included 2 case-cohort [46,47] and 8 population-based cohort studies [14-16,48-52], included in this review can be found in **Supplemental table 6.** Of the 9 studies included in meta-analysis, four studies were from Asia, three from North America, and two from Europe. One study included postmenopausal women only [50], whereas the other studies did not specify the menopausal status. Three studies reported estimates for men and women combined, however, reported that the interaction with sex was not significant. The baseline age of participants included in the 9 studies ranged from 32 to 80 years (median age was 53.87). The period of follow-up ranged from 4 to 15 years (**Supplemental table 6**). All the included observational studies reported data on phytoestrogens and risk of T2D, none with prospective glycemic traits. All 9 studies reported on dietary phytoestrogen intake, and some of these reported different types of phytoestrogens (overall isoflavones 7 studies; genistein 3 studies, daidzein 3 studies, soy products 3 studies, soy protein 2 studies, flavonoids 4 studies). In the study by Ko et al., additionally to dietary phytoestrogens, serum

levels of genistein and daidzein were measured [46]. The overall number of subjects in the 9 observational studies included in our meta-analysis was 212,796 with 9,721 incident T2D cases. Details on exposure and outcome assessment can be found in **Supplemental table 7.**

Association between phytoestrogens and glucose homeostasis from RCTs

Data from 17 RCTs (including 1,658 subjects), 15 RCTs (including 1,186 women) and data from 11 RCTs (including 909 women) contributed to the meta-analysis on effects of phytoestrogen supplementation on fasting serum glucose levels[27-35,38,39,41-45], fasting insulin[27-34,37-40,42-44] and HOMA-IR [27-30,33,34,39,40,42-44], respectively. As compared to placebo, phytoestrogen supplementation was associated with a reduction in serum glucose level [pooled mean difference of changes: -0.12 mmol/L (95%CI: -0.20 to -0.03)] (**Supplemental figure 1**). In the subgroup analysis by type of phytoestrogens, soy derived isoflavones were significantly associated with reduction in serum glucose level (pooled mean difference of changes: -0.24 mmol/L [95%CI: -0.42 to -0.06]), whereas no significant associations were observed between other subgroups of phytoestrogens (isoflavone mixture, isolated daidzein and genistein) and changes in glucose levels (**Figure 2**). Compared to placebo, overall phytoestrogen supplementation was not associated with a decrease in insulin levels, [pooled mean difference of changes: -0.99 pmol/L (95%CI: -4.65 to 2.67)] (**Supplemental figure 2**). In the subgroup analysis, isolated genistein was associated with a decrease in insulin levels [pooled mean difference of changes: -16.66 pmol/L (95%CI: -21.45 to -11.87)] (**Figure 3**), whereas isoflavone mix was associated with an increase in insulin levels [pooled mean difference of changes: 3.12 pmol/L (95%CI: 0.67 to 5.57)]. No association was observed between soy derived isoflavones and insulin levels. Furthermore, we observed a reduction in HOMA-IR in phytoestrogen users as compared to placebo [pooled mean difference of changes: -0.24 (95%CI: -0.45 to -0.03)] (**Supplemental Figure 3**). However, in the subgroup analysis there was an indication for an increased level of HOMA-IR with use of isoflavone mix [pooled mean difference of changes: 0.17 (95%CI: 0.04 to 0.31)], whereas there was significant decrease with isolated genistein as compared to placebo [pooled mean difference of changes: -0.83 (95%CI: -0.94 to -0.73)] (**Figure 4**).

Figure 2. Subgroup analysis by type of phytoestrogens and changes in serum glucose



WMD, weighted mean different. Mean difference refers to mean difference of changes between treatment groups. IV: fixed-effects model; D+L: random effects model; Error bars indicate 95% CIs. Solid vertical line presents no effect, the dotted line drawn though the diamond presents the summary measure with its confidence intervals (lateral tips of diamond). Heterogeneity assessment: I squared, P comes from Q statistics; in subgroup analysis we used multiple estimations from the same trail (for different phytoestrogen type), which may results in slight variations in overall estimates in this figure as compared to Supplemental Figure 1.; *Hutchins et al., 2013 (39): No significant relationships between gender, body weight, BMI, or percent fat mass and the changes, or lack of changes, in glucose, insulin, HOMA-IR, inflammatory or anti-inflammatory biomarkers were found; therefore, all further analyses were conducted based on the intervention

Figure 3. Subgroup analysis by type of phytoestrogens and changes in serum insulin

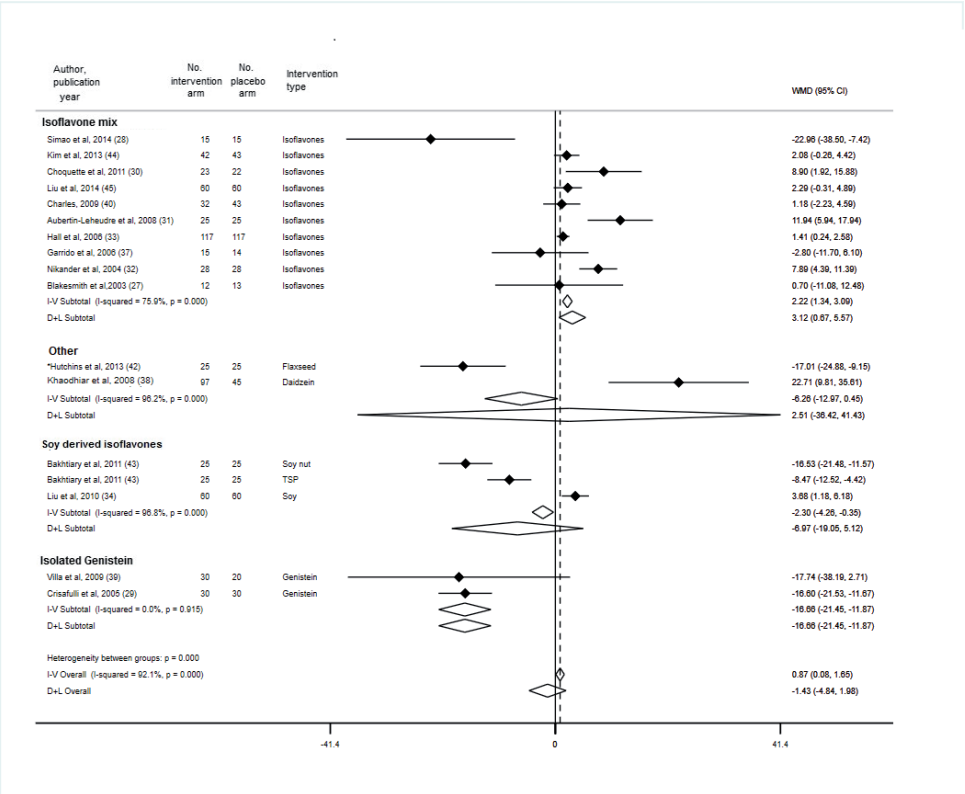


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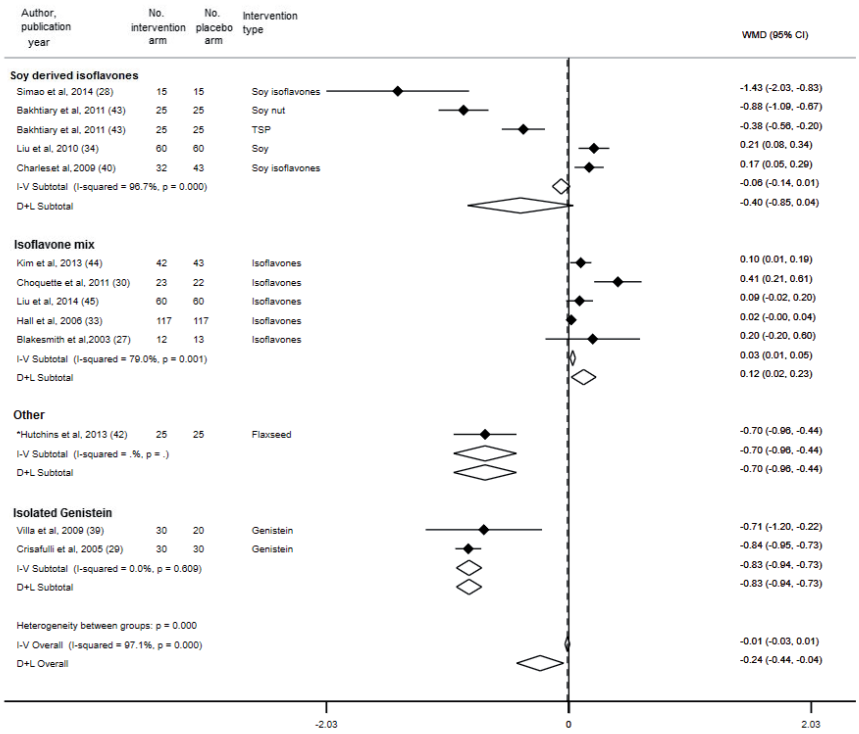
Figure 4. Subgroup analysis by type of phytoestrogens and changes in HOMA-IR



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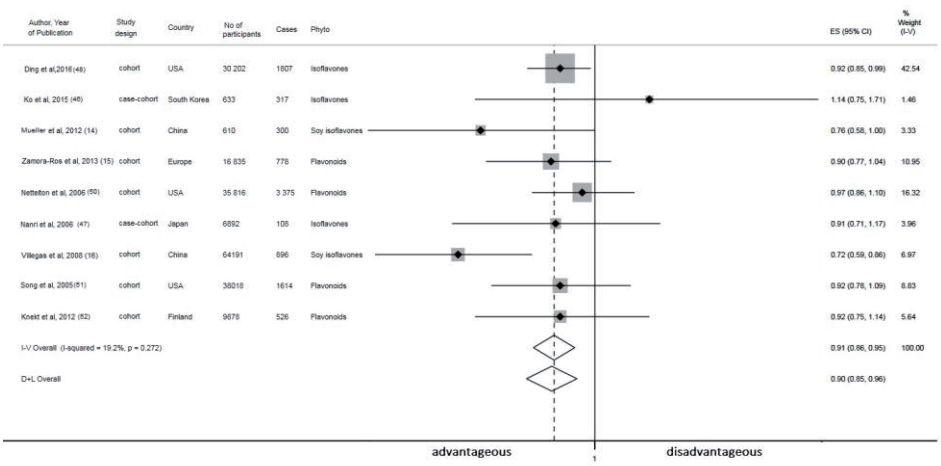
Association between phytoestrogens and risk of T2D from prospective observational studies

Data from 9 observational studies [14-16,46-48,50-52] contributed in the meta-analysis on phytoestrogen intake and risk of T2D. The meta-analysis of fully adjusted risk ratios, based

on 212,796 subjects and 9,721 incident cases of T2D, showed that women who reported high intake of any type of phytoestrogens had a lower risk of developing T2D (pooled RR= 0.90 [95% CI, 0.85 to 0.96] for highest versus the lowest quantiles of phytoestrogen intake) **(Figure 5)**. In addition to dietary intake, two longitudinal studies also measured phytoestrogen biomarkers in relation to incident T2D. Findings from the Nurses' Health Study, not included in our meta-analysis, showed that for each SD increase of urinary concentrations of total lignan metabolites, the odds ratio of developing T2D was 0.70 (95% CI 0.53-0.92) [49]. The study done by Ko et al, reported that higher plasma concentrations of genistein were associated with decreased risk of T2D (comparing extreme quintiles: OR=0.58, 95%CI 0.35-0.95) in women, while the no association was observed between plasma glycitein or daidzein and risk of T2D [46].

Figure 5. Meta-analysis of prospective population-based studies on the associations between dietary phytoestrogen intake and risk of type 2 diabetes in women



Size of data markers are proportional to the inverse of the variance of the odds ratio; CI confidence interval (bars); IV: fixed-effects model; D+L: random effects model; Solid vertical line presents no effect, the dotted line drawn though the diamond presents the summary measure with its confidence intervals (lateral tips of diamond). Heterogeneity assessment: I squared, P comes from Q statistic

Assessments of Bias, Heterogeneity and Sensitivity Analysis

Four RCTs showed high risk of bias in two domains, however, for most of the RCTs (n=14) the risk of bias could not be classified in one or more domains (**Supplemental table 2**). Most included observational studies were considered to be at low (n=8) and one of medium risk of bias (**Supplemental table 3**). The three meta-analyses of RCTs showed high between-

study heterogeneity, with an I^2 estimate exceeding 75% and $P_{Q\ statistic}$ <0.05 (**Figure 2, 3 and 4)**. High heterogeneity observed in our meta-analyses could be explained by differences between studies, including heterogeneous study populations, methods and effect estimates reported. We attempted to explore sources of heterogeneity contributing to our results, but none of the factors we considered (e.g., disease or menopausal status, route of administration, dosage or duration of the intervention (for RCTs), study location or design of studies) could explain the heterogeneity. Additionally, the heterogeneity was not explained by baseline characteristics of study participants, age and BMI. However, the quality of RCTs might be an important factor to influence such high heterogeneity observed; RCTs in low to medium risk of bias that reported insulin and HOMA-IR changes, showed lower heterogeneity as compared to RCTs in high risk of bias (**Table 1**). Meta-analysis of observational studies showed low heterogeneity with I^2 estimate 19.2% and $P_{Q\ statistic}$ =2.27. Stratification by type of phytoestrogens and difference between phytoestrogen intake in highest vs, lowest quantile yielded similar results as observed in the main analysis. Also, stratification by median BMI and age did not differ significantly from the main observations (**Table 2)**. Separate analysis excluding studies that reported pooled estimates of both sexes, and including only observational studies that investigated the association between phytoestrogen intake and T2D risk in female population was in line with the main findings (**Table 3**). In the leave-one out analysis for glucose our pooled estimates remained stable, indicating that the pooled results for glucose are not overly influenced by any single study. However, in the pooled analysis of phytoestrogen supplementation and insulin levels and HOMA-IR the summary effect size did not reach significance in all cases in the leave-one-out analysis, indicating no consistency (**Supplemental figure 4**). For the pooled analyses involving five or more studies, publication bias was assessed visually using Begg's funnel plots, which were approximately symmetrical. The Egger's test estimates were non-significant for all these analyses (p values ranging from 0.25 to 0.58) (**Supplemental figure 5 and 6**).

Discussion

In this systematic review and meta-analysis, we show that overall phytoestrogen supplementation is associated with reduction in fasting glucose in non-diabetic women. However, the results of clinical trials were not consistent for insulin and HOMA-IR and

indication for increased levels of these traits was observed with some specific types of phytoestrogens, such as use of isoflavone mix and isolated genistein. Findings from observational studies were consistent in showing higher dietary phytoestrogen intake to be associated with decreased risk of T2D in women.

Our findings of a protective effect of phytoestrogens among women without previous T2D are in line with other studies showing that phytoestrogens can improve HbA1c concentrations and insulin sensitivity in patients with metabolic syndrome and T2D [53-57]. Contrary to prior meta-analyses, which focused on both, men and women [17] , or on specific women populations (Asian postmenopausal women) [18], and included heterogeneous studies (e.g. participants taking glucose-lowering medications) [19], in our study we included (i) studies among women of any ethnicity and menopausal status, (ii) studies among women not taking glucose lowering medications as these can effect glucose/insulin levels and (iii) trials comparing any type of phytoestrogens against placebo (RCTs comparing intervention with lower dosage of phytoestrogens or estradiol/HRT were not included, also all studies that investigated combined interventions- exercise or diet with phytoestrogen supplementation, were not included). Additionally, our review did not restrict the search only to phytoestrogen supplementation, but we included also studies reporting dietary intake of phytoestrogens and phytoestrogens assessed in blood and urine. Therefore, our meta-analysis provides a more detailed assessment of the nature and magnitude of the association between composite and specific phytoestrogens, glucose homeostasis and T2D in women.

Potential mechanisms linking phytoestrogens and glucose metabolism and their potential role in T2D prevention have been extensively studied [7]. Phytoestrogens are thought to affect glucose metabolism via estrogen-dependent and non-estrogen dependent pathways [7]. Phytoestrogens modulate glucose and lipid metabolism directly (lipogenesis, lipolysis, adipogenesis) and indirectly modulate appetite and energy expenditure [7]. Phytoestrogens regulate glucose homeostasis-related metabolic processes at cellular levels in intestinal cells, pancreatic islet cells, hepatocytes and skeletal muscle cells [10,58,59]. Also, phytoestrogens increase expression of genes involved in glucose homeostasis and lipid metabolism [7], and suppress genes that affect gluconeogenesis[60]. Another possible protective mechanism in T2D is antioxidant activity of phytoestrogens [7], furthermore, clinical trials in humans found that isoflavone enriched soy products increased antioxidant

capacity [7]. It is also possible that phytoestrogen's anti-obesity features play a significant role in T2D prevention [61]. A study in normal-weight, postmenopausal women showed that the consumption of isoflavones was associated with lower body mass index and fasting insulin concentration [62].

To our knowledge, this is the first systematic review and meta-analysis including more than 213,000 women to comprehensively address the associations of phytoestrogens with glycemic traits and risk of developing T2D in women. However, there are several limitations that need to be taken into account. First, our overall findings may have been affected by publication bias. Despite conventional funnel plots and Egger test estimates indicate minimal publication bias, these approaches are limited by their qualitative nature. Second, while the quality of observational studies was in general high, the methodological quality of the RCTs varied considerably, which might have contributed to the heterogeneity we observed in the meta-analyses presented in this study. The factors that may have affected the quality of RCTs may include the composition of supplements or presence of menopausal symptoms, which is the main reason why women take supplements and which is also linked to adverse cardio-metabolic health [63,64]. Also, there were only four RCTs with more than 100 participants and only one RCT with a duration of the intervention of one year, which might undermine the precision of the estimates and limit our understanding about long term effects of phytoestrogen supplementation on glucose homeostasis. Third, pooled estimates for the association between phytoestrogen supplementation and insulin and HOMA-IR levels should be taken with caution as our leave-one-out sensitivity analysis showed that results were driven by individual studies. Fourth, the ability to meta-analyse studies on insulin and HOMA-IR are largely limited by the non-standardization of insulin assays. The mean insulin levels at baseline across RCTs demonstrate high variability, this is also the case for HOMA-IR, which includes insulin in its calculation. Fifth, a limitation of observational studies included in our meta-analysis is the use of food questionnaires to assess dietary intake of phytoestrogens. This method is prone to measurement error due to recall bias, incomplete inclusion of phytoestrogen-enriched food items in the questionnaire, and due to incomplete data on phytoestrogen composition of foods from food composition tables. Sixth, numerous factors may influence phytoestrogen metabolism and its plasma concentrations. However, because the outcome in all observational studies included in this systematic review was assessed prospectively, the subjective measure of dietary

phytoestrogen intake would likely lead to non-differential misclassification with respect to the outcome, and therefore would likely bias our estimates toward the null in our analysis. Considering the limited available evidence, prospective studies using objective biomarkers of phytoestrogen exposure are needed in order to further investigate potential protective role of phytoestrogens in prevention of T2D in women. Lastly, this review underscores a number of gaps in the literature concerning other types of phytoestrogens rather than isoflavone on their role in diabetes prevention. In light of these observations, the overall results of this study should be interpreted with caution.

This review may have several implications. Based on the available evidence, phytoestrogen-based remedies might be a safe choice with regard to T2D in treatment of menopausal symptoms in women. Additionally, our review addresses major literature gaps. It remains unclear if specific phytoestrogen-rich foods are more favorable in prevention of T2D. Findings from the Singapore Chinese Health Study, showed inverse associations between unsweetened soy products consumption and T2D risk, but in contrast to this, sweetened soybean drink consumption was positively associated with T2D risk (13). In the current review, we did not find differences in effects of different types of phytoestrogens risk of T2D, however, we have observed some differences in glucose homeostasis in regard to phytoestrogen type. In our subgroup analysis soy derived isoflavones were significantly associated with lower levels of glucose, while isolated genistein was associated with lower serum insulin and HOMA-IR. Furthermore, isoflavone mix significantly increased insulin and HOMA-IR levels. Isoflavone mixtures contain genistein, daidzein, and glycetin in various proportions due to variations of isoflavone composition in primary raw material. Therefore, when specific isoflavones (e.g. genistein) are administered alone they may have different metabolic effects as compared when a mixture of different types of isoflavones is administered. The content of genistein and daidzein is approximately equal, while glycetin is present in lower concentration in whole soy beans [19]. Genistein has ten-fold more potent estrogenic activity comparing to daidzein [65], whereas glycitein has the highest estrogenic potential in vivo[66]. Daidzein can be metabolised into equol, which has higher estrogenic potential than daidzein [67], while genistein and glycitein can be biodegraded into metabolites with no estrogenic activity [68]. However, our findings on subgroup analysis should be interpreted with caution as limited number of studies precluded our ability to perform comprehensive analysis. Further, it is known that in Asia fermented soy products are part of

the traditional diet, with isoflavone intake from 15 to 50 mg per day, with the highest intake in South-Eastern region [69], while in the European population isoflavones intake has been reported to be less than 2 mg per day [70]. The heterogeneity was moderate (I^2=42.1%, $P_{Qstatistics}$=0.16) for the association between phytoestrogens and risk of T2D, in the meta analysis including countries from Asia, whereas no heterogeneity was observed in the meta analysis of studies from outside of Asia. Baseline phytoestrogenintake in Asia is higher as compared to Western world; also, across Asia phytoestrogen intake varies across different countries [1], thus, there might be more variation and thereby more residual heterogeneity within this group. Further, there might be other factors contributing to higher heterogeneity in Asian studies (two studies were nested-case control and two were prospective cohort studies, whereas all non-Asian studies were prospective cohort) or diverse genetic populations which merit further investigation.

Emerging evidence is showing that soy products may be more effective in maintaining good health in equol-producing individuals [71]. The gut microbiome modifies phytoestrogens into metabolites that differ in biological activity from the parent compounds [71]. For example, Asian individuals have greater ability than non-Asians to produce equol, which is the metabolite of daidzein [1]. Existing trials, although did not reveal ethnic-differences on associations of phytoestrogens with glycemic traits, did not properly address this issue. Thus, it is necessary future trials and observational prospective studies investigate metabolites that are produced by phytoestrogens, and how these metabolites contribute to the relation of phytoestrogens to human health, and whether their levels and effects differ across populations. Further, future studies with adequate sample size investigating different types and dosages of phytoestrogens, and examining whether there are dose-effects are needed.

In conclusion, the available body of literature suggests that phytoestrogen dietary intake or supplementation might have a beneficial effect in prevention of insulin resistance and T2D among women. However, the intervention studies conducted up to date are of suboptimal quality and thus, further rigorous studies with long term follow-up are needed to determine the role of specific subgroups of phytoestrogens in diabetes prevention.

Table 1.Subgroup analysis

Subgroups by Study Characteristics		Number of studies	Difference, Mean (95 % CI)[1]	I^2 for heterogeneity[2]	*P-value* for heterogeneity[3]
Phytoestrogen use and mean serum glucose change					
Study population[a]	Healthy women	12	-0.1 (-0.19;-0.01)	97.3%	0.45
	Other women	5	-0.2 (-0.54;0.13)	96.1%	
Median number of participants[b]	≤60 women	8	-0.14 (-0.3;0.02)	93.2%	0.28
	>60 women	9	-0.1 (0.21;-0.003)	97.9%	
Menopausal status[c]	Postmenopausal	14	-0.07 (-0.15;0.01)	97.2%	0.17
	Other	3	-0.44 (-0.94;0.07)	91.5%	
Age[d]	≤55.15 y	9	-0.06 (-0.18;0.07)	96.6%	0.66
	>55.15y	8	-0.18 (-0.31;-0.05)	97.6%	
BMI[e]	≤25.77kg/m^2	9	-0.07 (-0.18;0.05)	97.5%	0.44
	>25.77kg/m^2	7	-0.23 (-0.43;-0.02)	97.1%	
Route of administration[f]	Tablet/capsule	14	-0.07 (-0.15;0.01)	97.2%	0.17
	Other	3	-0.44 (-0.94;0.07)	91.5%	
Dosage[g]	≤86mg/d	9	-0.17 (-0.36;0.02)	96.8%	0.29
	>86mg/d	7	-0.02 (-0.11;0.07)	97.2%	
	Other	1	NA	NA	
Intervention duration[h]	≤16 weeks	7	-0.09 (-0.24;0.07)	96.2%	0.42
	>16 weeks	10	-0.15 (-0.24;-0.06)	96.7%	
Location[i]	Asia	6	-0.07 (-0.15;0.01)	90.2%	0.81
	Other	11	-0.15 (-0.28;-0.02)	97.9%	
Risk of bias[J]	High risk of bias	8	-0.33 (-0.5;-0.16)	97.1%	0.62
	Low to medium risk of bias	9	0.03 (-0.06;0.12)	96.2%	
Design[k]	RCT Cross-over	4	0.01 (-0.31;0.03)	94.8%	0.33
	RCT	13	-0.15 (-0.26;-0.05)	97.4%	

[1]Mean difference refers to mean difference of changes between treatment groups in Serum glucose, insulin and HOMA-IR (subjects using phytoestrogens as compared with the subjects from control/placebo group)
[2]P value for heterogeneity was evaluated using random-effects meta-regression. P value was calculated between two/three groups that were considered to be source of heterogeneity, the groups are indicated in the table.;[a]Healthy women are considered premenopausal of postmenopausal women included in RCT, "other women" are women with metabolic syndrome, glucose intolerance, unrecognized diabetes (without antidiabetic medications), women treated for breast cancer in previous 6 months, osteopenic and obese women;[b]Median number of par cipants: calculated separately for each outcome;cMenopausal status: postmenopausal women vs. adult women; [d]Median age: calculated separately for each outcome; [d]Median BMI: calculated separately for each outcome (Liu et at., 2014 did not report BMI); [e]Route of administration includes tablets/capsules use and other routes of administration (shake, powder, flower); [f]Dosage (mean dosage based on all included RCTs was 80 mg/d); [g]Intervention duration: median intervention duration was calculated based on all included RCTs, we compared RCTs with duration of intervention ≤14 weeks and >14 weeks; [h] Location refers to study location, studies done in Asian ground vs. studies done in Europe, America and Australia; [j]Studies are considered to be in low risk of bias if allocation concealment, blinding of participants and outcome assessors were all coded yes, if a compliance assessment was done, and the number of dropouts and reasons for dropout were reported. In case that ≥3 quality criteria were not met, the study was classified as having high risk of bias; others were classified as having moderate risk of bias.[k]Study design refers to RCT vs. RCT cross-over design

Phytoestrogen use and mean serum insulin change

Subgroups by Study Characteristics		Number of studies	Difference, Mean (95 % CI)[1]	I^2 for heterogeneity[2]	*P-value* for heterogeneity[3]
Study population[a]	Healthy women	10	1.51 (-2.7;5.73)	89.1%	0.64
	Other women	5	-6.87 (-16.13;2.39)	95.7%	
Median number of participants[b]	≤50 women	6	-4.63 (-11.17;1.92)	94.8%	0.68
	>50 women	9	1.51 (-44.08;7.11)	90.2%	
Menopausal status[c]	Menopausal	12	0.98 (-2.76;4.71)	92.6%	0.20
	Other	3	-12.77 (-25.8;0.25)	74.3%	
Age[d]	≤56.61 y	8	-2.31 (-10.14;5.51)	92.1%	0.33
	>56.61 y	7	-0.28 (-4.85;4.29)	93.3%	
BMI[e]	≤26.25kg/m^2	8	1.51 (-1.95;4.97)	90%	0.13
	>26.25kg/m^2	7	-5.36 (-15.71;4.98)	91%	
Route of administration[f]	Tablet/capsule	9	2.51 (-4.15;9.17)	91.5%	0.23
	Other	6	-5.24 (-10.31;-0.17)	93.7%	
Dosage[g]	≤78mg/d	8	0.94 (-6.77;8.66)	93.2%	0.44
	>78mg/d	6	-1.45 (-5.59;2.69)	90.3%	
	Other	1	NA	NA	
Intervention duration[h]	≤12 weeks	8	-2.32 (-8.82;4.18)	93.2%	0.33
	>12 weeks	7	0.42 (-4.7;5.54)	91.8%	
Location[i]	Asia	4	-1.85 (-8.69;4.99)	94.1%	0.88
	Other	11	-0.86 (-6.12;4.41)	92.1%	
Risk of bias[j]	High risk of bias	6	-11.95 (-20.54;-3.35)	88%	0.19
	Low to medium risk of bias	9	3.91 (1.54;6.27)	76.2%	
Design[k]	RCT Cross-over	4	-1.02 (-8.2;6.17)	91.2%	0.29
	RCT	11	-1.13 (-6.43;4.18)	92.9%	

[1]Mean difference refers to mean difference of changes between treatment groups in Serum glucose, insulin and HOMA-IR (subjects using phytoestrogens as compared with the subjects from control/placebo group)
[2]P value for heterogeneity was evaluated using random-effects meta-regression. P value was calculated between two/three groups that were considered to be source of heterogeneity, the groups are indicated in the table.;[a]Healthy women are considered premenopausal of postmenopausal women included in RCT, "other women" are women with metabolic syndrome, glucose intolerance, unrecognized diabetes (without antidiabetic medications), women treated for breast cancer in previous 6 months, osteopenic and obese women;[b]Median number of par cipants: calculated separately for each outcome;cMenopausal status: postmenopausal women vs. adult women; [d]Median age: calculated separately for each outcome; [d]Median BMI: calculated separately for each outcome (Liu et at., 2014 did not report BMI); [e]Route of administration includes tablets/capsules use and other routes of administration (shake, powder, flower); [f]Dosage (mean dosage based on all included RCTs was 80 mg/d); [g]Intervention duration: median intervention duration was calculated based on all included RCTs, we compared RCTs with duration of intervention ≤14 weeks and >14 weeks; [h] Location refers to study location, studies done in Asian ground vs. studies done in Europe, America and Australia; [j]Studies are considered to be in low risk of bias if allocation concealment, blinding of participants and outcome assessors were all coded yes, if a compliance assessment was done, and the number of dropouts and reasons for dropout were reported. In case that ≥3 quality criteria were not met, the study was classified as having high risk of bias; others were classified as having moderate risk of bias.[k]Study design refers to RCT vs. RCT cross-over design

Phytoestrogen use and mean HOMA-IR change

Subgroups by Study Characteristics		Number of studies	Difference, Mean (95 % CI)[1]	I^2 for heterogeneity[2]	*P-value* for heterogeneity[3]
Study population[a]	Healthy women	4	-0.61 (-1.2;-0.02)	97.3%	0.27
	Other women	7	-0.08 (-0.35;0.20)	97.8%	
Median number of participants[b]	<60 women	6	.49 (-1.05;0.06)	.5%	0.11
	≥60 women	5	-0.02 (-0.18;0.13)	94.7%	
Menopausal status[c]	Menopausal	8	-0.14 (-0.37;0.09)	97.9%	0.15
	Other	3	-0.62 (-1.41;0.17)	91.5%	
Age, y[d]	≤60 y	6	-0.38 (-0.85;0.08)	.9%	0.17
	>60 y	5	-0.13 (-0.38;0.11)	96.4%	
BMI[e]	≤27.3kg/m^2	5	-0.09(-0.38;0.2)	98.4%	0.09
	>27.3kg/m^2	6	-0.44 (-0.9;0.02)	96.1%	
Route of administration[f]	Tablet/capsule	5	-0.16 (-0.371;0.39)	98.2%	0.20
	Other	6	-0.28 (-0.5;-0.05)	96%	
Dosage[g]	≤93mg/d	5	-0.51 (-1.1;0.08)	98%	0.35
	>93mg/d	5	0.02 (-0.17;0.22)	95.4%	
	Other	1	NA	NA	
Intervention duration[h]	<16 weeks	5	-0.43 (-0.82;-0.03)	96.5%	0.18
	≥16 weeks	6	-0.11 (-0.44;0.21)	98.2%	
Location[i]	Asia	3	-0.12 (-0.48;0.24)	97.8%	0.34
	Other	8	-0.32 (-0.65;0.02)	97.1%	
Risk of bias[j]	High risk of bias	6	-0.62 (-1.07;-0.17)	91.6%	0.10
	Low to medium risk of bias	5	0.1 (0.02;0.18)	75%	
Design[k]	RCT Cross-over	3	-0.17 (-0.66;0.33)	93.2%	0.09
	RCT	8	-0.3 (-0.64;0.04)	97.9%	

[1]Mean difference refers to mean difference of changes between treatment groups in Serum glucose, insulin and HOMA-IR (subjects using phytoestrogens as compared with the subjects from control/placebo group)
[2]P value for heterogeneity was evaluated using random-effects meta-regression. P value was calculated between two/three groups that were considered to be source of heterogeneity, the groups are indicated in the table.;[a]Healthy women are considered premenopausal of postmenopausal women included in RCT, "other women" are women with metabolic syndrome, glucose intolerance, unrecognized diabetes (without antidiabetic medications), women treated for breast cancer in previous 6 months, osteopenic and obese women;[b]Median number of par cipants: calculated separately for each outcome;cMenopausal status: postmenopausal women vs. adult women; [d]Median age: calculated separately for each outcome; [d]Median BMI: calculated separately for each outcome (Liu et at., 2014 did not report BMI); [e]Route of administration includes tablets/capsules use and other routes of administration (shake, powder, flower); [f]Dosage (mean dosage based on all included RCTs was 80 mg/d); [g]Intervention duration: median intervention duration was calculated based on all included RCTs, we compared RCTs with duration of intervention ≤14 weeks and >14 weeks; [h] Location refers to study location, studies done in Asian ground vs. studies done in Europe, America and Australia; [j]Studies are considered to be in low risk of bias if allocation concealment, blinding of participants and outcome assessors were all coded yes, if a compliance assessment was done, and the number of dropouts and reasons for dropout were reported. In case that ≥3 quality criteria were not met, the study was classified as having high risk of bias; others were classified as having moderate risk of bias.[k]Study design refers to RCT vs. RCT cross-over design

Table2. Subgroup analysis for phytoestrogen intake and risk of T2D

Subgroups by Study Characteristics		Number of studies	Number of participants	Number T2D cases	Relative risk (95% Confidence Interval)	*P-value* for heterogeneity[1]
Study population[a]	Only women	7	183 919	8 117	0.91 (0.83; 0.99)	0.65
	Men and women	3	28 927	1 604	0.88 (0.79;0.98)	
Study design[b]	Nested case-control	2	7950	425	0.97 (0.78;1.20)	0.45
	Cohort	7	204 896	9 296	0.89 (0.83;0.96)	
Type of phytoestrogens[c]	Isoflavones	5	106 006	3 428	0.86 (0.77;0.98)	0.30
	Flavonoids	4	106 840	6 293	0.93 (0.86;1.01)	
	Genistein	3	39 964	2 232	0.87 (0.73;1.05)	NA
	Daidzein	3	39 964	2 232	0.89 (0.84;0.95)	NA
	Soy products	3	72 997	1 304	0.81 (0.68;0.97)	NA
Location[d]	Asia	4	73 997	1 621	0.83 (0.87;0.98)	
	Other	5	138 849	8100	0.93 (0.88;0.98)	0.05
Difference between phytoestrogen intake in highest vs. lowest quantile	≤median (5.33 fold)	5	123 123	3 461	0.88 (0.78;1.00)	0.49
	>median (5.33 fold)	4	89 723	6 260	0.92(0.87;0.999)	
BMI	≤median (25.89 kg/m^2)	5	80 551	4 146	0.91 (0.86;0.97)	0.70
	>median (25.89 kg/m^2)	4	132 295	5 575	0.88 (0.78;1.00)	
Age	≤median (53.87y)	5	133 736	4 131	0.88 (0.79;0.98)	0.48
	>median (53.87y)	4	79 110	5 590	0.92 (0.87;0.98)	

[1]P value for heterogeneity was evaluated using random-effects meta-regression. P value was calculated between two/three groups that were considered to be source of heterogeneity, the groups are indicated in the table. (if more than 5 studies were included); [a]Study population: Studies done only in women: investigation performed only among female population, after excluding studies (Mueller et al, Zamora-Ros et al and Knekt et al) that reported overall results for male and female subjects but have stated that they tested the interaction term with sex; [b] Study design: Only prospective cohort and nested case-control studies were included; [c] Type of phytoestrogens: Soy products: estimated pooled together for soy beans, soy milk, soy flour, other soy products; [d]Location: Asia (South Korea, Japan and two studies from China; Other: Europe and two studies from USA)

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CHAPTER 4.2

Phytoestrogen supplementation and body composition in postmenopausal women: A systematic review and meta-analysis of randomized controlled trials

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Maturitas, Vol. 115, September 2018, Pages 74-83

ABSTRACT

BACKGROUND: Phytoestrogen-based medications are commonly used by menopausal women to relieve menopausal symptoms, especially among obese postmenopausal women. Substitution of animal with soy protein is often used in weight loss regimens, yet, the effect of phytoestrogens, the main constituent of soy foods, on body composition following regular diet is not completely understood.

METHODS: We conducted a systematic review and meta-analysis to investigate the associations between phytoestrogen supplementation and body weight and the main parameters of body composition in postmenopausal women. A literature search was done using 5 electronic databases from inception to April, 2018. Randomized controlled trials (RCTs) in postmenopausal women comparing phytoestrogen supplementation followed by usual diet and placebo were included in current meta-analysis.

RESULTS and CONCLUSIONS: From 5,932 references, we identified 23 RCTs that met our inclusion criteria, including 1,880 postmenopausal women. No association was observed between overall phytoestrogen supplementation followed by regular diet and body weight, body mass index, waist and hip circumference, total fat mass and percentage of body fat. However, the use of phytoestrogens supplementation was associated with a slight decrease in waist-hip ratio, pooled mean difference of changes of was -0.01cm (95%CI: -0.01 to -0.006). In subgroup analysis, we found a modest decrease in body weight with overall phytoestrogens supplement use as compared to placebo in healthy postmenopausal women [pooled mean difference of changes -0.28Kg (95%CI: -0.52 to -0.04)] and in small RCTs with median number of participants ≤66 [pooled mean difference of changes -0.49 Kg (95%CI: -0.87 to -0.11)]. In contrast, phytoestrogen supplementation was associated with increased body weight in postmenopausal women with preexisting metabolic disorders (prediabetes, type 2 diabetes, prehypertension and hyperlipidemia) [pooled mean difference of changes: 0.78 Kg (95%CI: 0.53 to 1.03)]. In addition, there were some indications that some types of phytoestrogens, such as Daidzein, but not soy products or isoflavone mix, could lead to modest adverse changes in body composition in menopausal women. Therefore, future studies should investigate the potential adverse effects of phytoestrogen supplementation followed with regular diet on body composition among postmenopausal women.

Introduction

Menopause is considered as the end of woman's reproductive life, generally defined as "cassation" of menstrual periods for twelve consecutive months[1]. Menopausal transition is characterized by hormonal disturbances, irregular menstrual periods and presence of menopausal symptoms[1]. The most challenging menopausal symptoms include hot flashes and night sweats, with 50.3% to 82.1% of postmenopausal reporting either mild, moderate or severe vasomotor symptoms[2,3]. In women, aging and menopause-induced estrogen deficiency could result in an increase in body weight and may lead to abdominal fat accumulation and decrease in lean mass during menopausal transition [4]. Overweight and obese menopausal women may also tend to have more prevalent [8] and more severe menopausal symptoms [5-8]. Thus, menopausal hormone therapy (MHT) remains the most effective treatment for menopausal vasomotor symptoms[9]. However, given the potentially undesirable health consequences of hormone therapy on cardiovascular health and breast cancer risk, the number of women choosing plant based-therapies as an alternative to treat menopausal symptoms is increasing [10].

The most commonly used herbal therapies may include "over the counter" phytoestrogen supplements, such as dietary soy isoflavones and soy extracts and herbal remedies such as red clover and black cohosh [10]. Phytoestrogens are a group of biologically active plant-derived compounds with estrogen-like properties[11]. Isoflavones (genisten and diadzein) and lignans are the most used phytoestrogens; while isoflavones can be abundantly found in soybeans, lignans are found in legumes, vegetables, fruits, flaxseed and whole grains[11]. Recent meta-analysis of clinical trials showed that specific phytoestrogen supplementation led to relief of menopausal symptoms [10]. Nevertheless, there is inconsistent evidence whether phytoestrogens could additionally help to reduce body weight and counteract the adverse changes that may occur in body composition in women after menopause. While few studies indicated that phytoestrogens may lead to modest improvements in body weight and the other parameters of body composition [12-14], there were few studies raising concerns that phytoestrogens could lead to adverse body composition changes, such as increase in weight [15-18] and body mass index (BMI)[16,18-20]. A meta-analysis of nine randomized trials (conducted in 2013) has suggested that isoflavone supplementation might reduce the body weight [21]. However, this study was limited only to non-Asian

postmenopausal women and by investigating only the changes in body weight and not the other parameters of body composition. Furthermore, only trials that investigated solely isoflavone supplementation, and not the other types of phytoestrogens, were included in that review.

Therefore, this comprehensive systematic review and meta-analysis of intervention studies aimed to evaluate the association between phytoestrogen supplementation followed with regular/normocaloric diet and body composition in postmenopausal women.

2. METHODS

2.1 Data Sources and Search Strategy

The Cochrane Handbook for Systematic Reviews of Interventions and PRISMA Statement were used to guide the conduct and reporting of this review [22]. A literature search was done using 5 electronic databases (Medline via Ovid, EMBASE, Web of Science Core Collection, Cochrane CENTRAL via Wiley and Google Scholar) from inception to April 2018 (date last searched). Additionally, we searched the reference lists of the included studies and relevant reviews. Details on the search strategy are provided in **Supplemental Table 1**.

2.2 Study Selection and Data Extraction

Studies were included if they met the following inclusion criteria: (i) were randomized placebo-controlled trials (ii) reported associations of phytoestrogen supplementation with total body weight, total fat mass (FM), percentage of body fat (PBF), BMI, waist circumference (WC), and waist to hip ratio (WHR), (iii) were performed among postmenopausal women and (iv) investigated phytoestrogen supplementation in intervention arm alone. RCTs investigated combination of exposures (e.g. hypocaloric diet or exercise with phytoestrogen supplementation) were not included in this study. Additionally, as we were interested to evaluate overall effect of phytoestrogens on body weight (and not its effect on weight loss), we decided to include RCTs irrespectively of the study aim; therefore, all eligible RCTs that reported baseline and end of study information on outcomes of interest were included in this review. Two reviewers independently evaluated the titles and abstracts according to the inclusion and exclusion criteria. For each potentially eligible study, two reviewers assessed the full-text. In cases of disagreement, a

decision was made by consensus or, if necessary, a third reviewer was consulted. A predesigned data extraction form was used to collect relevant information.

2.3 Assessing the Risk of Bias

The risk of bias within each individual study was evaluated by two reviewers. To assess the quality of RCTs, "The Cochrane Collaboration's tool" for assessing risk of bias was used [23]. Studies were judged to be at lower high risk of bias based on criteria to evaluate random sequence generation, allocation concealment, blinding of participants/personnel and outcome assessment, incomplete outcome data and selective reporting [23]. RCTs were considered to be in low risk of bias, if allocation concealment, blinding of participants and outcome assessors were all coded "*yes*", if a compliance assessment was done, and the number of dropouts and reasons for dropout were reported, otherwise the RCTs were considered to be at high risk of bias. If the risk of bias couldn't be determined in any of the segments (e.g. information not provided) the risk of bias was classified as unknown (**Supplemental Table 2)**.

2.4 Data Synthesis and Analysis

Summary outcomes measures were presented as mean differences (intervention minus control) of the treatment effects (differences in outcomes at the end of trial) between treatment groups in body weight, BMI, WC, HC, HC, FM and PBF. Estimates of weighted mean differences (WMDs) and 95% CIs were obtained using random-effect model. In case of cross-over trials, the data from the first period only were used. In addition, for sensitivity analysis, the estimates were reported using fixed effect models as shown in the forest plots. Heterogeneity was quantified using the I^2 statistic, classified as low (I^2 ≤25%), moderate (I^2 >25% and <75%), or high (I^2 ≥75%) [24]. Additionally, Q-statistic was used to assess the presence of heterogeneity. $P_{Q\ statistic}$ ≥0.05 was considered to indicate no significant heterogeneity among the included studies. Study characteristics including geographic location, "primary" aim of the study, median number of participants, type of phytoestrogens, median duration of intervention, route of phytoestrogen administration, time since menopause onset, and women's health status (healthy vs. women with preexisting chronic disease/non-healthy) were pre-specified as characteristics for assessment of heterogeneity and were evaluated using stratified analyses and random-

effects meta-regression, if 10 or more studies were included in the meta-analysis [25]. To assess the influence of each individual study to the overall estimates of the rest of the studies, leave-one-out sensitivity analysis was performed iteratively by removing one study at a time to confirm that the findings were not influenced by any single study. Publication bias was evaluated through a funnel plot and asymmetry was assessed using the Egger's test. All tests were two-tailed and p-values of 0.05 or less was considered as statistically significant. STATA release 14 (Stata Corp, College Station, Texas) was used for all statistical analyses.

3. Results

3.1 Characteristics of included RCTs

Overall 5,932 references were identified using the search strategy. After initial screening based on titles and abstracts, the full texts of 92 articles were retrieved and evaluated further. As shown in **Figure 1,** after full text assessment, 69 studies were excluded due to inappropriate study design, inappropriate population/exposure studies, or gender specific estimates missing. The remaining 23 RCTs [15-20,26-43] were included in the review and meta-analysis.

In total 1,880 postmenopausal women (1,130 in intervention arm and 750 in placebo arm) were included in the meta-analysis of 23 RCTs. Six trials were done in Asia, 6 in Europe, 5 in North America and 6 in South America. Most of the studies were done among healthy women (n=19), while four RCTs were conducted in women with metabolic syndrome, type 2 diabetes (T2D), sarcopenic obese, and one RCT in women with prehypertension. All women included in current review were postmenopausal and on average 4.19 to 15.9 years into menopause, and did not use MHT. The follow-up ranged from 8 to 48 weeks, with majority of RCTs lasting for 24 weeks (n=11). In nine included RCTs, the effect of phytoestrogen supplementation on parameters of body composition was primarily investigated, while the rest of RCTs investigated the other outcomes (eg. effect of phytoestrogen supplementation on bone mineral density, menopausal symptoms relief), but reported baseline and end study data on anthropometric parameters of interest. Detailed characteristics of included RCTs are presented in **Table 1**.

Figure 1. Flowchart of studies included in this review

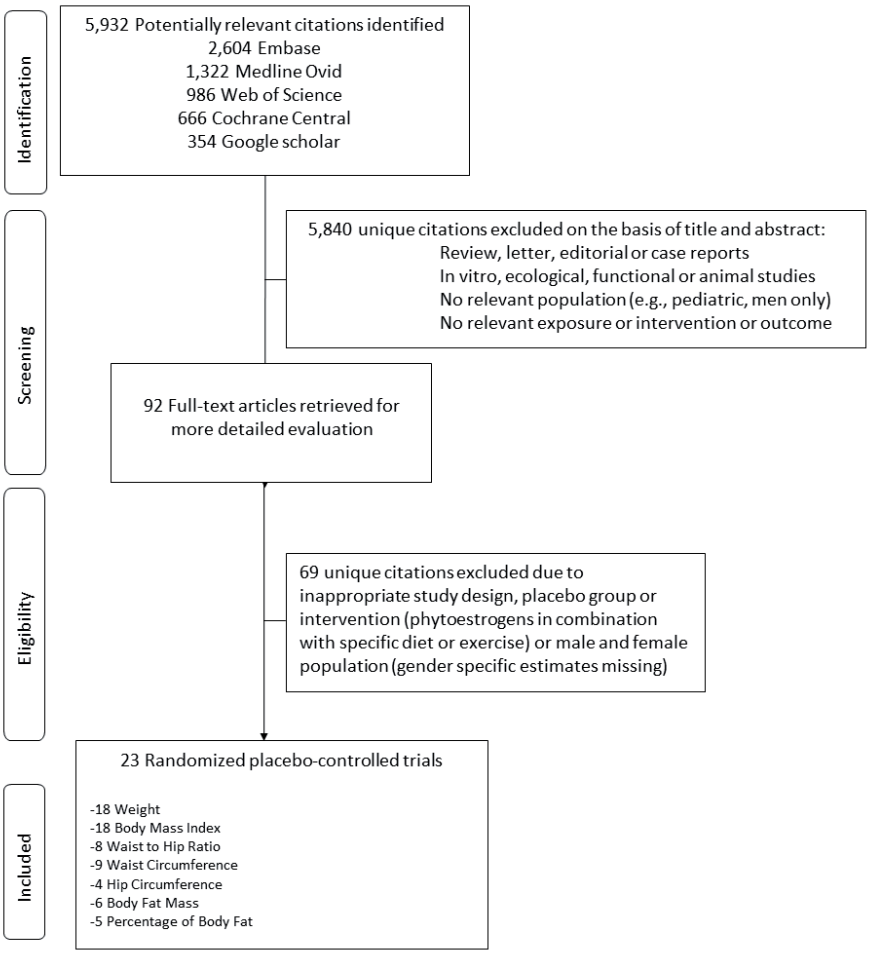


Table 1. Characteristics of studies included in meta-analysis

Lead Author, Publication Date	Location	Age group	Intervention form, therapy and daily dosage	Primary outcome of trial	Intervention period	Control	RCT design	Total trial participants	Mean years since menopause	Health status
Arjmandi et al, 2005	USA	<65	Snack bar, drink mix or cereal, 25mg of soy protein (60 mg of isoflavones)	Bone's metabolism	48 weeks	Placebo	Parallel	62	5.4	Healthy
*Aubertin-Leheudre et al, 2007	Canada	50-70 (66±5)	Capsule, 70 mg isoflavones	Body composition	24 weeks	Placebo	Parallel	18	NA	Sarcopenic obese
Aubertin-Leheudre et al, 2008	Canada	50-70 (66±5)	Capsule, 70 mg isoflavones	Clinical cardiovascular risk factors	24 weeks	Placebo	Parallel	50	6	Obese
*Bakhtiary et al, 2011	Iran	60-70 (64.35± 2.86)	Powder, Isoflavones, 117.2 mg	Body composition	12 weeks	Placebo	Parallel	50	15.9	Metabolic syndrome
Chiechi et al, 2002	Italy	39-60	Diet, isoflavones, 40-60mg	Serum lipids	24 weeks	Placebo	Parallel	67	4.9	Heathy
*Choquette et al, 2011	Canada	50-70 (58.7±5 .3)	Capsule, 70 mg isoflavones	Body composition	24 weeks	placebo	Parallel	55	9	Healthy
Colacurci et al, 2005	Italy	55.15± 3.85	Tablet, Genistein 60 mg, Daidzein 30 mg	Endothelial function	24 weeks	Placebo	Parallel	60	4.9	Healthy
Colli et al, 2012	Brazil	46-68 (55.2)	Flaxseed extraxt, 100mg ecoisolariciresinol diglucoside (SDG) or ground whole Flaxseed, 270 mg of SDG	Menopausal symptoms	24 weeks	Placebo	Parallel	53	NA	Healthy
Delmanto et al, 2013	Brazil	>45	Capsules, soy isoflavones, 100mg	Mammographic density and breast	40 weeks	Placebo	Parallel	80	6.85	Healthy

				parenchyma						
Engelbert et al, 2016	Germany	50-60	Capsule, Soy isoflavones, 117.4mg	LDL receptors and scavenger receptor CD36	12 weeks	Placebo	Parallel	170	NA	Healthy
Garrido et al,2006	Chile	45-60 (53.52± 3.52)	Capsule, isoflavones, 45 mg	Plasma lipids	12 weeks	Placebo	Parallel	29	1.5	Healthy
Hidalgo et al, 2005	Ecuador	>40	Capsule, red clover derived isoflavones, 40 mg	Menopausal symptoms	12 weeks	Placebo	Cross-over	53	NA	Healthy
Ho et al, 2007	Hong Kong	48-62 (54.25± 3.25)	Capsule, Isoflavones (genistein, daidzein, glycetein), 80 mg	Lipid profile	48 weeks	placebo	Parallel	203	4.13	Healthy
Khaodhiar et al, 2008	USA	38-60 (52.72± 5.22)	Capsule, Daidzein, 60mg	Menopausal symptoms	12 weeks	Placebo	Parallel	94	5.1	Healthy
Kim et al, 2013	South Korea	53.5/53.7	Capsules, isoflavones 70mg	Triglycerides and luteunizing hormone	12 weeks	Placebo	Parallel	85	3.6	Healthy
Liu et al, 2010	Hong Kong	48-70 (55.95± 4.1)	Flour, Isoflavones, 100mg	Glycemic control and insulin sensitivity	24 weeks	Placebo: low-fat milk protein	Parallel	180	5.9	Prediabetes/ untreated diabetes
*Liu et al, 2013	Hong Kong	48-65	Beverage powder, soy flour 40g and daidzein, 63mg	Body composition	24 weeks	Placebo	Parallel	180	9	Prehypertensive
*Maesta et al, 2006	Brazil	45-70	Tablets, soy protein, 25g (32 mg genistein, 15 daidzein, 3g glycitein)	Body composition	16 weeks	Placebo	Parallel	21	10.6	Healthy
*Orsatti et al, 2010	Brazil	45-70	Capsules, isoflavones, 100mg	Body composition	36 weeks	Placebo	Parallel	38	7.07	Healthy
*Sites et al, 2007	Italy	55.6	Shakes, 20g of soy protein + 160 mg of	Body composition	24 weeks	Placebo	Parallel	15	4.12	Healthy

			isoflavones							
Villa et al, 2009	Italy	53.91 (53.92± 3.94)	Tablets, 18 mg Genistein	Cardiovascular Risk Factors	24 weeks	Placebo	Parallel	50	NA	Healthy
*Weickert et al, 2006	Europe	Mean age 59	Cereal bars, isoflavones, 50mg	Body composition	8 weeks	Placebo	Cross-over	34	NA	Healthy
*Wu et al, 2006	Japan	45-60	Capsules Isoflavones,77mg	Body composition	1 year	Placebo	Parallel	66	3.7	Healthy

*RCTs that primarily investigated the association between phytoestrogen supplementation and body composition
NA: not available

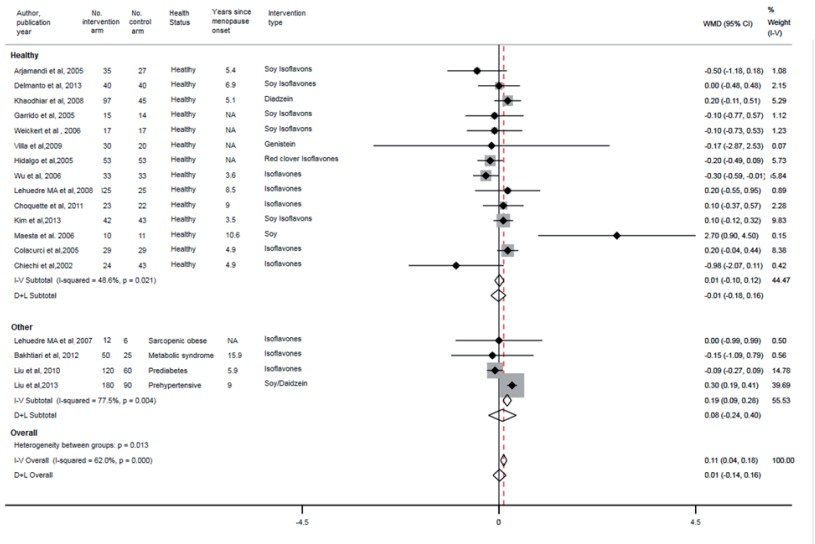
3.2 Association Between Phytoestrogen Supplementation and Parameters of Body Composition

Data from 18 RCTs, including 1,692 postmenopausal women, contributed to the meta-analysis on effects of phytoestrogen supplementation on body weight. Consumption of any type of phytoestrogen supplements, as compared to placebo, was not associated with significant decrease in body weight in postmenopausal women [pooled mean difference of changes: -0.14Kg (95%CI: -0.49 to 0.21)] (**Figure 2**). in subgroup analysis, a significant decrease in body weight with overall phytoestrogens supplement use as compared to placebo was found in healthy postmenopausal women [pooled mean difference of changes: -0.28 Kg (95%CI: -0.52 to -0.04)]. In contrast, phytoestrogen supplementation was associated with increased body weight in postmenopausal women with preexisting health disorders (prediabetes, T2D, prehypertension and hyperlipidemia), [pooled mean difference of changes: 0.78 Kg (95%CI: 0.53 to 1.03)] (**Figure 2**).

Based on the findings of 18 RCTs, including 1,456 postmenopausal women, overall phytoestrogen supplementation was not associated with BMI changes, [the pooled mean difference of BMI was 0.01 Kg/m^2 (95%CI: -0.14 to 0.16)] (**Figure 3**). In subgroup analysis on health status, the results remained similar, with no significant decrease in mean difference of BMI among healthy and postmenopausal women with preexisting health conditions (**Figure 3**). When stratified by type of phytoestrogen supplements, a significant increase was observed in body weight and BMI change with daidzein [pooled mean difference of changes: 0.92 Kg (95%CI: 0.24 to 1.59)] and [pooled mean difference of changes: 0.35

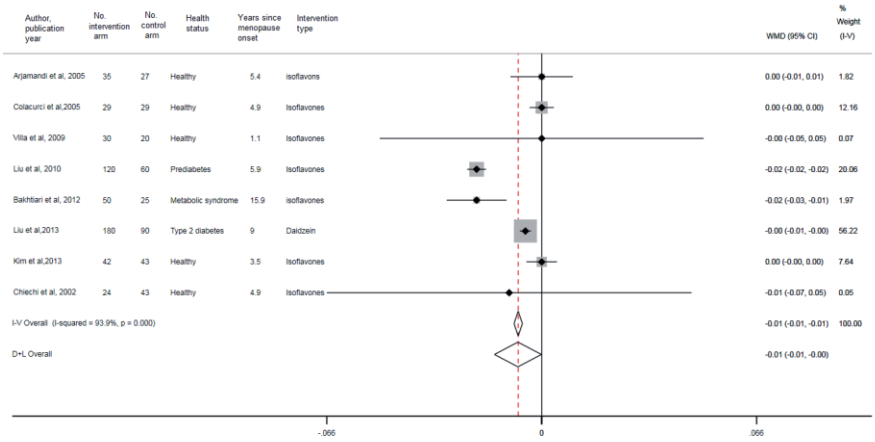
Kg/m^2 (95%CI: 0.17 to 0.52] respectively. Moreover, a significant decrease of body weight was found with isoflavone mix supplements [pooled mean difference of changes: -0.24 Kg (95%CI: -0.46 to-0.01)] (**Table 2**). The data from 8 RCTs including 847 postmenopausal women contributed to the meta-analysis on effects of phytoestrogen supplementation on WHR. Pooled mean WHR change was slightly and significantly decreased, [pooled mean difference of changes of was -0.01cm (95%CI: -0.01 to -0.006) (**Figure 4**). Data from 9 RCTs, including 824 postmenopausal women and 4 RCTs including 610 postmenopausal women investigated the association between phytoestrogen supplementation and waist and hip circumference changes, respectively. Consumption of any type of phytoestrogen supplements, as compared to placebo, was not associated with a reduction in changes of waist circumference and hip circumference [pooled mean differences of change were: 0.27cm (95%CI: -0.38 to 0.92) and 0.49cm (95% CI -0.20 to 1.17)] (**Supplemental figure 1**). Furthermore, pooled mean differences based on 6 RCTs, including 421 and 5 RCTs including 573 postmenopausal women showed no association between overall phytoestrogen use and changes in the body fat and percentage of body fat. Pooled mean difference of changes were -0.23kg (95%CI: -0.74 to 0.28) and -0.26% (95% CI -0.75 to 0.18), respectively (**Supplemental figure 2**).

Figure 3. The association between phytoestrogen intake and changes in BMI



Pooled mean difference is based on 18 RCTs, including 1,456 postmenopausal women (835 in intervention arm and 603 in control arm); WMD, weighted mean different. Mean difference refers to mean difference of changes between treatment groups. Size of data markers are proportional to the inverse of the variance of the effect estimate. P value comes from Q statistics.

Figure 4. The association between phytoestrogen supplementation and changes in waist to hip ratio



Pooled mean difference is based on 8 RCTs, including 847 postmenopausal women (510 in intervention arm and 337 in control arm); WMD, weighted mean different. Mean difference refers to mean difference of changes between treatment groups. Size of data markers are proportional to the inverse of the variance of the effect estimate. P value comes from Q statists

3.3 Assessments of Bias, Heterogeneity and Sensitivity Analysis

Two RCTs showed high risk of bias in two domains; however, for most of the RCTs (n=21), the risk of bias could not be classified in one or more domains (**Supplemental table 2**). The four of seven meta-analyses of RCTs showed high between-study heterogeneity, with an I^2 estimate exceeding 75% and $P_{Q\ statistic}$ <0.05, yet, in subgroup analysis by the type of phytoestrogens status, the heterogeneity varied from 0 to 97.2%. In the current systematic review, as shown in the stratified analyses, high heterogeneity might be attributed to the study quality, differences in the methodology of trials and study location. Although I^2 values varied across subgroup analysis, using "meta-regression method" , it was not possible to explain the observed heterogeneity made by any of parameters investigated (the "primary" goal of the study, health status, median years since menopause, route of phytoestrogen administration, type of phytoestrogens, duration of the intervention, number of study participants nor by study location or study quality) (**Table 1).** However, when stratified by median number of trial participants, in small RCTs with median number of participants ≤66 phytoestrogen supplementation was associated with significant decrease in body weight [pooled mean difference of changes -0.49Kg (95%CI: -0.87 to -0.11)](**Table 2).**

In a leave-one out sensitivity analysis, the pooled estimates were not influenced by any single study included in the analyzes (**Supplemental figure 3 a,b,c**). For the pooled analyses involving eight or more studies, publication bias was assessed visually using Begg's funnel plots. The funnel plot for pooled analysis of body weight changes was nonsymmetrical with Egger's p value of 0.005 indicating presence of publication bias. However, funnel plots for pooled analyses reporting changes in BMI and WHR were approximately symmetrical with non-significant Egger's test estimates for all of these analyses (p values were 0.19 and 0.97 respectively) (**Figure 5**).

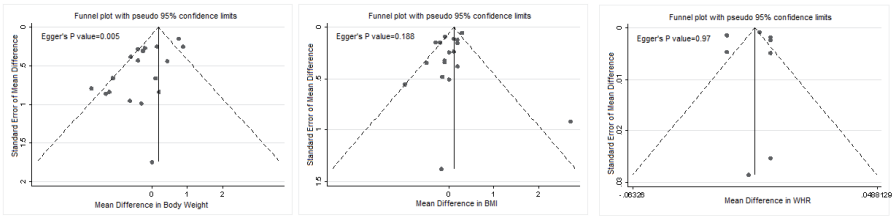
Table 2. The subgroup analyses by study characteristics

Subgroups by Study Characteristics		Number of studies	[1]Difference, Mean (95 % CI)	[2]I^2 for heterogeneity	[3]P value for heterogeneity
Phytoestrogen use and mean body weight change					
[a]Primary study goal of the RCT	Body composition	7	-0.04 (-0.68; 0.60)	68.7%	0.59
	Other	11	-0.21 (-0.64; 0.23)	62.3%	
[b]Median years since menopause onset	≤4.1 years	4	-0.23 (-0.6; 0.14)	16.5%	0.87
	>4.1 years	8	0.07 (-0.51; 0.64)	68.2%	
	Unknown	6	-0.29 (-0.63; 0.04)	0%	
[c]Route of administration	Tablet/capsule	10	-0.17 (-0.82; 0.47)	76.4%	0.52
	Diet	8	-0.17 (-0.42; 0.07)	0%	
[d]Intervention type	Soy products	6	-0.49 (-1.21;0.23)	65.4%	0.83
	Isoflavone mix	10	-0.24(-0.46; -0.01)	0%	
	Daidzein	2	0.92 (0.24; 1.59)	55%	
[e]Median number of study participants	≤66women	9	-0.49 (-0.87; -0.11)	0%	0.22
	>66 women	9	0.11 (-0.31; 0.52)	75.2%	
[f]Intervention duration	≤24 weeks	15	-0.10 (-0.51; 0.30)	69.5%	0.9
	> 24weeks	3	-0.32 (-1.02; 0.37)	53.2%	
[g]Location	Asia	7	0.22 (-0.25; 0.69)	76.8%	0.48
	Europe	4	-0.35 (-0.78; 0.07)	11%	
	North America	4	-0.6 (-1.44; 0.24)	0%	
	South America	3	-0.48 (-1.13; 0.17)	0%	
[h]Risk of bias	High	2	-0.85 (-1.93; 0.22)	0%	0.39
	Low to medium	16	-0.09 (-0.45; 0.27)	69.6%	

Phytoestrogen use and mean Body Mass Index change					
[a]Primary study goal of the RCT	Body composition	7	0.002 (-0.26;0.44)	74.4%	0.49
	Other	11	0.01(-0.13; 0.13)	24.6%	
[b]Median years since menopause onset	≤7.45 years	8	-0.03 (-0.2; 0.14)	53.9%	0.51
	>7.45 years	4	-0.08 (-0.5; 0.33)	0%	
	Unknown	6	-0.16(-0.40; 0.08)	0%	
[c]Route of administration	Tablet/capsule	10	0.03 (-0.17; 0.23)	47.9%	0.23
	Diet	8	-0.02 (-0.26; 0.22)	73.1%	
[d]Intervention type	Soy products	5	-0.15 (-0.76; 0.47)	68.3%	0.64
	Isoflavone mix	9	0.01 (-0.10; 0.11)	0%	
	Daidzein	2	0.35 (0.17; 0.52)	25.1%	
	Genistein	1	-0.17 (-2.87, 2.53)	NA	
[e]Median number of study participants	≤60 women	9	0.07 (-0.2; 0.35)	36%	0.19
	>60 women	9	-0.02 (-0.21; 0.17)	75.2%	
[f]Intervention duration	≤20 weeks	9	-0.03 (-0.24; 0.19)	74%	0.31
	> 20 weeks	9	0.05 (-0.16; 0.26)	38.1%	
[g]Location	Asia	5	0.01 (-0.24;0.26)	82.7%	0.48
	Europe	4	-0.06 (-0.51; 0.39)	38.2%	
	North America	5	0.09 (-0.13; 0.31)	0%	
	South America	4	0.1 (-0.44; 0.63)	69.6%	
[h]Risk of bias	High	2	0.18 (-0.06; 0.41)	0%	0.81
	Low to medium	16	-0.01 (-0.17; 0.16)	65.8%	

[1]Mean difference refers to mean difference of changes between treatment groups in body weight and BMI (subjects using phytoestrogens as compared with the subjects from control/placebo group)
[2]I^2 for heterogeneity was calculated using fixed- effects models
[3]P value for heterogeneity was evaluated using random-effects meta-regression (in case that more than 8 studies was meta-analyzed).
[a]Some of RCTs investigated the effect of phytoestrogens on body composition, the others investigated other outcomes but they reported changes in anthropometric parameters at baseline and at the end of the studies
[b]Median years since menopause, number of years since menopause onset, unknown: no information
[c]Route of administration includes tablets/capsules use and other routes of administration (shake, powder, flower)
[d]Type of phytoestrogens includes use of soy derived isoflavones/soy protein + isoflavones, extracts of soy isoflavones/isoflavone mixture, daidzein/genistein supplements.
[e]Number of study participants: median number of participants calculated for each outcome separately
[f]Intervention duration: median intervention duration was calculated for each outcome separately
[g] Location refers to study location, studies done in Asian ground and the other location (studies done in Europe, America and Australia)
[h]Studies are judged to be at lower high risk of bias based on criteria to evaluate random sequence generation, allocation concealment, blinding of participants/personnel and outcome assessment, incomplete outcome data and selective reporting

Figure 5. Funnel plots for RCTs included in the main analysis



The dotted lines show 95% confidence intervals around the overall summary estimate calculated using a random effect model

4. Discussion

4.1 Summary of Evidence

In this systematic review and meta-analysis, phytoestrogen supplementation (compared to placebo and followed by usual diet) was associated with a slight decrease in WHR, and was not associated with changes in body weight, BMI, WC, HC, FM and PBF in postmenopausal women. There was also, an indication that type of phytoestrogens, and health status may play a role in modifying the effect of phytoestrogens in reducing body weight. For instance, a significant decrease in changes of body weight was observed among healthy postmenopausal women and when isoflavone mixture supplements were used. Contrary, body weight was increased in women with underlying metabolic disorders and/or with use of isoflavones rich in daidzein. Furthermore, trials with small sample size showed improved body weight effects in women associated with phytoestrogens, but this effect was not observed in trials with larger samples.

4.2 Interpretation of Findings

In contrast to our findings, a meta-analysis published in 2013, reported a significant decrease in body weight with soy isoflavone supplementation [pooled mean difference of -0.52 kg (95%CI: -0.89 to -0.134)][21]. Although, we did not observe overall significant associations between phytoestrogen supplementation and improvements in body composition parameters, we concluded that type of phytoestrogens and underlying

metabolic status of women may play a role in modifying the effectiveness of phytoestrogens in reducing the body weight. There may be several factors that may have yield the differences in our findings on phytoestrogen supplementation and changes in body weight in comparison with previous review. First, we included all types of phytoestrogen supplements, while the prior meta-analysis investigated solely the effect of soy derived phytoestrogens and not the other types of phytoestrogen supplements (e.g. daidzein/ genistein enriched formulations, soy products). Second, they included only non-Asian postmenopausal women, while in the current review seventeen non-Asian trials (European, North and South American) and six of Asian trials were included. Finally, the findings of the previous review were based on 9 RCTs and 578 postmenopausal women (272 in intervention trial and 256 in control arm) while we included 23 RCTs and 1,880 postmenopausal women (1,130 in intervention arm and 750 in placebo arm). Overall, there are some indications that certain types of phytoestrogens may be beneficial in reducing body weight, these findings are supported with experimental data and merit further investigation.

4.3 Biological mechanisms/plausibility

Adipose tissue is highly responsive to estrogen, and both, human and mouse fat tissue express estrogen receptor (ER) α and ERβ [44]. The mechanisms by which dietary soy and phytoestrogens may reduce adiposity are not fully understood. Phytoestrogens may affect the body composition directly binding estrogen receptors (mainly ER α), by mediating the action of hormones thought to be involved in the regulation of body composition (adiponectin, ghrelin, insulin, leptin) or by altering the metabolic activity of adipocytes[45]. Indeed, experimental studies suggested that phytoestrogens could be useful in treating or preventing increased adiposity after menopause onset[46]. Findings from animal studies reported that fat abundance observed in mice exposed to dietary phytoestrogens correlated with enzymes which could be the key regulators of fatty acid oxidation (AMP-activated protein kinase and Acetyl-CoA carboxylase)[47]. Ovariectomized mice fed with a soy-rich diet have reduced weight and had less adipose deposition than those fed on a soy-free diet[48]. Genistein as the most abundant phytoestrogen in soy food, was extensively studied. Indeed, genistein reversed the truncal fat accumulation in ovariectomized rodent models [44,46]. Moreover, i*n vitro* studies using isolated rat adipocytes genistein was found to inhibit

the conversion of acetate into lipid, inhibit basal lipogenesis, inhibit the conversion of glucose to lipids more than estradiol, and increase basal lipolysis[49]. Although, genistein may cause adipose changes in mice in concentrations that are within the range of those reported in humans under various nutritional conditions, it is not clear whether genistein could have antilipogenic effects in humans.[44].

4.4 Strengths and Limitations

To our knowledge, this is the first systematic review and meta-analysis including 1,880 postmenopausal women to comprehensively address the associations of phytoestrogens with anthropometric parameters in women. Only clinical trials investigating phytoestrogen supplementation following usual/regular diet were included; therefore, studies that combined phytoestrogen supplementation with hypocaloric diets or in combination with exercise were not taken into consideration. Furthermore, we included clinical trials that did not primary investigate body composition but have reported outcomes of interest at baseline as well as at the end of trial. In subgroup analysis, those studies which did not primary investigate body composition were excluded and the results remained stable.

However, there are several limitations that need to be mentioned. First, in this study, we found a trend for the possibility of publication for studies investigating body weight changes. Despite the findings of funnel plots and Egger's test, the estimates could indicate minimal publication bias for RCTs investigating BMI and WHR, these approaches are potentially limited by their qualitative nature; therefore, findings on BMI and WHR may have been affected by publication bias as well. Second, the quality of included RCTs in this review was limited because the majority of included RCTs could not be classified in one or more domains, which might have contributed to the heterogeneity that has been observed in the meta-analyses presented in this study (quality and composition of supplements). Furthermore, in included trials women were on average 4.19 to 15.9 years into menopause. Early postmenopausal period is characterized by pronounced changes in body composition, with an increase in both overall and intra-abdominal adiposity[50]. In fact, in subgroup analysis by median time since menopause, pooled effect on body weight, although non-significant, was larger and in opposite direction compared to women with longer menopause time. In addition, only four RCTs with more than 100 participants and only four RCT with a duration of the intervention of ≥6 months were found, which might undermine

the precision of the estimates and may limit the understanding of long-term effects of phytoestrogen supplementation on body composition in women. Considering these observations, the overall results of this study should be interpreted with caution. Finally, most of trials were published before 2013, with only one recent RCT investigating this topic after 2013. Thus, there may be some differences in formulation and quality of supplements of recent data compared to supplements was used ≥5 years ago.

4.5 Scientific Implications

Inconsistent findings across different trials included in this review may be a consequence of variations in study protocols(differences in dose, duration, route of administration and composition of phytoestrogens used) and baseline characteristics of women studied (various comorbidities, years since menopause onset, the capacity of individuals to produce equol and the genetic susceptibility). Therefore, further well-designed clinical trials should clarify which type and dose of phytoestrogens may have favorable effect on body composition in women, in particular, soy protein isolates, isoflavone mixture and isolated genistein and daidzein effectiveness should be compared. The time since menopause onset, metabolic status and body composition at baseline should be the most important women's characteristics to account for when investigating the association between phytoestrogens and body composition. Additionally, due to variations in phytoestrogen metabolism among individuals, phytoestrogen metabolites (urinary concentrations) shall be measured to reduce measurement errors and address the issue of serum phytoestrogen concentrations over the study period.

4.6 Conclusions

The European Menopause and Andropause Society (EMAS) suggested non-hormonal management of menopausal symptoms as an option for women who cannot or do not wish to take MHT[9]. However, this review raises an important concern regarding the body weight changes with phytoestrogen supplementation. Based on current literature, phytoestrogen supplements followed by usual diet were not associated with changes in body weight. However, the type of phytoestrogens and underlying disease in women may play an important role in modifying the effectiveness of phytoestrogens in reducing body weight and may even lead to increase in body weight. Therefore, until obtaining more evidence in favor of beneficial role of phytoestrogens in reducing body weight, it might be much safer to combine phytoestrogen supplements (especially daidzein rich formulations) with

hypocaloric diet/enhanced physical activity to maintain normal body weight during the supplementation period.

Supplement available online at:

https://www.sciencedirect.com/science/article/pii/S037851221830324 4?via%3Dihub#sec0115

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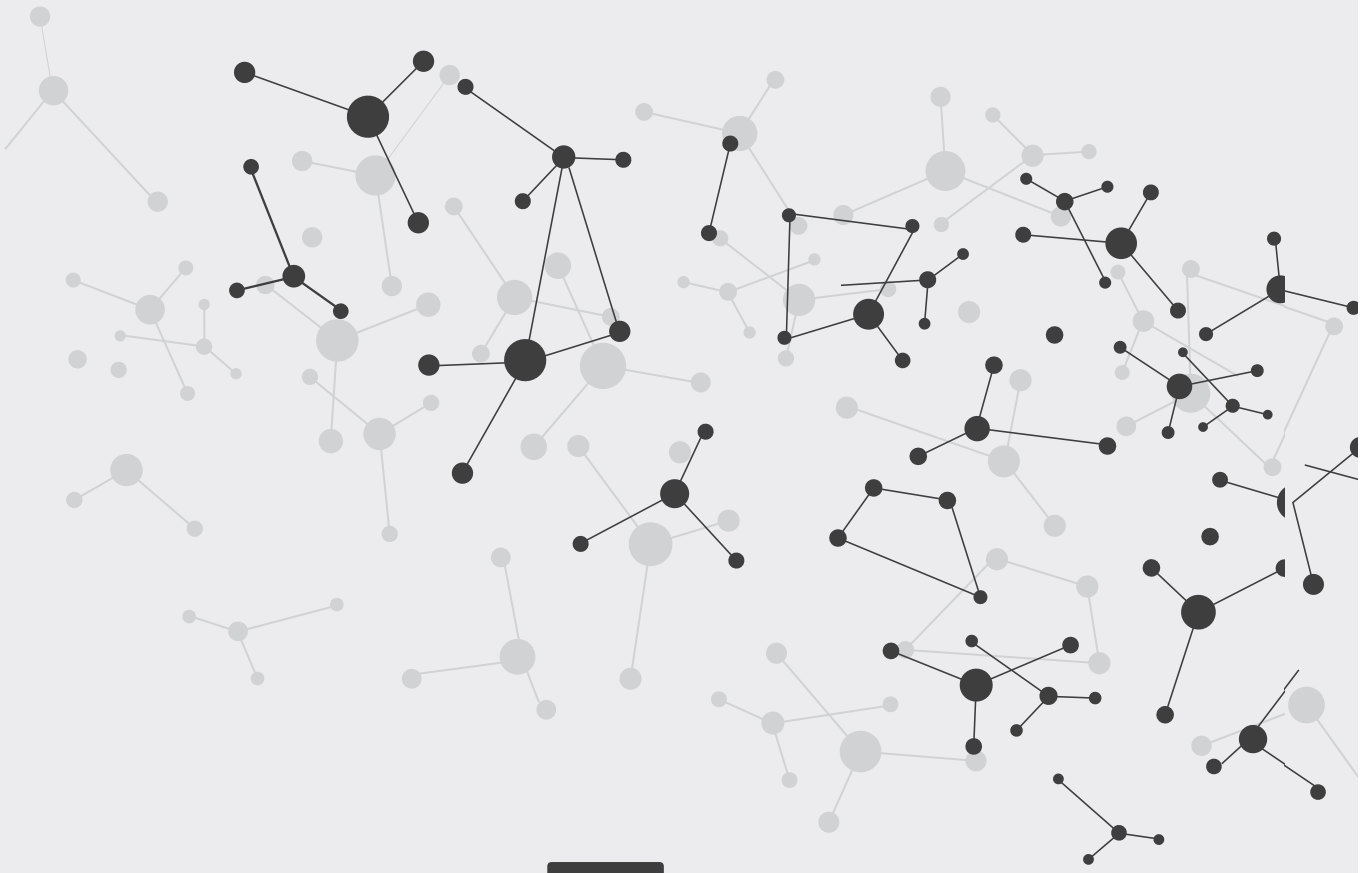
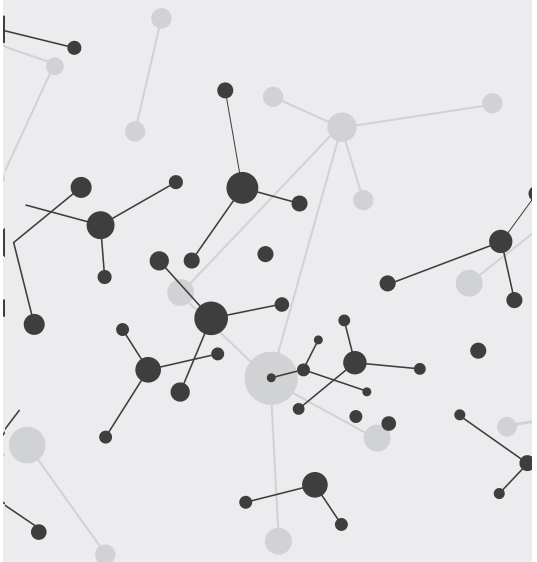


Chapter 5

General Discussion

General Discussion



The main aim of this thesis was to study the associations of endogenous sex hormone levels, and of treatment modalities and nutritional factors altering sex steroid hormone homeostasis and function with various cardiometabolic outcomes. In particular, we have studied the associations between: (i) endogenous serum sex hormones and amino-terminal pro-B-type natriuretic peptide (NT-proBNP) levels in individuals without cardiovascular disease (CVD), (ii) endogenous estradiol and testosterone and carotid plaque composition and risk of stroke in elderly men and postmenopausal women with carotid atherosclerosis, (iii) endogenous testosterone and sex hormone-binding globulin (SHBG) and risk of incident type 2 diabetes (T2D), and (iv) serum dehydroepiandrosterone (DHEA) and prognosis of T2D. We have also conducted a series of systematic reviews and meta-analyses to investigate the associations of progestin only contraceptive (POC) use and hormone therapy (HT) use with various cardiometabolic outcomes in women. Furthermore, we investigated the role of phytoestrogen supplementation in modifying body composition, glucose homeostasis and T2D risk in women. Detailed findings and discussion points from each of these studies are reported in previous chapters.

In this chapter, we will summarize the main findings from this thesis, discuss the most important methodological issues and finally, we will report the public health and clinical implications of our findings and provide directions for future research.

MAIN FINDINGS

Endogenous Sex Hormones and Cardiometabolic Risk

NT-proBNP

NT-proBNP has a well-documented prognostic value for cardiovascular disease and sex-hormones are suggested to modulate NT-proBNP levels. We have performed the first and most comprehensive study to examine the associations of estradiol, androgens and SHBG with NT-proBNP levels in postmenopausal women. In a cross-sectional analysis among 4,112 postmenopausal women free of clinical CVDs (coronary heart disease, heart failure and stroke), lower levels of serum androgens (testosterone, free androgen index, DHEA and DHEAs) and higher level of SHBG were associated with higher levels of serum NT-proBNP, irrespectively of known confounders. We further investigated the association between

DHEAs and NT-proBNP to explore whether the association was causal. In a larger sample from the same population (RS) among 7,390 men and women free of CVD, we applied Mendelian randomization analysis using genetic risk score of DHEAs (based on previously published GWAS) as an instrumental variable. Genetically predisposed higher levels of DHEAs were associated with lower NT-proBNP concentrations, thus providing evidence for a potential causal, inverse association between DHEAs and NT-proBNP.

Stroke

Observational studies reported a clear age and sex interaction in stroke prevalence, incidence, and mortality. While premenopausal women experience fewer strokes as compared to age-matched men, after the menopause stroke rates are higher in women as compared to their male counterparts. Due to dramatic changes in the female sex hormone concentrations around the menopause onset, shifting the balance from estradiol to testosterone predominance, estradiol hormone-dependent mechanisms have been suggested as protective and androgen-related pathways as harmful with respect to stroke.[1]. However, limited evidence exists on the association between endogenous estradiol and testosterone and risk of stroke, particularly in high-risk populations for development of stroke, such as individuals with carotid atherosclerosis. In a cross-sectional analysis in men and women with carotid atherosclerosis, we showed sex differences in the association between estradiol and carotid plaque composition assessed by magnetic resonance imaging. Total estradiol was positively associated with higher prevalence of lipid core in both men and women; and with the presence of intraplaque hemorrhage in women but not men. Similarly, the prospective analysis showed higher estradiol levels to be associated with increased risk of stroke in women, but not men. Carotid plaque thickness and the other morphological characteristics of carotid plaques, however, did not affect the direction and magnitude of the association between total estradiol and risk of stroke in women. Therefore, it may be that other mechanisms besides estradiol potential effects on plaque composition may link estradiol with risk of stroke in this high-risk population of women. No consistent association was observed between testosterone and plaque composition and risk of stroke in either sex.

Diabetes

Androgens such as DHEA and testosterone, as well as carrier blood proteins (e.g., sex hormone binding globulin-SHBG) involved in the transport of sex steroids in plasma and the regulation of their availability, might be key players in the pathophysiology of T2D in both men and women, yet the evidence comes from observational studies with limited sample sizes[2-4]. We performed two longitudinal studies to examine the associations of circulating androgen exposure and SHBG with risk of T2D in a sex-specific manner, as well as the prognostic value of DHEA and its main derivate DHEAs in T2D. Including more than 150,000 men and women and more than 4,000 T2D cases from The Health Improvement Network (THIN), we found that serum testosterone concentrations above 1.5nmol/L in women, and below 20nmol/L in men, were associated with a linearly increased risk of T2D. Reduced SHBG levels in both sexes, but particularly in women, significantly increased the risk of T2D. In 1,130 men and women with T2D from population based Rotterdam study, we found that, irrespective of sex, high levels of serum DHEA were associated with lower risk of developing diabetes complications, including chronic kidney disease, hypertension, initiation of insulin therapy and all-cause mortality. We did not observe an association between serum DHEA and risk of stroke among these individuals. Also, serum DHEAs was not associated with any of the investigated diabetic complications. Our findings support the notion that androgens may impact the risk of T2D and its complications in both men and women, yet showing different effects between the sexes

Exogenous Sex hormone Use and Cardiometabolic Risk

When hormone therapy (HT) was introduced it was hypothesized to reduce CVD risk. Earlier observational studies suggested protective effects of HT in terms of heart disease and mortality[5-8]. Subsequently, randomized clinical trials were designed and initiated to confirm whether HT could truly prevent heart disease besides treating menopausal symptoms [9]. The first results from trials: Women's Health Initiative (WHI) [10] and Heart and Estrogen/Progestin Replacement Study (HERS) [11], were contrary to what was expected, reporting increased risk of stroke, heart disease and even breast cancer in women given HT compared with placebo. Since the initial publication of the WHI results, the data have been

reanalysed and several studies have attempted to delineate the elements that could shed light into the conflicting findings on HT and CVD risks, proposing several contributing factors. These include the timing of HT initiation (the so-called "timing hypothesis"), dose of HT, the route of administration (e.g. oral, transdermal), and the CVD risk profile of the women. The timing hypothesis suggests that the difference in clinical effects depends on whether HT is initiated close to the onset of menopause (<6 years) or several years after [12]. We aimed to perform a comprehensive analysis on whether HT can increase the risk of CVD and whether these effects could differ by dose, duration, route and timing of initiation of treatment. Based on data from more than 2,5 million menopausal women, we show that medium to high dose (>0.625mg/day) oral HT was not associated with increased risk of heart disease, and that beneficial cardioprotective effects are actually observed with low doses of oral and transdermal HT. Also, there were some indications that vaginal HT may decrease MI and stroke risk, but the evidence is limited and requires further investigation. Supporting the notion of protective low dose vs. the harmful high dosage HT effect, oral HT and transdermal HT composed of estrogen alone or in combination formulation, appeared to increase the risk of VTE and stroke in a dose-dependent manner. In women with increased baseline thromboembolic risk, transdermal estradiol alone or combined with micronized progesterone appeared to be safer with regard to CVD risk as compared to oral HT formulations. Also, due to greater absolute risks of coronary heart disease, stroke and VTE, late HT initiation (10 years since the menopause onset or >60years old) shall be recommended for the shortest time possible and in lowest possible dose and preferably transdermal low dose HT (<50 µg/d of estrogen) should be advised.

Similarly, in young women (during their reproductive period) the use of oral contraceptives (COCs) has been associated with increased relative risks of venous thromboembolic events, stroke and myocardial infarction[13]. It has been postulated that the adverse cardiometabolic profile seen with COCs is due to the high estrogen content of COC. Therefore, the estrogen dose has been reduced substantially, with a simultaneous decrease in the associated risk of thromboembolic events. Also, new oral contraceptives with progestin-only content have been developed, which are considered to be safer[13]. However, evidence whether POCs affect the various cardiometabolic outcomes is scarce. The result from the systematic review we performed on this topic, based on nineteen observational studies and 62,088

women, showed that use of oral POCs was not associated with an excess risk of VTE, MI, stroke and hypertension. We found limited evidence that DMPA is associated with an increased risk of VTE, while intrauterine application of levonorgestrel was associated with a decreased risk of VTE. There was also an indication for an increased risk of diabetes with injectable POCs, albeit non-significant.

Phytoestrogens Supplementation and Metabolic Risk in Women

Phytoestrogens, plant-derived compounds with estrogen-like biological activity, have been suggested to improve women's health[14]. Phytoestrogens are commonly used in the traditional Asian diet, while in the Western world they are mostly used as supplemental therapy by menopausal women in order to alleviate menopausal symptoms. In women, aging and menopause are associated with an increase in body weight and abdominal fat accumulation, which subsequently may increase the risk of T2D[15]. Emerging evidence indicated that estradiol signalling can increase the risk of diabetes in postmenopausal women[3], It has been suggested that phytoestrogens, however, can inhibit the estradiol-induced adverse effects on T2D because of the ability of these compounds to compete with estradiol to bind its receptors as antagonists, as well as to act via estrogen-independent pathways [16]. We hypothesized that body composition and metabolic status might determine the effect of phytoestrogens. Therefore, we aimed to summarize the existing literature on the association between phytoestrogen intake and body weight and the most important parameters of body composition in postmenopausal women. Furthermore, we explored the association between phytoestrogen intake and glucose homeostasis/diabetes risk in adult women. Based on findings from 23 RCTs and 1,180 postmenopausal women, phytoestrogen supplementation (compared to placebo and followed by usual diet) was associated with a slight decrease in waist to hip ratio but it was not associated with changes in body weight, BMI, WC, HC, FM and PBF. There was an indication that the type of phytoestrogens and the health status may play a role in modifying the effect of phytoestrogens in reducing body weight. For instance, a significant decrease in change of body weight was observed among healthy postmenopausal women when isoflavone mixture supplements were used as compared to placebo. In contrast, body weight was increased in women with underlying metabolic disorders and/or with use of isoflavones rich in daidzein. Although the reasons for these differential effects are not clear, this

observation may be due to differences in time from onset of menopause among women included in the analysis: women that are closer to menopause onset are prone to increased waist fat accumulation and increase in body weight. Also, various phytoestrogen subgroups may have different antilipogenic effects in humans. Furthermore, our findings based on 18 RCTs (n=1,687 individuals) and 9 prospective population-based studies (n=212,796 individuals) have indicated a beneficial role of phytoestrogens in glucose homeostasis and T2D risk. In particular, higher dietary phytoestrogen intake was associated with a 10% lower risk of developing T2D in adult women. Overall, we show that phytoestrogen supplementation is associated with a reduction in fasting glucose in non-diabetic women. However, the results of clinical trials were not consistent for insulin and HOMA-IR. Nevertheless, an indication for increased levels of these traits was observed with some specific types of phytoestrogens, such as use of isoflavone mix and isolated genistein, confirming our suggestion that beneficial phytoestrogen effects may significantly vary with the type of phytoestrogens investigated.

Methodological Considerations

Selection Bias and Generalizability of the Results

Studies included in Chapter 2.1, 2.2, 2.3 and 2.5 were performed in the Rotterdam Study (RS), a large prospective population-based cohort, while a study included in Chapter 2.4 was carried out within the Health Improvement Network (THIN) database, a large primary care database in the UK that comprises data from over 700 general practices (14 million patients). The participation in the RS is voluntary; to be included in the study subjects are invited to the research centre where their health status is examined in details. This may introduce the healthy volunteer bias. Participants are supposed to visit the research centre and to complete the health status assessment, thus, subjects that are hospitalized, disabled or frail are less likely to be included in the study. It may be that CVD risk factors and chronic diseases are more prevalent among non-participants. Therefore, our results on stroke risk reported in Chapter 2.3 and on T2D complications reported in Chapter 2.5 might have been underestimated. For example, In Chapter 2.3 we found differences between participants who attend magnetic resonance imaging (MRI) visits and who did not attend. The majority of subjects who did not perform MRI had contraindications or physical limitations (38.73 %), therefore they might have been sicker comparing to the participants who were eligible to

attend MRI. However, it has been shown that using a selected source population in a cohort study usually leads to bias towards the null[17]. Yet, this is not likely to influence our findings from Chapter 2.1 and 2.2 as we have included only individuals without prevalent CVD (stroke, coronary heart disease and heart failure). Another important point to be discussed is the generalizability of our findings. Subjects are included in the RS based on the postal code therefore, they can be considered as a random sample of the general population. However, the RS population is homogeneous, mostly consisting of middle aged and elderly middle socioeconomic class Caucasians, and thus results may only be generalizable to similar populations.

The nature of a large general practice (GP) databases such as THIN confers another types of biases. The THIN database holds data on demographic characteristics of the patients and represents real primary care recording practice and contains extensive and reliable clinical (clinical diagnosis, physical measurement, laboratory results) and prescribing data. Also, it is broadly representative of the UK population structure [18] and has been utilized for numerous epidemiological studies, including studies on diabetes[19,20] and sex hormones[21,22]. In particular, the data collected within THIN are routinely collected for clinical management (i.e., not research), and therefore detailed clinical phenotyping in studies in GP databases is not possible. Also, health conditions may be misdiagnosed or miscoded in GP records. However, minor medical events are more likely to be missed than medically significant diagnoses or events. In our study the clinical diagnosis of T2D by the GP was the outcome of interest. In the UK, the Quality Outcome Framework (QOF) in general practices ensures high quality data on important comorbidities such as cardiovascular disease, hypertension and diabetes [23] . Within the database, diagnostic codes for T2D were identified based on Read codes, a hierarchical coding system to record signs, symptoms, procedures and diagnosis in primary care [24].

Sex Hormones Measurements

In studies embedded within the RS and THIN database important limitations regarding sex hormone measurements have been raised. First, in RS sex hormones serum levels were assessed only at baseline visit (RSI-3, RSII-1 and RSIII-1), therefore we did not have repeated sex hormones measure, however, the studies presented in Chapters 2.1, 2.2, 2.3 and 2.5

were carried out in middle-aged and elderly subjects, and in older age hormone levels are more stable over time[25]. However, this limited us to perform only cross-sectional analyses and therefore we could not draw conclusions with regards to causality. Second, serum levels of total testosterone (TT) were measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS), which at the moment of measurement was considered to be the gold standard method[26]. However, total estradiol/17-β estradiol (TE) levels were measured with a radioimmunoassay and sex hormone-binding globulin (SHBG) with the Immulite platform (Diagnostics Products Corporation Breda, the Netherlands). The minimum detection limit for TE was 18.35pmol/liter. Undetectable estradiol was scored as 18.35, which is considered suboptimal, particularly in postmenopausal women. Therefore, the analysis was done by categorizing the values into 0 if estradiol levels were ≤18.35 pmol/L and 1 if the values were >18.35 pmol/L while in men estradiol was used as a continuous variable. However, dichotomisation of continuous variables may also have some advantages and might improve performance of models when it has biological interpretation. In our case 32.7% women had undetectable estradiol levels, therefore dichotomisation in this case is equivalent to comparing middle and high tertile with the lowest tertile, which is commonly used in observational analyses. Third, there were no measures of bioavailable estradiol and testosteron. We have used the free androgen index, calculated as (T/SHBG)*100, as a surrogate measure of bioavailable testosterone (BT), however, we still did not have bioavailable estradiol measurements which could have strengthened our results.

Finally, in the THIN database, in men, physiologically higher testosterone concentrations do not represent a challenge for quantification by either radioimmunoassay (RIA) or tandem mass spectrometry techniques. However, in women, low circulating concentrations pose significant analytical and quantification difficulties for standard RIAs. The consensus is that today measurements should be performed by liquid chromatography-tandem mass spectrometry to improve quantification and avoid cross reactivity[27]. Furthermore, we have no information on the time of blood sampling; in men, serum testosterone levels exhibit a circadian variation with peak values in the morning and Endocrine Society guidelines emphasize that morning samples are crucial to accurately diagnose hypogonadism[28]. However, the testosterone levels measurements were indicated by a specialist, and thus, we may conclude that good clinical practice is followed in the majority of the cases and that morning samples were assessed in order to diagnose the hypogonadism.

Confounding

Confounding can be caused by variables that are associated with both outcome and exposure and are not in the causal pathway between exposure and outcome. In all of our analyses we adjusted for potential confounding factors, which were selected based on the literature, or if potential confounding factors lead to change in effect estimate for more than 10%[29]. Controlling for potential confounding variables may limit bias, but this occurs only if all potential confounders are perfectly measured and if their association with the exposure of interest is perfectly characterized[30]. Some confounding factors used in the analyses in studies in Chapter 2 were self-reported and assessed with questionnaires (e.g. smoking, alcohol consumption, age at menopause). Although, the questionnaires used were validated, they are still subjective and therefore prone to information bias, such as recall, response bias or interviewer bias. However, the misclassifications that may have occurred in any of the variables used in the models is not related to the outcomes (NT-proBNP, stroke nor T2D) and therefore is probably non-differential, and thus might have not affected the associations of interest. We have tried to control for confounding by using multivariable modelling, thus adjusting for several potential confounding factors, but we cannot rule out that residual/unmeasured confounding may still be present. This may lead to over- or underestimation of true effect estimate, depending how the confounding factor is related to both the exposure and the outcome. For example, we did not measure estradiol levels using the gold standard method, therefore, we cannot exclude the presence of residual confounding in serum NT-proBNP and carotid plaque composition and stroke risk, as presented in Chapters 2.1 and 2.3. Also, the biology behind our findings presented in Chapter 2.2 is largely unknown and therefore may be driven by residual confounding. In Chapter 2.5 we present a study conducted within the THIN database based on GP records, and therefore, the hormone measurements used as an exposure in our study are obtained from men and women with a clinical indication for serum measurement. This obviously introduces a potential confounding by indication. Subjects with the indication may have clinical manifestations of hypo- or hypogonadism which is a proxy of severity of hormonal disturbances. However, these limitations may be ameliorated by the large patient numbers

and the clear and potent gradient towards sex-specific diabetes risk we have observed in the study population. Also, in women this was possible to be explored since we had information on prevalent PCOS. Thus adjusting the main model for prevalent PCOS results did not substantially change. In Chapter 3.1 some of the studies included in our meta-analysis did not specify the type and dosage of POC, and the type and dosage of POC varied considerably. Hence, the pooled estimates may be confounded by variations in POC formulations. Also, in Chapter 3.2 among all of the included studies age and underlying comorbidities were not appropriately addressed in regard of CVD risk and they may have led to overestimation of HT effect on CVD outcomes.

Genetic Variants as Instruments for Strengthening Causal Inference in Observational Studies

Inferring causation in observational studies is often challenging or impossible, as observed associations can be due to other than causal explanations, with residual confounding being the most important concern[31]. Although randomized controlled trials (RCTs) are considered as a standard approach to causal inference, they are often not performed for being non-feasible or unethical. Therefore, the Mendelian randomization (MR) approach is becoming increasingly popular to overcome the issue of unobserved confounding in observational studies. MR approach allows estimation of causal effect from observational data in the presence of confounding factors. The method is considered as a 'natural' RCT since it uses selected common genetic variants related to a specific exposure of interest as an instrumental variable (IV) to evaluate causality between exposure and outcome. Since genotypes are assorted randomly during meiosis, MR addresses the issue of reverse causality. In addition, the distribution of genetic variants is thought to be unrelated to confounders, a common source of false positive results in epidemiological studies[32]. In Chapter 2.1 we report an inverse cross-sectional association between serum DHEAs and NT-proBNP in postmenopausal women without CVD. To further investigate potential causal association between DHEAs and NT-proBNP we used the MR approach of identified genetic variants combined into genetic risk score (GRS) as an IV. In Chapter 2.2 we provide some evidence for potential inverse causal association between DHEAs and NT-proBNP in middle-aged and elderly men and women without CVD. A valid MR study requires that the three core assumptions have been met: IV must be reproducibly and strongly associated with the

exposure, it must not be associated with confounders and it is only associated with the outcome through the exposure (i.e., it is independent of the outcome given the exposure). In our particular example, we decided to use the GRS rather than assessing the link between each SNP individually and serum NT-proBNP. GRS is supposed to avoid weak instrument bias, and also, increases the power and simplicity of the study[33]. However, as the biological effects of all the variants in an allele score may not be well known, the instrumental variable assumptions may not be satisfied for all the variants[33]. Indeed, the strength of the GRS as an instrument, measured by the F statistic was satisfactory overall. In men however, F statistics was close to 10 indicating that in males, DHEAs GRS might be a weak instrument. However, we consider this could be due to low power, as pointed out by our power calculation analysis in men. We have also formally tested whether there is a difference between the observational and IV estimates as the primary outcome of interest using Durbin–Wu–Hausman/endogeneity test and we have confirmed that there was no difference between observational and causal estimates. Another important assumption of Mendelian randomization is that the genetic variant must mediate its effect on outcome only via the risk factor, i.e., the genetic variant shows no pleiotropic effects. This assumption cannot be proven formally in practice because of incomplete knowledge of the underlying biology. However, recent MR extension MR Egger regression helps to control for biases though horizontal pleiotropy. Our results indicated minimal pleiotropy, therefore, indicating the robustness of our findings.

Heterogeneity and Publication Bias

Systematic review is a comprehensive high-level summary of previously published research that is using systematic explicit methods to identify, select, and critically appraise evidence relevant to a research question[34]. While the meta-analysis is usually the final step in a systematic review and it is a statistical procedure for assembling the results of several studies into a single summary estimate[35]. However, pooling the results together from various studies may be challenging due to differences in between studies characteristics (e.g. study populations, design, and exposure and outcome definitions). Assessment of the consistency of effects across various studies is an essential part of a meta-analysis. Unless we know how consistent the results of studies are, we cannot determine the generalisability of the findings of the meta-analysis[36]. The Cochran's chi-squared test

(Cochran's Q) is the most commonly used test to examine the null hypothesis that all studies are evaluating the same effect. The most common metric for measuring the magnitude of the between-study heterogeneity is the I^2, which is easily interpretable and does not depend on the number of the studies. It ranges between 0% and 100% and is typically considered low for I2 < 25%, modest for 25–50%, and high for >50%[36]. In the meta-analyses included in this thesis the heterogeneity between studies varied from 0 to 98%. In cases that more than 8 studies were included in the meta-analysis we performed stratified analysis and meta-regression to explore such high heterogeneity, involving various study-level characteristics (e.g. age, menopausal status, type of intervention). In our meta-analyses high heterogeneity could be explained by some of study-level characteristics, and even more importantly we concluded that the quality of included studies contributed to high heterogeneity in some of our analyses. It is important to mention that when stratifying some of the study characteristics we often did not have information on that specific study characteristics among all studies included in analysis. For example, in Chapter 4.1 we have stratified the analysis based on time since menopause onset. Yet, 30% of the trials did not report the time since menopause onset, and therefore, we could not include these trials in our analysis which may have influenced our results. Thus, we emphasized that our results shall be interpreted with caution. Furthermore, to investigate the consistency of our findings we performed the leave-one-out sensitivity analyses by iteratively removing one study at a time to confirm that our findings were not driven by any single study. In most of the analyses the results showed similar and consistent results indicating the robustness of our findings.

Another important methodological issue to be addressed is the publication bias. Publication bias is the term for what occurs whenever the research that appears in the published literature is systematically unrepresentative of all of the completed studies[37]. In simple words, publication bias occurs when the outcome of an experiment or research study influences the decision whether to publish (e.g., negative/non-significant results are less likely to be accepted for publication and even less likely to be submitted to the journal). In this thesis publication bias was evaluated through a funnel plot and asymmetry was assessed using the Egger's test. We have detected an indication of publication bias in only one of all meta-analyses we have performed (in Chapter 4.1 in case of RCTs that

investigated the association between phytoestrogen supplementation and body weight), implications of which we discuss in detail in the limitation section of the Chapter 4.1. Although the conventional funnel plots and Egger's test estimated indicated minimal publication bias in the rest of the analyses, these approaches are limited by their qualitative nature. Despite the efforts to perform a systematic search of both, published and unpublished literature, we still cannot exclude the possibility of publication bias from underreporting of negative findings. Lastly, although the publication bias presents perhaps the greatest threat to the validity of meta-analysis methods, it is still not an argument against the use of a meta-analysis. Publication bias exists in the literature irrespective of whether systematic review or other methodology is used to summarize research[37]. We have performed a series of meta-analyses and we found a trend for the possibility of publication bias solely for studies investigating the association between phytoestrogen supplements and body weight changes (in Chapter 4.1). This was a meta-analysis of clinical trials, and indeed, among clinical trials nearly one-third of the results ultimately do not get published[38].

Clinical and Public Health Implications of Findings and Directions for Future Research

The most important findings from this thesis and clinical implications are summarized in **Figure 1**. Our findings from **Chapter 2.1** support the hypothesis that higher serum androgens, and not estradiol, are responsible for lower natriuretic peptides levels in postmenopausal women. NT-proBNP levels are used as a diagnostic tool in HF, but also associate with risk of T2D and CVD. Our findings could indicate that androgens might, at least in part, be responsible for the change in risk of developing cardiometabolic outcomes after menopause. Therefore, future observational studies and clinical trials should investigate whether NPs levels or treatment modalities targeting androgen-related mechanisms are associated with development of heart failure in menopausal women. Also, it has been suggested that NT-proBNP levels increase with increasing age in healthy individuals, and are higher in females, age- and gender-specific cut-offs when defining upper reference values have been suggested[39]. Therefore, it is important that future studies take this into account when addressing the remaining issues. In **Chapter 2.2** we showed the causal association between serum DHEAs, the most abundant sex steroid, and NT-proBNP

levels suggesting that altering the serum DHEAs might play an important role in prevention and management of chronic heart failure. However, this requires further investigation; primary, in vitro activity-based metabolomic profiling may be used to assess the biological basis of our findings and explain potential metabolic pathways that link DHEAs and natriuretic peptide levels. Furthermore, currently there are no genetic variant identified for DHEA, and thus large scale GWAS on DHEA and its derivate DHEAs could help to better understand the genetic basis of sex hormones and to identify potential biological pathways that could be used as target to develop novel therapies to prevent heart failure.

In **Chapter 2.3** we report that endogenous estradiol may lead to the development of vulnerable carotid plaque composition and increase risk of stroke in postmenopausal women with carotid atherosclerosis. HT use in menopausal women is associated with increased serum estradiol levels. Hence, HT in postmenopausal women should be prescribed with caution, especially among those women who already have been diagnosed with carotid atherosclerosis. Future population-based observational studies are needed to reinforce our findings, and to examine whether serum estradiol, in women with carotid atherosclerosis could be further used as a biomarker to identify women who are at further increased risk of developing stroke. Also, future studies should investigate the risk of ischemic and haemorrhagic stroke separately, as due to limited number of stroke cases we could not properly study this. In **Chapter 2.4** we conducted the largest longitudinal study to confirm a sexually dimorphic role for androgens in mediating the risk of T2D. Serum testosterone concentrations above 1.5nmol/L in women, and below 20nmol/L in men, were associated with a linearly increased risk of diabetes. Reduced SHBG levels in both sexes, but particularly in women, significantly increase the risk of T2D. These data further define androgens as a novel metabolic risk factor in men and women, but potential mechanisms underpinning these observations remain to be clarified. We suggest that women with androgen excess and men with androgen deficiency should be systematically screened for T2D. Future clinical studies shall investigate if reducing androgens in women, and increasing androgens in men, will improve overall metabolic health and risk of progression to overt T2D. In **Chapter 2.5** we further demonstrated the role of sex hormones in the prognosis of T2D. We have shown that DHEA metabolism may play a role in the prognosis of diabetes. However, future experimental studies should examine the underlying biology that links

DHEA and T2D complications, and also observational studies or clinical trials are needed to test whether medications or lifestyle factors that alter DHEA metabolism can be effectively used in prevention of diabetes complications.

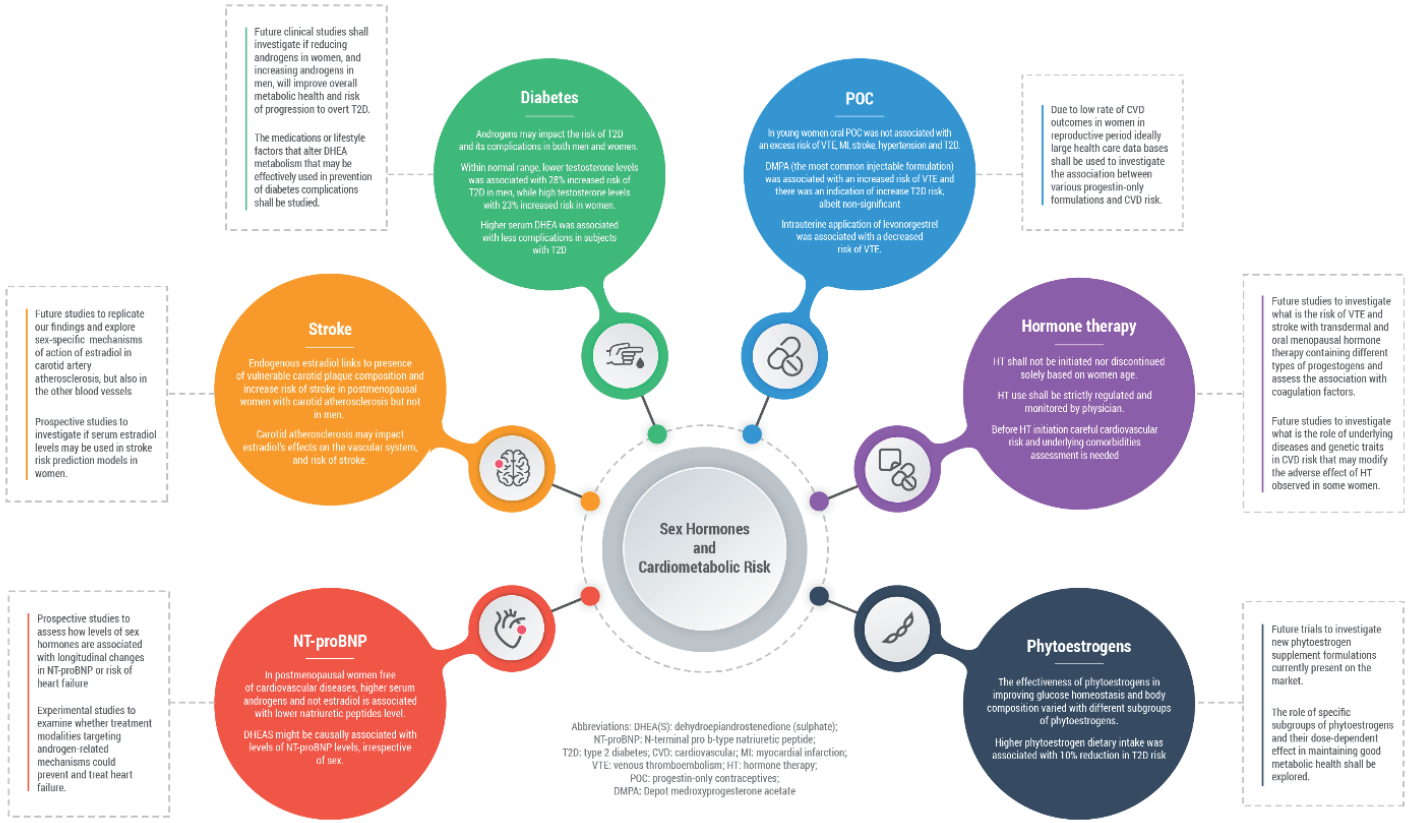
In Chapters 3.1 and 3.2 we presented two systematic reviews and meta-analyses on the role of exogenous sex hormones on cardiometabolic outcomes in women. Our findings indicate that HT use should be strictly regulated and monitored by the physician. Before HT initiation careful CVD risk assessment is needed. Also in women further from menopause, certain comorbidities (such as carotid/coronary atherosclerosis) may increase the risk of adverse outcomes, and thus careful assessment of baseline health status is advised before prescribing HT. However, future large observational studies should investigate what is the role of specific underling diseases and predisposing factors that may contribute to adverse cardiovascular outcomes in HT users. Ideally, large general practice data bases may be used to study this issue.

In young women oral POC use was not associated with an excess risk of VTE, MI, stroke, hypertension and T2D. Therefore, it may be safe to be used. However, we found limited evidence that depo-medroxyprogesterone acetate (DMPA), the most common injectable formulation, is associated with an increased risk of VTE, while intrauterine application of levonorgestrel was associated with a decreased risk of VTE. There was also an indication for an increased risk of diabetes with injectable POCs, albeit non-significant. The regimes of POC reported varied considerably, and therefore, it remains unclear what the effect of specific the type and dosages of POCs on various cardiometabolic outcomes is. Clearly, rigorous large population-based studies or based on data from health care registries are needed to further explore the remaining issues.

Finally, in Chapters 4.1 and 4.2 we demonstrated that higher phytoestrogen dietary intake may reduce T2D risk and may improve glucose homeostasis in women free of diabetes. We also highlight the efficacy of phytoestrogens in reducing body weight varied by type of phytoestrogens and by underlying metabolic status of women. However, due to the suboptimal quality of studies and trials included in our meta-analyses we give detailed instructions about which remaining issues future studies should address before we can make firm conclusions. We suggest caution when using some types of phytoestrogen

supplements in menopause due to their potential effect on body weight. Until future studies investigate the long-term effect of specific types of phytoestrogen supplements (e.g. daidzein enrich formulations) on body composition and potential women's characteristics that may affect their efficacy, we advise reducing calories intake or enhancing physical activity during supplementation period. The main concern exists in women who are near menopause onset, as they may be in higher risk to experience deleterious effects, due to changes in weight and fat distribution that naturally occur during the menopausal transition. Also, the potential of phytoestrogen rich food/supplements in glucose metabolism and risk of diabetes merits further investigation. Due to broad variations in subject's abilities to metabolize phytoestrogens, future studies should carefully explore the associations between blood/urine levels of phytoestrogen metabolites and parameters of glucose metabolism and T2D risk. Also, future studies with adequate sample size investigating different types and dosages of phytoestrogens and examining whether there are dose-effects are needed.

Figure 1. Thesis summary and implications for future research



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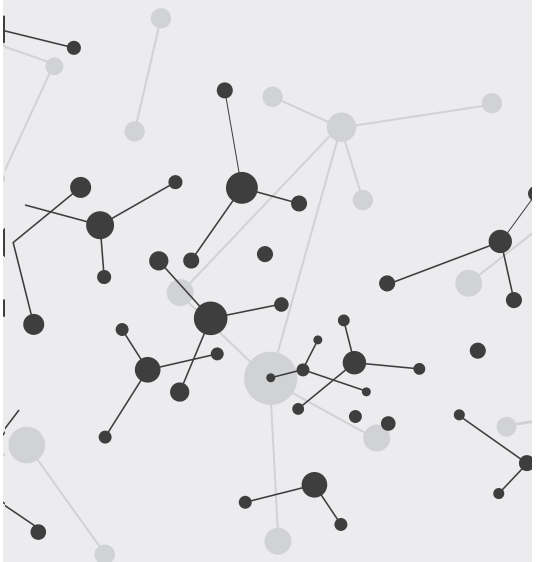
Chapter 6

Summary / Samenvatting



English Summary

In **Chapter 1**, we describe the rationale of the research presented in this thesis. Sexual dimorphism exists in cardiometabolic disease susceptibility such as CVD and T2D. Until now, sex differences in traditional cardiovascular risk factors have been extensively investigated in order to search the underlying mechanisms. However, the current evidence does not explain the complex pathophysiology of sex differences in cardiometabolic outcomes, and despite improvements in prevention and treatment CVD remains the leading cause of death for women worldwide. In addition to life style differences between genders (e.g. smoking and alcohol intake habits, physical activity) the biological differences among men and women may contribute to sex differences in cardiometabolic health. Aging-related sex hormones changes have been suggested to be an important player in modifying CVD risk over the life span in both genders and especially in women. In this thesis we performed original data analyses and systematic reviews and meta-analyses to investigate the associations between endogenous and exogenous sex hormones, phytoestrogens and various cardiometabolic diseases. In **Chapter 2** we performed five original studies to investigate the associations between endogenous sex hormones and cardiometabolic risk in men and women. In **Chapter 2.1** we used data from 4,112 postmenopausal women of the Rotterdam Study cohort and showed that androgens (testosterone, FAI (define?), DHEAs and DHEA, but not estradiol) might be responsible for lower cardioprotective natriuretic peptides levels in postmenopausal women. In **Chapter 2.2** we further analysed the data on DHEAs and NT-proBNP in both male and female participant of the Rotterdam Study and reported that genetically predisposed higher DHEAs was associated with lower NT-proBNP levels. In **Chapter 2.3** we report that endogenous estradiol may lead to the development of vulnerable carotid plaque composition and increase risk of stroke in postmenopausal women with carotid atherosclerosis but not in men. Hence, as hormone therapy (HT) use in menopausal women increases serum estradiol levels, we emphasized that with regards to stroke risk, HT in postmenopausal women should be taken with caution, especially among those women who already have diagnosis of carotid atherosclerosis. In **Chapter 2.4** we provide evidence of a sexually dimorphic role for androgens in mediating the risk of T2D in a large longitudinal study of more than 150,000 participants. We report that women with androgen excess and men with androgen deficiency are associated with increased risk of

T2D. Also, we report that even within the normal range, testosterone levels could impact the risk of diabetes. In Chapter 2.5 we further demonstrated the role of endogenous sex hormones in the prognosis of T2D. We showed that DHEA metabolism may play a role in the prognosis of diabetes. However, future studies are needed to understand the underlying mechanisms and to test whether medications or lifestyle factors that alter DHEA metabolism can be effectively used in the prevention of diabetes complications. In Chapter 3 we performed a systematic search of the literature to investigate the associations between exogenous sex hormones, in particular contraception and HT use, and cardiometabolic risk in women. In Chapter 3.1 based on the systemic review of the literature including 19 observational studies and 62,088 women, we report that in young women oral progestin-only contraceptive use (POC) was not associated with an excess risk of venous thromboembolism (VTE), myocardial infarction, stroke, hypertension and T2D. However, we found limited evidence that depot medroxyprogesterone acetate, the most common injectable formulation, is associated with an increased risk of VTE, while intrauterine application of levonorgestrel was associated with a decreased risk of VTE. There was also an indication for an increased risk of diabetes with injectable POCs, albeit non-significant. The regimes of POC reported in studies varied considerably, therefore, it remains unclear what the effects are of specific type and dosages of POCs on various cardiometabolic outcomes, and future studies are needed to address these issues. In Chapter 3.2 based on data from more than 2.5 million postmenopausal women, we concluded that HT may impact cardiovascular health and its use should thus be strictly regulated and monitored by the physician. Use of low dose oral and transdermal HT appears to be safe with regard to CVD risk in women in menopausal transition and within the first years (e.g. 10 years) after menopause onset. In women with increased baseline thromboembolic risk, transdermal estradiol alone or with micronized progesterone is suggested as first-line treatment. In case that HT is initiated more than 10 years since the menopause onset (>60 years old) due to greater absolute risks of coronary heart disease, stroke and venous thromboembolism, it should be used for the shortest time possible and in lowest possible dose, and preferably transdermal administration should be recommended. However, individualized treatment approach including baseline CVD risk assessment should be applied when prescribing HT. HT should not be initiated or terminated solely on the basis of a woman's age. Future large observational studies should

investigate what the role of specific underling diseases and predisposing factors is that may contribute to adverse cardiovascular outcomes in HT users.

Finally, in **Chapter 4** we summarized the existing literature on associations between phytoestrogen dietary intake/supplementation and metabolic risk in women. In **Chapter 4.1** summarizing data from 23 clinical trials and 1,880 postmenopausal women, overall we did not find an association between phytoestrogen supplements and changes in body weight and other parameters of body composition. However, body weight increased in postmenopausal women with pre-existing metabolic disorders (prediabetes, type 2 diabetes (T2D), prehypertension and hyperlipidemia) while in healthy menopausal women we observed that a reduced body weight associated with phytoestrogens. Also, there is some evidence that supplements containing an isoflavone mixture could have minimal beneficial effect, while daidzein-enriched isoflavones may lead to increased body weight. Future studies should now investigate the long term effect of specific types of phytoestrogen supplements on body composition and women characteristics that may affect their efficacy; we advise reducing calories intake or enhancing physical activity during supplementation period. In **Chapter 4.2**, based on findings from 18 RCTs (1,687 women) and 9 prospective population-based studies (212,796 women) we demonstrated that higher phytoestrogen supplementation may improve glucose homeostasis, and that high dietary intake of phytoestrogens may reduce T2D risk. However, the findings varied by types of phytoestrogens and we consider that future studies with long term follow-up are needed to determine the role of specific subgroups of phytoestrogens in diabetes prevention. In **Chapter 5**, we provide a general discussion of our findings and give some implications for clinical practice putting our research in broader context. Furthermore, this chapter describes major methodological considerations and limitations of our studies and gives suggestions for future research.

Nederlandse samenvatting

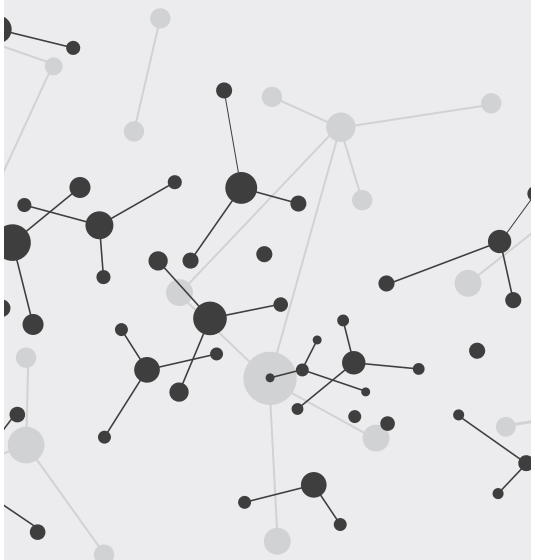
In Chapter 1, wordt de rationale achter het onderzoek in dit proefschrift uiteengezet. Er zijn geslachtsbepaalde verschillen in cardiovasculaire en metabole gezondheidsrisico's, zoals voor hart- en vaatziekten (cardiovasculaire ziekten) en voor diabetes type 2. Tot op heden worden de mechanismen voor deze geslachtsgerelateerde verschillen uitgebreid onderzocht. Helaas schiet de huidige kennis tekort en blijven, ondanks de verbeteringen die tot stand zijn gekomen met betrekking tot preventie en behandeling, hart- en vaatziekten de voornaamste oorzaak van overlijden van vrouwen over de hele wereld. Behalve verschillen in levensstijl, zoals roken, drinkgedrag en lichamelijke activiteit, kunnen biologische verschillen een rol spelen in de geslachtsbepaalde gezondheidsrisico's. Met name verouderingsgerelateerde veranderingen in geslachtshormonen worden een grote rol toebedeeld bij het bepalen van het risico voor hart- en vaatziekten, in mannen, maar vooral in vrouwen. In dit proefschrift is met behulp van originele gegevensanalyses, systematische literatuurstudies en meta-analyses de relatie tussen endogene (inwendige) en exogene (uitwendig gegeven) geslachtshormonen, phyto-oestrogenen (in plantaardig voedsel voorkomende stoffen die lijken op oestrogeen) en verschillende metabole en cardiovasculaire aandoeningen onderzocht. In Chapter 2 zijn 5 originele data analyses opgenomen die de relatie tussen endogene geslachtshormonen en genoemde gezondheidsrisico's hebben onderzocht in beide geslachten. In Chapter 2.1 zijn er gegevens gebruikt van 4.112 vrouwen na de menopauze (postmenopauzaal) binnen de Rotterdam Study gebruikt. Ze laten zien dat androgenen ('mannelijke' geslachtshormonen zoals testosteron, FAI, DHEA, maar niet oestrogeen) verantwoordelijk zijn voor lagere niveau's van zogenaamde natriuretische peptiden in, die geacht worden tegen cardiovasculaire ziekten te beschermen, in postmenopauzale vrouwen. In Chapter 2.2 zijn de DHEAS en het natriuretische peptide NT-proBNP verder onderzocht in zowel mannelijke als vrouwelijke deelnemers aan de Rotterdam Study, en daarin is aangetoond dat een genetische aanleg voor hogere DHAES gepaard gaat met lagere NT-proBNP spiegels. In Chapter 2.3 beschrijven we dat endogeen oestradiol de ontwikkeling van de risicovolle breekbare plaques en risico op beroertes verhoogt in vrouwen met aderverkalking in de halsslagader, maar niet in mannen. Daarom zou in deze groep vrouwen hormoontherapie, die vaak wordt toegepast om symptomen van de menopauze te bestrijden, risicovol kunnen zijn. In

Chapter 2.4 raporteren we dat androgenen een andere rol hebben in beide geslachten voor wat betreft het risico om diabetes type 2 te ontwikkelen, zoals aangetoond wordt in een studie met meer dan 150.000 deelnemers. Er is te zien dat in vrouwen een verhoogd androgeen hormoonspiegel en in mannen juist een verlaagde spiegel het risico verhoogt. Tevens wordt beschreven dat zelfs normale spiegels van invloed kunnen zijn. In Chapter 2.5 wordt ingegaan op de rol van endogene geslachtshormonen op de prognose bij diabetes, waarbij DHEA metabolisme een rol lijkt te spelen. Er is echter verder onderzoek nodig om de mechanismen te begrijpen. Tevens dient getest te worden of medicijnen en levensstijl die DHEA metabolisme beïnvloeden kunnen worden aangewend om complicaties bij diabetes te voorkomen. In Chapter 3 is een systematisch literatuuronderzoek verricht om de effecten van exogene geslachtshormonen, met name afkomstig van anticonceptie medicatie of hormoonvervangende therapie, op het risico op cardiovasculaire ziekten en diabetes te onderzoeken. In Chapter 3.1 werden 19 observationele studies waarbij een totaal van 62.088 vrouwen betrokken waren gebruikt om aan te tonen dat in jonge vrouwen anticonceptiva met alleen progestins geen extra risico op emboliën, hartinfarct, beroerte, hoge bloeddruk of diabetes veroorzaken. Medroxyprogesterone acetate, een injecteerbare vorm van anitconceptiva, veroorzaakt mogelijk wel een verhoogd risico op emboliën, terwijl intrauterine toediening van levonorgestrel (zoals in een spiraaltje) juist een verminderd risico teweegbrengt. Injecteerbare progestins geven mogelijke een verhoogd risico voor diabetes, maar dit is niet statistisch significant. De behandelingsregimes varieerden behoorlijk, en daarom moeten toekomstige studies ontrafelen wat de invloed van het type en de dosis van de progestin-bevattende anticonceptiva is. In Chapter 3.2 kon op basis van gegevens van meer dan 2,5 miljoen postmenopauzale vrouwen geconcludeerd worden dat hormoonvervangende therapie de cardiovasculaire gezondheid kan beïnvloeden. Daarom moet een arts deze therapie strikt begeleiden. Een lage dosis is veilig vanaf de vroege menopauze tot aan 10 jaar na de start. Daarna wordt door het door veroudering veroorzaakte verhoogde risico op hart- en vaatziekten een bepalende factor die ertoe dwingt een zo kort mogelijke behandeling met een zo laag mogelijke dosis toe te passen. Toediening door het aanbrengen op de huid is hierbij geïndiceerd. Een persoonlijk risicoprofiel is bij de instelling aan te raden, en men dient niet af te gaan op de leeftijd alleen. Grote observationele studies zijn nodig om de exacte invloed van onderliggende ziekten en risicofactoren verder vast te leggen.

Tot slot, in Chapter 4 is literatuuronderzoek verricht om verbanden tussen phyto-oestrogenen in het dieet en metabool risico in vrouwen te onderzoeken. In Chapter 4.1 zijn gegevens gebruikt van 23 klinische studies met in totaal 1.880 postmenopauzale vrouwen. Er werd geen verband gevonden tussen phyto-oestrogeen inname en lichaamsgewicht of andere relevante variabelen van lichaamssamenstelling. Echter, in vrouwen met bestaande metabole stoornissen (prediabetes, diabetes, prehypertensie en verhoogde lipiden) werd een stijgend lichaamsgewicht waargenomen, terwijl in gezonde postmenopauzale vrouwen juist een verlaging van het gewicht was te zien bij het gebruik van phyto-oestrogenen. Qua samenstelling lijken mengelingen van isoflavonen minimaal gunstig, terwijl daidzein-verrijkte isoflavonen juist het lichaamsgewicht laten toenemen. In toekomstige studies moeten de effecten van de verschillende phyto-oestrogenen beter onderzocht worden, alsmede invloed van de lichaamskarakteristieken van de vrouwen. Een vermindering van het aantal calorieën in het dieet en verbetering van lichaamsactiviteit strekt voorlopig tot de aanbeveling tijdens phyto-oestrogenen dieet. In Chapter 4.2 is op grond van 18 klinische interventiestudies (met 1.687 vrouwen) en 9 prospectieve studies (in 212.796 vrouwen) aan te tonen dat een hogere phyto-oestrogenen inname de glucosehuishouding verbetert en het risico op diabetes vermindert. Echter varieert de uitkomst naar gelang het type phyto-oestrogeen, hetgeen weer uitnodigt tot het onderzoeken van deze verschillende types in toekomstige studies. Chapter 5 voorziet in een algehele bespreking van de bevindingen, met daarin de gevolgen voor de klinische praktijk. Verder beschrijft dit hoofdstuk de methodologische beperkingen en overwegingen, en biedt het suggesties aan voor toekomstig onderzoek.

Chapter 7

Appendices



PhD Portfolio
List of Publications
About the Author
Acknowledgments

PhD portfolio

Name of PhD Student:	Marija Glišić
Research School:	Netherlands Institute for Health Sciences
Erasmus MC Department:	Epidemiology and Internal Medicine
PhD Period:	August 2016-October 2018
Promotors:	Prof. dr. Oscar H. Franco and Prof. dr A.H. Jan Danser
Co-promotors:	Dr Taulant Muka and Ass. Prof Anton J.M. Roks

PhD training (Courses and Workshops)	Year	ECTS
1. Master of science in Health Sciences (NIHES)	2016/17	**70**
Study Design		4.3
Biostatistical Methods I: Basic Principles		5.7
Biostatistical Methods II: Classical Regression Models		4.3
English Language		1.4
Introduction to Medical Writing		2
Clinical Epidemiology		5.7
Methodologic Topics in Epidemiologic Research		1.4
Principles of Research in Medicine and Epidemiology		0.7
The Practice of Epidemiologic Analyss		0.7
Clinical Decision Analysis		0.7
Social Epidemiology		0.7
Methods of Public Health Research		0.7
Markers and Prognostic Research		0.7
Women's Health		0.9
Pharmaco-epidemiology and Drug Safety		1.9
Advanced Topics in Clinical Trials		1.9
Planning and Evaluation of Screening		1.4
Advanced topics in Decision-making Medicine		2.4
Systematical literature retrieval in PubMed	2018	
Systematical literature retrieval in other databases	2018	
2. Research Integrity	2017	0.3

Attended Conferences		
Dutch Pharmacology Day, Utrecht, Poster presentation	2018	1.2
Science Days, Antwerp, Oral presentation	2018	1.5
European Congress on Menopause and Andropause, Amsterdam, two	2017	3
Oral presentations	2017	1.2
European Society of Cardiology Congress, Barcelona, Poster	2017	1.2
presentation	2017	0.7
European Association of Study of Diabetes2017 annual meeting, Lisbon, Poster presentation		
Insulin Receptor Meeting, Nice		

Teaching		
1.Teaching assistant at Bachelor course: Cardiovascular regulation by the autonomic nervous system	2017	0.2
2. Teaching assistant at Master course: Clinical Translation of Epidemiology	2017	0.2
3. Teaching assistant at Bachelor course Clinical Technology, NIHES and Delft University	2017 2016	0.2 0.2
4. Teaching assistant at Master course Biostatistical Methods I: Basic Principles		
5. Teaching assistant at Master course Biostatistical Methods I: Basic Principles		
Attended Seminars		
Seminars of the Department of Epidemiology	2016-17	0.2
2020 meetings	2016-17	0.2
Cardiovascular Group Meetings	2016-17	0.6
Pharmacology & Vascular Medicine Group Meetings	2016-17	1.2
Other		
Peer review of articles for scientific journals	2016-2018	1
Research visit to MRC Epidemiology Unit, Cambridge University	July 2018-September 2018	4

*1 ECTS corresponds to 25 to 30 hours of work

List of Publications

•Glisic M, Mujaj B, Rueda-Ochoa OL, Asllanaj E, Laven JS, Kavousi M, Ikram MK, Vernooij MW, Ikram MA, Franco OH, Bos D, Muka T. Associations of endogenous estradiol and testosterone levels with plaque composition and risk of stroke in subjects with carotid atherosclerosis. Circ Res. 2017 Nov 2. PMID: 29097437

•Glisic M, Rojas LZ, Asllanaj E, Vargas KG, Kavousi M, Ikram MA, Fauser BCJM, Laven JSE, Muka T, Franco OH. Sex steroids, sex hormone-binding globulin and levels of N-terminal pro-brain natriuretic peptide in postmenopausal women. Int J Cardiol. 2018 Mar 6. pii: S0167-5273(17)33426-5. doi: 10.1016/j.ijcard.2018.03.008.

•Glisic M, Shahzad S, Tsoli S, Chadni M, Asllanaj E, Rojas LZ, Brown E, Chowdhury R, Muka T, Franco OH. Association between progestin-only contraceptive use and cardiometabolic outcomes: A systematic review and meta-analysis. Eur J Prev Cardiol. 2018 Jan 1:2047487318774847. doi: 10.1177/2047487318774847

•Milic J, Glisic M, Voortman T, PletschBorba T, Asllanaja E, Rojas L.Z., Troup J, C. Kiefte-de Jong J, van Beeck E, Muka T, Franco O.H. Menopause, ageing, and alcohol use disorders in women. https://doi.org/10.1016/j.maturitas.2018.03.006

•Rojas LZ, Glisic M, Pletsch-Borba L, Echeverría LE, Bramer WM, Bano A, Stringa N, Zaciragic A, Kraja B, Asllanaj E, Chowdhury R, Morillo CA, Rueda-Ochoa OL, Franco OH, Muka T. Electrocardiographic abnormalities in Chagas disease in the general population: A systematic review and meta-analysis PLoS Negl Trop Dis. 2018 Jun 13;12(6):e0006567. doi: 10.1371/journal.pntd.0006567.

•Glisic M, Kastrati N,* Gonzalez-Jaramillo M*, Bramer WM, Ahmadizar F, PhD, Chowdhury R, Danser, AHJ, Roks AM, Voortman T, Franco OH, Muka T. Association between phytoestrogens, glucose homeostasis and risk of diabetes in women: a systematic review and meta-analysis (accepted for publication in Advances in Nutrition)

•Glisic M, Kastrati N*, Musa J*, Milic J, Asllanaj E, Portilla Fernandez E, Nano J, Ochoa Rosales C, Amiri M , Kraja B, Bano A, Bramer WM, Roks AJM, Danser AHJ, Franco OH , Muka T. Phytoestrogen supplementation and body composition in postmenopausal women: A systematic review and meta-analysis of randomized controlled trials. Maturitas 115 (2018) 74-83.

•Asllanaj E, Bano A, Glisic M, Jaspers L, Ikram M.A, Laven J.S.E, Vőlzke H, Muka T, Franco O.H. Age at natural menopause and life expectancy with and without type 2 diabetes. (accepted for publication in Menopause)

•Asllanaj E*, Ochoa-Rosales C*, Glisic M, Nano J, Muka T, Franco O.H. Chromatin landscape and Epigenetic biomarkers for clinical diagnosis and prognosis of Type 2 Diabetes mellitus (Book chapter, submitted to Elsevier)

•Zaimi I, Asllanaj E, Glisic M, Ikram A, Laven JSE, Kavousi M, Muka T, Franco OH. Endogenous dehydroepiandrosterone levels and its derivatives modifies the association between total estradiol and type 2 diabetes risk in postmenopausal women: The Rotterdam Study. Maturitas, Volume 103, September 2017, Pages 93-94. https://doi.org/10.1016/j.maturitas.2017.06.017

•A Hendriks, Kis, Zsuzsanna, Glisic M, Bramer, Wichor, Szili-Torok, T. P291Imaging guided versus non-imaging guided ventricular tachycardia ablation - a meta-analysis. EP Europace. 20. i41-i41. 10.1093/europace/euy015.103.

*denotes equal contribution

About the Author

Marija Glišić was born in Loznica, Serbia on September 4th, 1988. In 2003, she completed the high school at "Gymnasium Vuk Karadzic" in Loznica, Serbia. In 2014, Marija obtained her MD degree at the University of Belgrade. During medical school, in 2012, she won the ERAWEB Scholarship and went to the University of Rotterdam in the Netherlands for the first time, to perform 10-month training in epidemiology. In 2013 and 2014, Marija won CEEPUS Scholarships and visited Medical University of Graz where she performed clinical and research internships in the field of radiology. Marija started her PhD in Epidemiology of Heart Failure at University of Belgrade, in 2016 she returned to Erasmus Medical Center as a PhD exchange student under ERAWEB scholarship. In 2017 she obtained a Master of Science degree in Health Sciences. Her master thesis, supervised by Prof. Oscar H. Franco and Dr. Taulant Muka, was on the association between sex hormones and carotid plaque composition and risk of stroke. In September 2017, Marija continued her studies with PhD in Cardiovascular Epidemiology group and Division of Vascular medicine at Erasmus MC. She worked on cardiometabolic health looking at the associations between endogenous and exogenous sex hormones and estrogen-like compounds and various cardiometabolic outcomes. Before defending her PhD, Marija spent three months as a visiting researcher at the MRC Epidemiology Unit, at University of Cambridge School of Clinical Medicine.

Acknowledgments

I cannot believe I came to the moment to write this section. I have completed this thesis in "only" two years, however, this process took a lot of extra hours and sacrifice and therefore much more of actual time. Honestly, I would not recommend anyone to repeat this. The completion of this thesis would have not been possible without the contribution of many of my colleagues who were involved in different stages of my PhD project. I take the opportunity to express my gratitude as follows:

First of all, I want to thank you **Taulant**. I admit, you were not my favourite person in the past two years and I am aware that the moment I have the diploma in my hands I will be able to appreciate the pain you put me through. The truth is that you have been an amazing supervisor and I learnt a lot from you in the past two years. Thank you for having a faith in me and for the spare time you spent reviewing my manuscripts or responding to my doubts. When I first met you in 2012 I knew you are nerdy and stubborn enough to show the world that we (Balkan people) are as good as, or to be completely honest- better than the rest of the world ☺. I am sure I would never make this done without your help. Now when I made it, I will stop complaining and we can finally be friends again.

Secondly, I want to thank you **Oscar** for giving me the opportunity to show what I am capable of doing and for being such a great mentor. Thank you for your motivational speeches and all advice/lessonss not only on science, but even more importantly regarding life. Thank you for every time when you were not completely honest with me in order to cheer me up, yes I could recognize it, and it is highly appreciated. I am sure that each of your students feels the same about you and that is certainly something that makes a great mentor and something you should be proud of.

Jan and **Anton**, thank you both for having a faith in Oscar's word. Without the opportunity you have provided me with completion of this project would not be possible. Jan thank you for being so strict and responsible when it was the most important, without you we probably wouldn't be here today. Anton, thank you for patience when explaining us the principles of experimental work and for your positive attitude in helpless moments. The experience I have gained working at the lab is precious; I truly hope we will find a way to collaborate in the future.

Special thanks to the members of reading and small committees. Dear **Prof. Geleijnse**, **Prof. van Rossum**, **Prof. Kardys**, **Dr. van den Brink** and **Prof. Laven**, I am very grateful for your expertise in assessment of my thesis and for joining my defense ceremony.

Dear **Kate**, it has been six years since we met, and meanwhile I have met many beautiful souls, still you are one of my favourite! **Irma**, isto vazi I za tebe. Koliko god se trudila ne mogu da pronadjem nikoga slicnog tebi da je do sada bio u mom zivotu. Thank you so much for organizing the defense despite your busy schedules. I am super proud of what both of you have achieved so far and I am completely sure we will be crossing each other's paths in some more cheerful environment. Te sakam Kate, a tebe volim Irma!!!

I also want to thank to **Prof. Ken** and **Prof. Nick** for hosting me at MRC Epidemiology Unit. The time I have spent at Cambridge although very intensive and challenging, is for sure one of the most beautiful periods during my PhD journey. Special thanks to **Felix** and **Alex** for your help with statistical analyses and very amusing coffee breaks.

I also need to thank to all of my **co-authors** for their valuable inputs and for wonderful collaborations we have had. Also, thanks to **all of amazing colleagues** from the Epidemiology and Internal medicine departments. You are way too many to mention here by name, I have enjoyed our coffee/cookie breaks and fruitful discussions we were engaged in. Special thanks to my officemates and colleagues that have been working with me in various projects: **Eralda**, **Lyda**, **Chantal**, **Jana, Eliana, Zhangling, Trudy, Carolina, Valentina, Alice, Silvana, Mohsen**, **Oscar Layonel**, **Magda**, **Paula**, **Katrina**, **Rugina**, **Kajvan**, **Ehsan**, **Renee**, **Dominique** and **Estrellita.** I wish you all lots of success in the future! I am sure we will work on common projects in the future. **Eralda**, I will especially miss our depressive talks late at night while enjoying sunsets from our cosy office. Also, thanks to **Nano** and **Richard** for IT support! Many thanks to **Usha**, **Emillie** and **Ingrid** for their unselfish help and patience when teaching me various lab techniques. A big thanks to **Mardin** and **Anna** for lovely collaboration on cell culture experiments, we have learnt a lot from each other! Also, many thanks to **Wichor**, **Prof. Chowdhury** and **Dr. Nirantharakumar** for wonderful collaborations.

Special thanks to **Prof. Arfan** and **Maryam** for all of your help and understanding. Also, thanks to **Yolanda** and **Andreas** for your patience and help with administrative and financial

issues! And finally, a big big thanks to **Mirjam**, **Birgitte** and **Gabrielle,** ladies, without your help I would be totally lost.

Finally, I need to apologize and thank to you **Albana**, you are probably the person who suffered the most handling all frustrations caused by your brother. Also, thank you for taking care of my diet and giving me practical human interrelations advices. Yet, most of all thank you for being a great friend to me, you will be greatly missed!

Слађо, **Тања**, **Мики**, **Бане**, морам да вам се захвалим сто ме нисте заборавили и што нисте престали да ме волите упркос томе што сам била отуђена. Тањчи, хвала сто си нашла времена да ме посетиш у туђини и учиниш да ми буде бар мало лакше. Хвала на свим болесним/смешним порукама, психотерапијама и подршци. Слађо, хвала што си се трансформисала у вјештицу у мом одсуству, ти си мој највољенији пројекат. Хвала и сто си увек пронашла начин да дођеш да ме видиш. Бане, хвала ти за све мотивационе поруке које су ме сваки пут подсећале колико те волим. Ипак, никад ти нећу опростити ако ово буде једина књига коју ниси прочитао. Милоше, хвала што си увек био окрутни али оптимистични глас разума који ме је враћао на земљу кад је било потребно. Ова књига је пуна мудрости о женама, додуше, мудрости о женама у менопаузи, теби ћу ипак опростити ако не нађеш времена да је прочиташ.

Секо и **Пеђа**, хвала што се нисте променили након свих ових година. Хвала што сте ме тешили и у безнаднежним моментима и што сте пронашли начина да ме насмејете и охрабрите и кад сте знали да је све отишло дођавола. Голе, посебно хвала за логистичке и административне подухвате које си морала да обављаш од 2012. године, без тебе не би било могуће борити се против система. **Мама**, **тата** и **бако** хвала за све жртве које сте морали да поднесете паааа од кад сам се упустила у авантуру звану медицина. Хвала што сте прикривали колико вам смета што сам далеко, што сте увек препуштали одлуке само мени и што сте успели да преживите свако од наших опраштања на аеродрому. Без ваше подршке ништа не би било могуће!

Стеване, шта бих ја без тебе. Хвала што си био уз мене кад је било најлепше и најтеже, што си ме волео упркос лошем холандском времену, километрима који су нас делили и упркос мени. Хвала што си дао све од себе да улепшаш не само све ове месеце од кад си у мом животу, него и моје радове, и хвала што си преживео дизајнирање омота

ове књиге. Тешки пројекти су тек пред нама али сада бар знам да си спреман да се носиш са њима. P.S. Пројекти које имам на уму најмање укључују дизајнирање!

Marija