TAULANT MUKA

WOMEN'S HEALTH
Implications of Diet and Cardiometabolic Risk Factors

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Implications of Diet and Cardiometabolic Risk Factors

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Chapter 2

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Chaker*, L., Falla*, A., van der Lee S. J*., **Muka, T**., Imo, D., Jaspers, L., Colpani, V., Mendis, S., Chowdhury, R., Bramer, W. M., Pazoki, R. & Franco, O. H. The global impact of non-communicable diseases on macro-economic productivity: a systematic review. *Eur J Epidemiol* **30**, 357-395, (2015).

Chapter 3

Muka, T., Kiefte-de Jong, J. C., Hofman, A., Dehghan, A., Rivadeneira, F. & Franco, O. H. Polyunsaturated fatty acids and serum C-reactive protein: the Rotterdam study. *Am J Epidemiol* **181**, 846-856, (2015).

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Vitezova A.*, **Muka T***., Zillikens M.C., Uitterlinden AG, Hofman A., Rivadeneira F., Kiefte- de Jong J.C, Franco O.H. Vitamin D and body composition in the elderly: the Rotterdam Study. Clinical Nutrition, in press.

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Chapter 4

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Muka T., Vargas KG*., Japser L*., Wen K., Dhana K., Vitezova A., Nano J., Brahimaj A., Colpani V., Bano A., Kraja B., Zaciragic A., Bramer WM., van Dijk GM., Franco OH. Estrogen receptor β actions in the female cardiovascular system: a systematic review of animal and human studies. Maturitas 86, 28-43 (2016).

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Muka T*., Oliver-Williams C*., Colpani V., Kunutsor S., Chowdhury S., Chodhury R., Kavousi M*., Franco OH*. Association of vasomotor and other menopausal symptoms with risk of cardiovascular disease: a systematic review and meta-analysis. PLOS One (*under review*).

Muka T*., Oliver-Williams C*., Kunutsor S., Laven JSE., Fause BCJM., Chodhury R., Kavousi M*., Franco OH*. Association of age at menopause and duration from onset of menopause with cardiovascular outcomes, intermediate vascular traits and all-cause mortality: a systematic review and meta-analysis of observational studies. JAMA Cardiology (*under revision*).

Franco OH., Chowdhury C*., Troup J*., Voortman T., Kunutsor S., Kavousi M., Oliver-Williams C*., **Muka T***. Effects of natural and plant-based therapies on menopausal symptoms: a systematic review and meta-analysis. JAMA (*under revision*)

*Author contributed equally

To my aunts Melpo and Vasilika

&

to my parents and sister

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Chapter 1

General Introduction

INTRODUCTION

Non-communicable diseases (NCDs) are the leading cause of mortality and morbidity worldwide. Cardiovascular disease (CVD) is the main cause of death and its burden is growing[1]. By 2020 it is estimated that CVD will account for 73% of total global mortality and 56% of total morbidity[1,2]. Additionally, type 2 diabetes (T2D), which is associated with a marked increase (by two to four) in the risk of CVD[3], is fast becoming a global epidemic. The International Diabetes Federation (IDF) has projected that the number of people with T2D in the world will increase from 382 million in 2013 to 592 million by 2035[4].

Economic Impact of Non-Communicable Diseases

NCDs, including CVD and T2D, have a large economic impact, leading to decreases in working-age population participation in the labor force due to disability, and therefore undercut productivity, which, in turn, affects economic growth. NCDs have been estimated to reduce economic growth by about 5–10%[5]. Moreover, because NCDs are chronic conditions that require expensive treatment regimens and prolonged individual care by specialized healthcare services, they boost healthcare outlays[6]. Despite the understanding of the financial burden of NCDs, the impact of NCDs on macro-economic outcomes in various geographical regions is unclear. Exploring the studies that investigate the impact of NCDs on healthcare expenditures and national income can help shape nations' future healthcare plans and strategies by better informing policy makers and healthcare planners about the emerging costs of NCDs.

Obesity as a Major Determinant of NCDs

In 400 BC, the Greek physician Hippocrates observed that "Sudden death is more common in those who are naturally fat than in the lean." Today, obesity is established as an underlying risk factor for several NCDs, including CVD and T2D. Obesity increases the risk for cardiometabolic diseases through other risk factors[7]. The latter include major risk factors, including hypercholestrerolemia, hypertension and hyperglycemia, as well as emerging risk factors, such as inflammation[7]. Increased adipose tissue mass contributes to an increase in circulating levels of proinflammatory cytokines, due to hypertrophied adipocytes and adipose tissue-resident immune cells (primarily lymphocytes

and macrophages). These proinflammatory cytokines may lead to vascular dysfunction and insulin resistance (**Figure 1**) [8-10]. Moreover, obesity is associated with nonalcoholic fatty liver disease (NAFLD), which is characterized by an increase in intrahepatic triglyceride and is associated with increased risk for T2D and CVD (**Figure 1**) [11]. Obesity can impair both physical and psychological health and wellbeing[12,13]. The net effect of the increase in fat mass and the enlarged fat cells is an increased mortality risk, which results in a decrease in life expectancy, estimated to be up to 13 years[14]. Over the last decade, the prevalence of obesity in many countries has more than doubled[15]. Recently, an accumulating body of evidence suggests that regional fat distribution is more important in understanding the association of obesity with NCDs than is overall body fat mass[16-18]. Android obesity, which is characterized by the accumulation of body fat in the upper truncal region, is more common in men, and has been associated with an increased risk of atherosclerosis, T2D, CVD and total mortality[19-23]. Contrary to this, gynoid obesity, defined as an accumulation of body fat in hips and thighs, is more common in women, and has been independently associated with a reduced risk of metabolic complications[16,17]. Accumulation of abdominal fat increases with advancing age, and during the menopause transition, women are more prone to increase the accumulation of central fat[24]. Although epidemiological studies have identified potentially modifiable factors that affect body fat distribution, such as physical activity and cigarette smoking[25-27], the role of dietary intake, particularly dietary fat, and its effect on the distribution of body fat remains unclear[28-32]. Furthermore, identifying biomarkers that are associated with body fat distribution may have clinical utility in identifying individuals at high risk of metabolic disturbances later in life who could benefit from effective preventive interventions.

The Role of Nutrition in Cardiometabolic Health

According to the World Health Organization (WHO), CVD and its risk factors are closely related to unbalanced diet and other lifestyle factors[33]. Nevertheless, the optimal diet for prevention of cardiometabolic risk factors and the incidence of CVD and T2D remains uncertain. Epidemiological studies have identified nutritional factors that affect cardiometabolic health, such as salt, carbohydrates, fiber, fish, fruits and vegetables[34-38]. However, the role of other nutritional factors, including dietary fat and vitamin D in cardiometabolic health, in particular in inflammation and obesity remains controversial[28-32]. Several observational and experimental studies showed the beneficial effects of polyunsaturated fatty acids (PUFAs) in CVD, in particular of n-3 PUFAs[39,40]. PUFAs may exert their cardioprotective effect by reducing thrombosis, decreasing arrhythmias and by stabilizing atherosclerotic plaques[41]. The current dietary guidelines for prevention and management of CVD and other chronic diseases encourage high consumption of both classes of PUFAs, n-3 and n-6, as a replacement for other types of fatty acids, such as saturated fatty acids[42,43]. Despite the consistency of favorable recommendations regarding dietary PUFAs, there are concerns that high amount of n-6 PUFAs in the diet may contribute to increased inflammation[44,45].

Furthermore, in Western countries, the consumption of plant derived n-3 PUFAs has almost doubled during the last decades, moving from 1 to 1.9 g/day[46]. Thus far, there is little controversy on the topic of the beneficial effects of marine-derived n-3 PUFAs supplementation on chronic

systemic inflammation[47], but very little is known about the effects of plant derived n-3 PUFAs on inflammation[48]. Furthermore, different types of fatty acid have different rates of oxidation depending on their chemical structure[49,50]. Impaired fat oxidation has been associated with the development of obesity[51]. However, no conclusion may yet be made on whether different dietary fatty acids also affect the distribution of body fat.

Vitamin D is another nutritional factor that is emerging as more important to skeletal, cardiovascular, and other health outcomes[52]. Vitamin D is obtained from diet and dietary supplements; however, its main source in the body is its production in the skin under the influence of solar ultraviolet radiation[53]. Vitamin D deficiency is especially common among elderly people (its prevalence is usually reported to be above 40%), who often have less sun exposure because of reduced outdoor activity, and the limited capacity of their skin to produce vitamin D metabolites[53]. In addition to its role in skeletal health, vitamin D has also physiological functions in non-skeletal tissues, where local synthesis influences pathways that are integral to cardiovascular function and disease, including inflammation, thrombosis and the renin-angiotensin system[54]. Indeed, observational studies have linked vitamin D deficiency with an increased risk of CVD and randomized controlled trials have reported a beneficial effect of vitamin D supplementation in reducing the risk of CVD[55-57]. Moreover, it is known that circulating levels of serum 25(OH)D are lower in obese individuals, however, it is not clear whether vitamin D status is associated with body fat distribution and the directionality of previously observed associations[58].

Biomarkers and Cardiometabolic health

Primary prevention of CVD and T2D are public health priorities[59]. Scientific evidence shows that both CVD and T2D are life course diseases that begin with the evolution of risk factors that, in turn, contribute to the development of subclinical disease[60,61]. Subclinical disease culminates in overt CVD and T2D, which affect the prognosis and the risk of morbidity and mortality among diagnosed patients[62,63]. Therefore, clinical assessment and early identification and treatment of risk factors are needed to accelerate disease prevention and morbidity improvement[64]. Measurement of various biomarkers is one tool that can be used to better document health risks. Clinicians may use relevant biomarkers to assess early changes in subclinical disease status preceding diagnosis of disease and biomarkers may also be used to identify people with a higher susceptibility to cardio metabolic diseases. Thus, measurement of biomarkers may help to identify novel pathways and subgroups in the population with greater susceptibility to cardometabolic diseases[65].

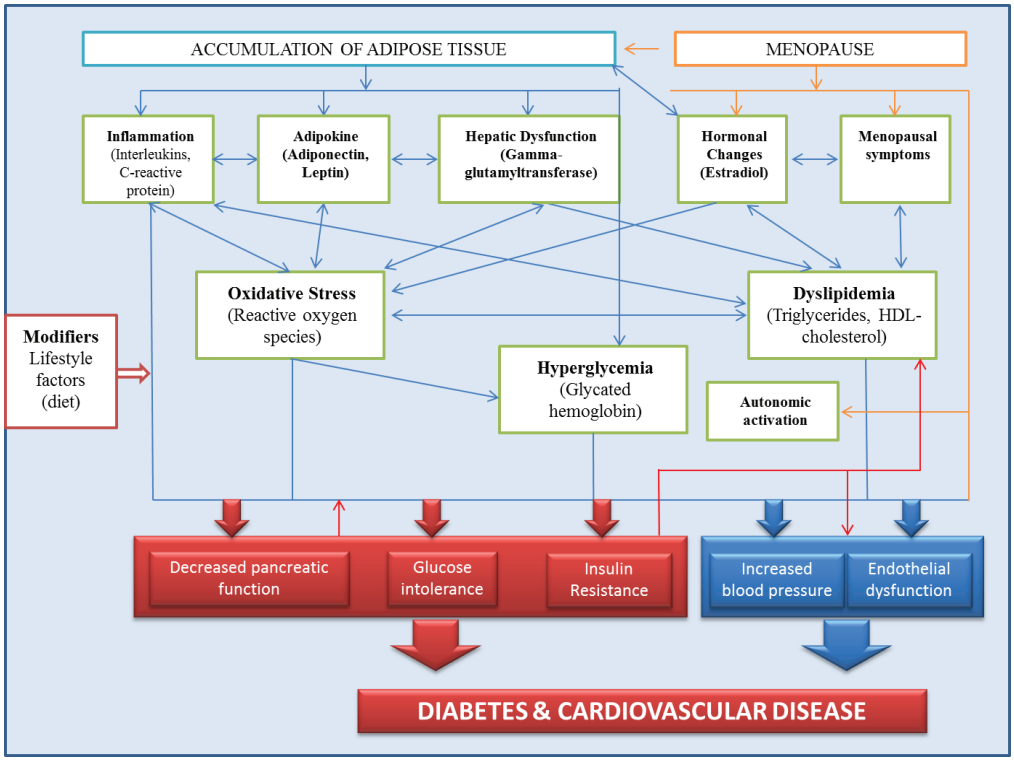
Extensive data have been accumulated to support the use of specific biomarkers for early identification of cardiometabolic risk, including blood pressure, serum cholesterol levels and glucose[66,67]. However, the current scientific knowledge does not completely explain the complex pathophysiology underlying cardiometabolic diseases so researchers still search for other pathways. Novel biomarkers of cardiometabolic health have been suggested, such as gamma-glutammyltransaferase (GGT) and endogenous sex hormones. GGT is a marker of alcohol consumption and liver disease and has been associated with obesity-related outcomes, including hypertension, dyslipidemia, insulin resistance, CVD and cancer [68-73].GGT plays a pivotal role

in oxidative stress [74,75], which may play a causal role in the development of obesity[76]. To date, however, very little attention has been given to GGT within normal range and its role in obesity and/or body fat distribution among healthy individuals. Furthermore, observationally, higher levels of gamma-glutamyltransferase (GGT) are consistently associated with increased risk of pre-diabetes and T2D [77-83]. However, to date, controversy exists whether these effects are causal, confounded or a consequence of reverse causation. If the associations from observational studies are found to be causal, GGT-lowering therapies could be used in T2D prevention.

Cardiovascular Health in Women

Despite improvements in prevention and treatment, CVD remains the leading cause of death for women worldwide[84]. Compared with age-matched men, premenopausal women have a lower risk of coronary heart disease (CHD) which gradually increases after menopause so that by their sixth decade of life, women have the same incidence of CHD as men[85]. Therefore, early recognition of women at high risk for CVD and timely implementation of appropriate lifestyle or therapeutic interventions are of tremendous public health importance. The disparity between the incidence of CVD among women in pre- and post-menopause has been largely ascribed to the changes in hormonal patterns during the menopause transition, including a marked decline in endogenous estradiol levels, leading to a period of relative excess of androgen[86]. The menopausal shift in

Figure 1. Underlying mechanisms linking adiposity, menopause and its related changes with type 2 diabetes and cardiovascular disease.



hormonal balance contributes to an increase in visceral adiposity and other cardiometabolic risk factors[87]. Therefore, women who have a premature or early menopause may live more years of their lives at an increased risk of adverse cardiovascular outcomes[88-903–5]. The relation between early menopause and adverse cardiovascular outcomes highlights the need to evaluate the role of both menopausal age and time since onset of menopause as risk factors for CVD. Furthermore, during their menopausal transition, up to 80% of women experience vasomotor symptoms (VMS), which typically include hot flushes and night sweats [91], and are known to impair the quality of life[92,93]. A growing body of evidence suggests a link between VMS and cardiovascular risk profile[94-99], however, whether a link really exists remains unclear. **Figure 1** describes the mechanisms linking menopause and its related hormonal changes with cardiometabolic outcomes. Furthermore, during the menopause, women get treatment for menopausal symptoms, including hormone replacement therapy (HT). Recent evidence show that use of HT may have a potentially negative impact on cardiovascular health[100]. Therefore, it is of importance identifying other medical therapies that may be used to treat menopausal symptoms avoiding the adverse cardiovascular effects induced by the use of HT. There is a broad range of natural and plant-based therapies that purport to have a positive effect on menopausal symptoms and that are used by 40-50% of women in Western countries as complimentary therapies to manage menopausal symptoms[101,102]. These therapies include the oral use of phytoestrogens such as dietary isoflavones and soy extracts; herbal remedies such as red clover and black cohosh; and non-biological treatments, such as acupuncture and yoga. However, whether these therapies can help to manage menopausal symptoms remains unclear given that there in not yet a comprehensive assessment of the impact of natural and plant-based therapies on the presence and severity of menopausal symptoms.

General Aim of This Thesis

The first objective of this thesis was to identify nutritional factors and biomarkers that can play a role in cardiometabolic health. A second aim was to identify determinants of adverse cardiometabolic health in women.

STUDY DESIGN

Systematic reviews and meta-analysis

Part of the research described in chapter 2 and 4 of this thesis are systematic reviews and meta-analyses of the literature. Systematic searches of electronic medical databases were performed to retrieve scientific articles on the topic of interest. Two independent reviewers screened the retrieved titles and abstracts and selected eligible studies. Reference lists of the included studies were screened to identify additional relevant studies. Heterogeneity permitting, we sought to pool the results using a random effects meta-analysis model. Heterogeneity was assessed using the Cochrane χ^2 statistic and the I^2 statistic. Publication bias was evaluated through a funnel plot and Egger's test. Study quality (for cohort and case-control studies) was assessed based on the Newcastle–Ottawa Scale(NOS)[103] using three pre-defined domains, namely: selection of participants (population

representativeness), comparability (adjustment for confounders), and ascertainment of outcomes of interest. Further details on the methods can be found in the specific chapters.

Rotterdam Study

The studies presented in chapter 3, 4 and 5 of this thesis were carried out within the framework of the Rotterdam Study, a population-based prospective cohort study, which started in 1990 in the Ommoord district, in the city of Rotterdam, The Netherlands. Details regarding the design, objectives, and methods of the Rotterdam Study have been described in detail elsewhere[104]. In brief, in 1990, all inhabitants of a well-defined district of Rotterdam were invited to participate in the study, of whom 7,983 agreed (78.1%). In 2000, an additional 3011 participants were enrolled (RS-II), consisting of all persons living in the study district who had turned 55 years of age between 1991 and 2000.). The third cohort was formed in 2006 and included 3932 participants 45 years and older (RS-III). Follow up examinations were performed approximately every 3-5 years (**Figure 2**)[104]. There were no eligibility criteria to enter the Rotterdam Study cohorts except the minimum age and residential area based on zip codes. Dietary intake used in this thesis was assessed by using a validated semi-quantitative food frequency questionnaire (FFQ) at baseline (1990-1993). Participants completed a checklist at home about foods and drinks they had consumed at least twice a month during the preceding year, as well as dietary habits and prescribed diets.

Figure 2. Diagram of examination cycles of the Rotterdam Study (RS)*

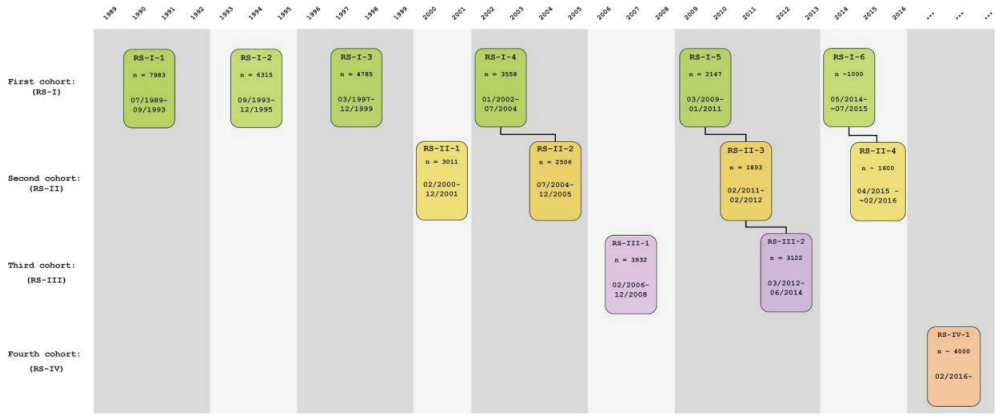


*Diagram of examination cycles of the Rotterdam Study (RS). RS-I-1 refers to the baseline examination of the original cohort (pilot phase 07/1989–12/1989; cohort recruitment 01/1990–09/1993). RS-I-2, RS-I-3, RS-I-4, RS-I-5, and RS-I-6 refer to re-examinations of the original cohort members. RS-II-1 refers to the extension of the cohort with persons in the study district that became 55 years since the start of the study or those of 55 years or over that migrated into the study district. RS-II-2, RS-II-3, and RS-II-4 refer to re-examinations of the extension cohort. RS-III-1 refers to the baseline examination of all persons aged 45 years and over living in the study district that had not been examined already (i.e., mainly comprising those aged 45–60 years). RS-III-2 refers to the first re-examination of this third cohort. Examination RS-I-4 and RS-II-2 were conducted as one project and feature an identical research program. Similarly, examinations RS-I-5, RS-II-3, and RS-III-2 share the same program items. Also, examinations RS-I-6 and RS-II-4 are conducted as one project. RS-IV-1 refers to the baseline visit of a new cohort, to be established in February 2016.

Table 1 General outline of this thesis and the waves of the Rotterdam Study included in the analysis presented in each chapter

Chapter	Exposure	Exposure assessment	Outcome	Outcome assessment	Systematic Review/ Original Data Analysis
2.1	Non-communicable diseases	NA	Health care spending and national income	NA	Systematic Review
2.2	Non-communicable diseases	NA	Macro-economic productivity	NA	Systematic Review
3.1	Dietary polyunsaturated fatty acids	RSI-I*	Inflammation (C-reactive protein)	RSI-I and RSI-3	Original data analysis (Rotterdam Study)
3.2	Dietary fat composition	RSI-1	Body fat (total fat, android fat, gynoid fat and android/ gynoid fat ratio)	RSI-4	Original data analysis (Rotterdam Study and Calcium Intake Fracture Outcome Study)
3.3	Plasma vitamin D levels	RSI-3	Body fat (total fat, android fat, gynoid fat and android/ gynoid fat ratio)	RSI-4	Original data analysis (Rotterdam Study)
3.4	Plasma gamma-glutamyltransferase levels	RSI-3	Body fat (total fat, android fat, gynoid fat and android/ gynoid fat ratio)	RSI-4	Original data analysis (Rotterdam Study)
3.5	Plasma gamma-glutamyltransferase levels	RSI-3, RSII-1 and RSIII-1	Glycemic traits and type 2 diabetes	Until January 1st 2012	Original data analysis (Rotterdam Study)
4.1	Endogenous sex hormones (estradiol, sex hormone-binding globulin, testosterone)	RSI-3 and RSII-1	Incident type 2 diabetes	Until January 1st 2012	Original data analysis (Rotterdam Study) and Systematic Review
4.2	Estrogen receptor beta	NA	Intermediate cardiovascular risk factors and cardiovascular outcomes	NA	Systematic Review
4.3	Vasomotor symptoms	NA	Intermediate cardiovascular risk factors	NA	Systematic Review
4.4	Menopausal symptoms	NA	Cardiovascular outcomes	NA	Systematic Review
4.5	Age of menopause	NA	Intermediate cardiovascular risk factors and cardiovascular outcomes	NA	Systematic Review
4.6	Natural and plant-based therapies	NA	Menopausal Symptoms	NA	Systematic Review

*Roman numeral for the cohort and Arabic numeral for the visit. NA, non-applicable; RS, Rotterdam Study. RS-I-1 refers to the baseline examination of the original cohort (pilot phase 07/1989–12/1989; cohort recruitment 01/1990–09/1993). RS-I-2, RS-I-3, RS-I-4, RS-I-5, and RS-I-6 refer to re-examinations of the original cohort members. RS-II-1 and RS-III-1 refers to the extensions of the cohort.

Next, during their visit to the research center, they underwent a standardized interview with a trained dietician based on the food and drink checklist, using a computerized validated 170-item semi-quantitative FFQ, taking into account seasonal variations in fruit, vegetable and fish intake. Nutrient intakes were calculated as the frequency of intake multiplied by the nutrient composition of the specified portion size. Nutrient estimates in the first visit of the first cohort (RSI-1) were based on the Dutch Food Composition Table of 1993 [105]. A validation study including 80 participants of the Rotterdam Study, showed that the FFQ is a suitable instrument for ranking individuals by fat and fiber intakes [106]. Except the first visit of the first cohort, all blood samples were drawn in the morning (≤ 11:00 am) and were taken after the participants had fasted for 8 hours. Total body composition was assessed by DXA using total body-fat beam densitometer (GE Lunar Corp, Madison, WI, USA for RS and Hologic Corp, Waltham, MA, USA for CAIFOS). **Table 1** summarizes the rounds of the Rotterdam Study that were used in the studies included in this thesis.

Outline of This Thesis

The objectives are addressed in several studies presented in this thesis (**Table 1**). Chapter 2 of this thesis describes the systematic reviews of the literature on economic impact of NCDs. In chapter 3.1 and 3.2 we studied the association of dietary fat intake with inflammation and body composition whereas in chapter 3.3, we examined whether vitamin D plays a role in body fat. We studied the association between gamma-glutammyltransaferase (GGT) within normal range and regional body fat distribution and whether levels of GGT could be causally associated with glycemic traits, prediabetes and T2D in Chapter 3.4 and 3.5, respectively. Chapter 4 focuses on cardiometabolic health in women. In chapter 4.1 we present an original data analysis and comprehensive review on the association of endogenous sex hormones levels with the risk of T2D in women. Chapters 4.2 to 4.5 provide comprehensive reviews and meta-analysis on the role of estrogen receptor beta in the female cardiovascular system, and the association of menopausal symptoms and age of menopause with intermediate CVD risk factors and cardiometabolic outcomes. Chapter 4.6 presents a systematic review and meta-analysis on the impact of plant-based and natural therapies on menopausal symptoms. Lastly, chapter 5 provides an overview of the main findings and conclusions from these studies and discusses implications and suggestions for future research.

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Chapter 2

Economic Impact of Non-Communicable Diseases

2.1 The Global Impact of Non-Communicable Diseases on Healthcare Spending and National Income: a Systematic Review

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ABSTRACT

IMPORTANCE: The impact of non-communicable diseases (NCDs) in populations extends beyond ill-health and mortality with large financial consequences.

OBJECTIVE: To systematically review and meta-analyze studies evaluating the impact of NCDs (including coronary heart disease, stroke, type 2 diabetes mellitus, cancer (lung, colon, cervical and breast), chronic obstructive pulmonary disease (COPD) and chronic kidney disease) at the macro-economic level: healthcare spending and national income.

DATA SOURCES: Medical databases (Medline, Embase and Google Scholar) up to January 20th 2014. For further identification of suitable studies, we searched reference lists of included studies and contacted experts in the field.

STUDY SELECTION: We included randomized controlled trials, systematic reviews, cohorts, case-control, cross-sectional, modeling and ecological studies carried out in adults assessing the economic consequences of NCDs on healthcare spending and national income without language restrictions. All abstracts and full text selection was done by two independent reviewers. Any disagreements were resolved through consensus or consultation of a third reviewer.

DATA EXTRACTION: Data were extracted by two independent reviewers using a pre-designed data collection form.

MAIN OUTCOME AND MEASURES: Studies evaluating the impact of at least one of the selected NCDs on at least one of the following outcome measures: healthcare expenditure, national

income, hospital spending, gross domestic product (GDP), gross national product (GNP), net national income (NNI), adjusted national income (NNI), total costs, direct costs, indirect costs, inpatient costs, outpatient costs, per capita healthcare spending, aggregate economic outcome, capital loss in production levels in a country, economic growth, GDP per capita (per capita income), percentage change in GDP, intensive growth, extensive growth, employment, direct governmental expenditure and non-governmental expenditure.

RESULTS: From 4364 references, 153 studies met our inclusion criteria. Most of the studies were focused on healthcare related costs of NCDs. 30 studies reported the economic impact of NCDs on healthcare budgets and 13 on national income. Healthcare expenditure for cardiovascular disease (12-16.5%) was the highest; other NCDs ranged between 0.7%-7.4%. NCD-related health costs vary across the countries, regions, and according to type of NCD. Additionally, there is an increase in costs with increased severity and years lived with the disease. Low- and middle-income countries were the focus of just 16 papers, which suggests an information shortage concerning the true economic burden of NCDs in these countries.

CONCLUSIONS AND RELEVANCE: NCDs pose a significant financial burden on healthcare budgets and nations' welfare, which is likely to increase over time. However further work is required to standardize more consistently the methods available to assess the economic impact of NCDs and to involve (hitherto under-addressed) LMI populations across the globe.

INTRODUCTION

Due to lifestyle and environmental change, healthcare improvements and improved potential to survive until old age, non-communicable diseases (NCDs) (including coronary heart disease (CHD), stroke, chronic obstructive pulmonary disease (COPD), cancer, type 2 diabetes mellitus (DM) and chronic kidney disease (CKD) are currently the leading cause of adult death and disability worldwide[1]. The global burden of NCDs is expected to rise further as a result of an increasing global population and demographic shifts, especially increases in the older population. Indeed, the global population above the age of 60, the age group most affected by NCDs, is expected to double between 2000 and 2050[2].

Most NCDs are chronic conditions that require expensive treatment regimens and prolonged individual care by increasingly specialized healthcare services. NCDs also detrimentally impact on national income, socio-economic development and economic growth[3] through productivity losses, prolonged disability and increases in health and social care expenditure. Historically, high-income countries (HIC) experience the greatest economic consequences of NCDs. Yet, as a result of economic growth, epidemiological transition, ageing populations and healthcare system development, many low- and middle-income countries (LMIC) are now also experiencing a greater impact of NCDs. LMICs also suffer a substantial burden of NCD-related risk factors such as tobacco use, heavy alcohol consumption or unhealthy diet among their impoverished population groups.[4] However, policy programs that respond to the increasing burden of NCDs in many LMIC remain limited. [5] To date, however, little work has been done to systematically appraise the current evidence on the economic burden of NCDs globally. Exploring the studies that investigate the impact of NCDs on healthcare expenditure and national income can help shape future healthcare plans and strategies by better informing policy makers and healthcare planners about the emerging costs of NCDs.

We aimed to systematically review the literature evaluating the financial burden of six major NCDs (CHD, stroke, cancer (lung, colon, cervical and breast), COPD, DM and CKD) at the macro-economic level in order to quantify: (i,) the costs related to NCDs (direct, indirect, aggregate, over time and by disease severity); (ii) the per capita healthcare expenditure on NCDs; and (iii) national economic loss due to NCDs; and (iv) the overall aggregate economic impact of the NCDs on national income and healthcare spending.

METHODS

Search strategy and inclusion criteria

We conducted a systematic search of electronic medical databases (Medline, Embase and Google Scholar) until November 6th 2014 (date last searched) to retrieve scientific articles assessing the consequences of NCDs at the macro-economic level specifically the impact on national income and healthcare expenditure (including: health expenditure, national income, hospital spending, gross domestic product (GDP), gross national product (GNP), net national income (NNI), adjusted

national income (NNI), healthcare costs, direct costs, indirect costs, per capita healthcare spending, medical costs, non-medical costs, aggregate economic outcome, capital loss in production levels in a country, economic growth, percent rate of increase in GDP, GDP per capita (per capita income), intensive growth, extensive growth, employment, direct governmental expenditure and non-governmental expenditure) (see **Appendix 1** in the Supplement). The step-wise inclusion and exclusion procedure outlined in **Figure 1** was followed. Eligible study designs included randomized controlled trials (RCTs), cohort, case-control, cross-sectional, systematic reviews, ecological studies and modeling studies. We included studies evaluating the impact of at least one NCDs selected (CHD, stroke, COPD, type 2 diabetes mellitus, cancer (lung, colon, breast, and cervical), and CKD) on at least one measure of the impact on national income and healthcare expenditure (as specified above). Only studies carried out in adults (>18 years old) were included and we specified no language or date restrictions.

Study selection

Two independent reviewers screened the abstracts retrieved by the search strategy and selected eligible studies. Any disagreements between the two reviewers were resolved through consensus or consultation of a third independent reviewer. The references of the retrieved studies were scanned to identify additional relevant publications that were missed by the initial search strategy. Authors of the included studies were contacted in order to identify additional publications.

Data extraction

A predesigned data collection form was prepared to extract the relevant information from the included full texts, including study design, WHO region, characteristics of the study participants, NCDs details and economic measures reported.

Quality evaluation

We used the Newcastle- Ottawa Scale (NOS) to evaluate the quality of cross-sectional, case-control and cohort studies included in this review. [6] NOS scale assesses the quality of the articles in three domains of selection, comparability and exposure. Within the selection category, four items are assessed and a maximum of one star can be awarded to each item. Two stars can be awarded to the one item within the comparability category. Finally, one star can be awarded to each of the four items in the exposure category. A score will be made by adding up the number of stars and thus, NOS scale can have a maximum of nine stars in total. We used this scale for quality assessment of case-control and cohort studies. For cross-sectional and descriptive studies we used an adapted version of NOS scale (See **Appendix 2** in the Supplement). No quality score was applied to the modeling studies.

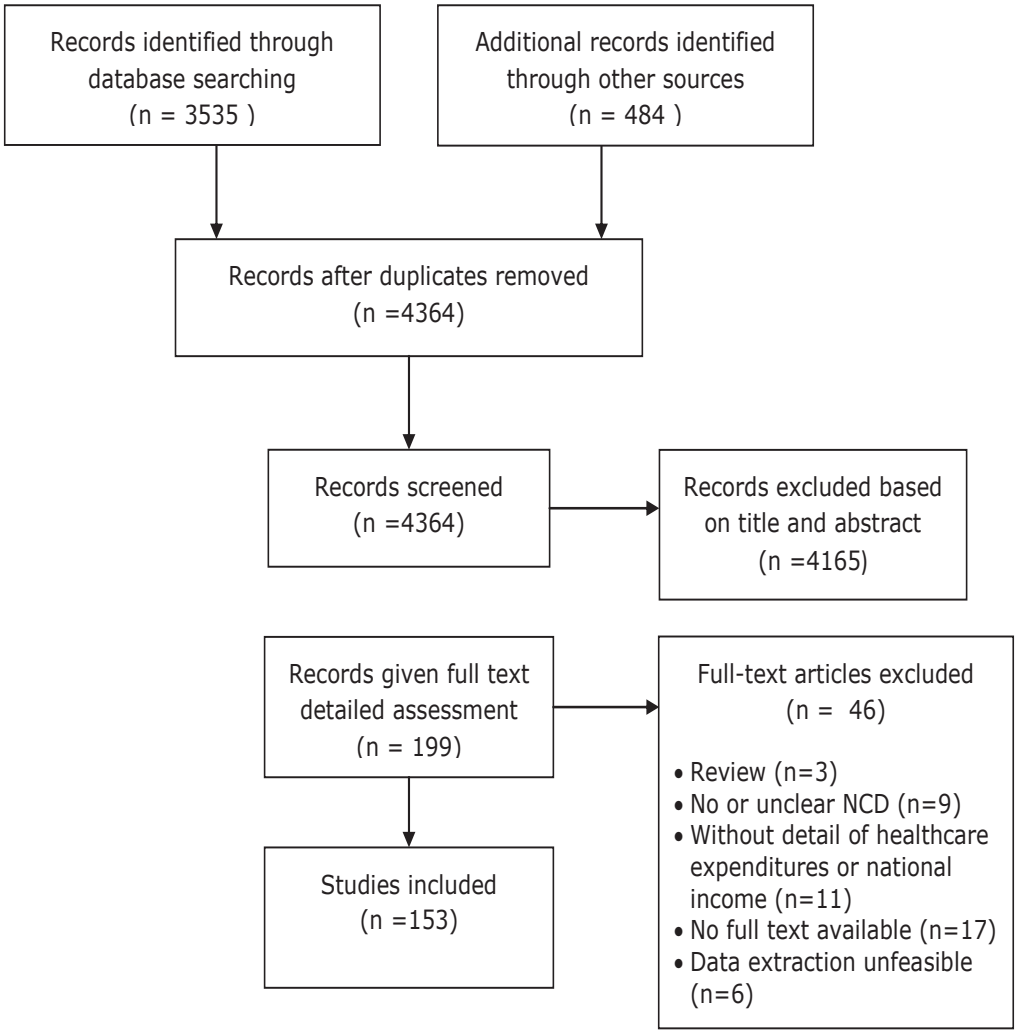
Statistical methods

Heterogeneity permitting, we sought to pool the results using a random effects meta-analysis model. If pooled, results were expressed as the pooled estimate and the corresponding 95% confidence intervals. All costs presented are converted in USD 2013.

RESULTS

In total, we identified 4364 potentially relevant citations (**Figure 1**). Based on the title and abstracts, full texts of 199 articles were selected for detailed evaluation. Of those, 153 articles met our eligibility criteria and were therefore included in the analysis (**Table 1**).

Figure 1. Flowchart of Studies for the Global Impact of Non-communicable Diseases on Healthcare Spending and National Income



General characteristics of the included studies

A wide geographical distribution was observed in the reviewed studies. The majority of the studies (n=68) were from the American WHO region (mainly USA and Canada), 57 studies were from the European, 19 from the Western Pacific, two from the South-East Asian, three and from the African WHO region and the Eastern Mediterranean respectively, whereas two studies were conducted in multiple regions. The majority (n=137) of the reviewed studies were conducted in HIC, ten in upper middle-income countries, and five were conducted in low-income countries whereas one study included countries from the three income categories. Therefore, the GDP variation across the studies reviewed was narrow. The studies identified were mainly observational studies, having a retrospective or longitudinal design; 22 studies were modeling studies and only one study was an RCT. Medical records were the most frequently used method to select participants. In many cases, these medical records were linked to socio-economic databases to extract employment data. Adjustment for age, gender, ethnicity, co-morbidities/existing conditions, and geographic regions was usually applied.

Of the 153 studies included in this review, 40 studies focused on the economic impact of CHD and stroke (cardiovascular disease), 32 on COPD, five on CKD, 24 on DM, 45 on cancer and 7 studies provide evidence on economic impact of a combination of NCDs (Table 1). Most of the studies investigated the economic impact of NCDs among people aged 45 years and over.

Table 1. General Characteristics of the Studies Included in this Review

Lead Author	Period of Surveillance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs	Quality score*
Abudagga, A; et al.[117] 2013	2011-2012	USA	AMR	Cohort	17382	Both	COPD	7
Ademi, Z; et al.[11] 2013	2004-2006	Australia	WPR	Cohort	2873	Both	CVD	8
Aljunid, S; et al.[77] 2010	2007-2008	Malaysia	WPR	Modeling	444	Female	Cervical cancer	NA
Anis, AH; et al.[7] 2000	1994-1995	Canada	AMR	Modeling	352	Both	COPD, CVD	NA
Baker, MS; et al.[15] 1991	1974-1981	USA	AMR	Longitudinal	125831	Both	Lung & breast cancer	6
Bakerly, N; et al.[118] 2009	2003-2004	UK	EUR	Cohort	225	Both	COPD	4
Ballesta, M; et al.[119] 2006	1999	Spain	EUR	Descriptive observational	517	Both	DM	5
Baumeister, SE; et al.[21] 2009	1994-2005	Germany	EUR	Combined	4856	Both	CKD	6

Table 1. General Characteristics of the Studies Included in this Review (continued)

Lead Author	Period of Surveil-lance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs	Quality score*
Beaulieu, N; et al.[87] 2009	2009	Worldwide	NA	Modeling	NA	Both	Lung, colorectal, cervical & breast cancer	NA
Biorac, N; et al.[41] 2009	2007	Serbia	EUR	Cohort	99	Both	DM	2
Blanchette, CM; et al.[50] 2008	2004	USA	AMR	Cohort	6243	Both	COPD	7
Blanchette, CM; et al.[120] 2012	1987-2007	USA	AMR	Cross-sectional	644	Both	COPD	7
Bonastre, J; et al.[121] 2012	1998-2008	France	EUR	Cohort	290	Female	Breast cancer	8
Boncz, I; et al.[74] 2010		Hungary	EUR	Survey	NA	Both	Colorectal, cervical & breast cancer	5
Bottacchi, E; et al.[28] 2012	2004-2007	Italy	EUR	Cost of illness (cohort)	800	Both	Stroke	6
Bouvier, V; et al.[122] 2003	1997-1998	France	EUR	Survey	142	Both	Colorectal cancer	6
Broekx, S; et al.[36] 2011	1997-2004	Belgium	EUR	Cost of illness (cross sectional)	20439	Female	Breast cancer	3
Brown, ML; et al.[123] 1999	1990-1994	USA	AMR	Modeling	NA	Both	Colorectal cancer	NA
Caro, JJ; et al.[124] 2006	1990-1995	France	EUR	Cohort	18704	Both	Stroke	6
Chang, S; et al.[125] 2004	1998-2000	USA	AMR	Retrospective cohort	2858	Both	Lung & colorectal cancer	9
Chirikos, TN; et al.[126] 2008	1991-1999	USA	AMR	Cohort	80421	Both	Lung cancer	7
Chittleboroug, CR; et al.[79] 2009	1997-2002	Australia	WPR	Cross-sectional	2352	Both	COPD	7
Chodick, G; et al.[46] 2005	1999-2001	Israel	EUR	Cohort	24632	Both	DM	7
Chouaid, C; et al.[127] 2004	1998-1999	France	EUR	Modeling	428	Both	Lung cancer	NA
Christensen, MC; Munro, V;[128] 2008	2004-2005	UK	EUR	Cohort	1016	Both	Stroke	5

Table 1. General Characteristics of the Studies Included in this Review (continued)

Lead Author	Period of Surveillance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs	Quality score*
Claesson, L; et al.[47] 2000	1993-1994	Sweden	EUR	RCT	249	Both	Stroke	8
Clerc, L; et al.[29] 2008	2004-2005	France	EUR	Cohort	384	Both	Colorectal cancer	4
Cocquyt, V; et al.[14] 2003	1997-1998	Belgium	EUR	Modeling	118	Female	Breast cancer	NA
Costantino, ME; et al.[129] 2014	2008-2009	USA	AMR	Retrospective Cohort	389550	Both	DM	6
Corrao, G; et al.[130] 2014	2011	Italy	EUR	Cohort	26949	Both	Heart Failure	7
Dahlberg, L; et al.[131] 2009	2005-2006	Sweden	EUR	Cohort	53	Female	Breast cancer	6
Dalal, AA; et al.[45] 2011	2006-2009	USA	AMR	Cohort	42166	Both	COPD	4
Dalal, AA; et al.[132] 2011	2003-2008	USA	AMR	Cohort	4594	Both	COPD	4
Darkow, T; et al.[34] 2007	2001-2004	USA	AMR	Cohort	1349	Both	COPD	6
Davari, M; et al.[75] 2012	2005-2010	Iran	EMR	Cross-sectional	435	Both	Colorectal cancer	2
Degli Esposti, L; et al.[133] 2013	2009	Italy	EUR	Retrospective	21586	Both	DM	5
Dewey, HM; et al.[85] 2001	1997	Australia	WPR	Cost of illness	275	Both	Stroke	4
Dewey, HM; et al.[72] 2003	1997	Australia	WPR	Cost of illness	NA	NA	Stroke	4
Di Salvo, TG; et al.[134] 1996	1991-1992	USA	AMR	Cohort	292	Both	AMI	4
Domingo, C; et al.[135] 2006	NA	Spain	EUR	Cohort	124	Both	COPD	4
D'Souza, AO; et al.[136] 2014	2003-2007	USA	AMR	Retrospective	40884	Both	COPD	7
Elrayah-Eliadarous, H; et al.[137] 2010	2005	Sudan	EMR	Retrospective	822	NA	DM	3
Fernandez De Bobadilla, J; et al.[138] 2008	2006	Spain	EUR	Cohort	2858	Both	Stroke	4
Ferrandinda, G; et al.[139] 2010	2000-2007	Italy	EUR	Cohort	351	Female	Cervical cancer	6

Table 1. General Characteristics of the Studies Included in this Review (continued)

Lead Author	Period of Surveil-lance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs	Quality score*
Fireman, BH; et al.[61] 1997	1987-1991	USA	AMR	Survey	21977	Both	Lung, colorectal & breast cancer	8
Fletcher, MJ; et al.[14] 2009	2009	Cross-country	NA	Cross-sectional	2426	Both	COPD	1
Gil, A; et al.[76] 2007	1999-2002	Spain	EUR	Retrospective	16604	Female	Cervical cancer	6
Gruber, EV; et al.[141] 2012	2009	Germany	EUR	Modeling	14000000	Female	Breast cancer	NA
Havlovicova, M; et al.[142] 2001	1997	Czech Republic	EUR	Cohort	224	Both	Stroke	6
Hilleman, DE; et al.[51] 2000	1993-1998	USA	AMR	Cohort (phamarco-economic analysis)	413	Both	COPD	4
Hodgson, TA; Cohen, AJ[70] 1999	1995	USA	AMR	Survey	NA	NA	CVD, CHD	NA
Hogan, P; et al.[81] 2003	2002	USA	AMR	Survey/ modeling	NA	Both	DM	NA
Hu, S; et al.[143] 2013	2010-2011	China	WPR	Cross-sectional	63	Both	Stroke	1
Hutchinson, A; et al.[52] 2010	2001-2002	Australia	WPR	Cohort	80	Both	COPD	6
Jansson, SA; et al.[49] 2002	1998-1999	Sweden	EUR	Cohort	212	Both	COPD	6
Jaworski, R; et al.[9] 2012	2005	Poland	EUR	Cohort	2593	Both	CAD	3
Jensen, MB; et al.[53] 2013	2004-2006	Denmark	EUR	Cohort	546	Both	COPD	7
Jonsson, B; et al.[68] 2002	1999	Europe	EUR	Retrospective	6996	Both	DM	3
Kabadi, GS; et al.[144] 2014	2005-2006	Tanzanie	AFR	Prospective	16	Both	Stroke	6
Kang, HY; et al.[145] 2011	2002-2004	Korea	SEAR	Modeling	NA	Both	Stroke	NA
Kang, S; et al.[16] 2012	2005-2008	Australia	WPR	Cohort	210	Both	Lung cancer	6
Kangas, T; et al.[146] 1996	1987-1989	Finland	EUR	Cross-sectional	NA	NA	DM	4
Kerrigan, M; et al.[147] 2005	1996-1998	USA	AMR	Case-control	346	Both	Colorectal cancer	9

Table 1. General Characteristics of the Studies Included in this Review (continued)

Lead Author	Period of Surveil-lance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs	Quality score*
Kim, TH; et al.[148] 2012	2005	Korea	WPR	Cohort	3125	Both	DM	3
Kirigia, JM; et al.[80] 2009	2000	Africa	AFR	Cost of illness	NA	Both	DM	NA
Klever-Deichert, G; et al.[84] 1999	1996	Germany	EUR	Modeling	NA	Both	CVD	NA
Kolominsky-Rabas, PL; et al.[149] 2006	1994-2003	Germany	EUR	Cost of illness	2458	Both	Stroke	5
Kumar, A; et al.[23] 2008	2005-2005	India	SEAR	Cross-sectional	819	Both	DM	2
Kuwabara, H; et al.[150] 2009	2003	Japan	WPR	Modeling	3490	Both	Breast cancer	NA
Laliberte, F; et al.[151] 2009	2000-2006	USA	AMR	Retrospective	91069	Both	CKD	7
Lamerato, L; et al.[152] 2006	1996-2002	USA	AMR	Cohort	1616	Female	Breast cancer	7
Lamping, DL; et al.[22] 2000	1995-1996	UK	EUR	Cohort	221	Both	CKD	6
Lang, K; et al.[63] 2009	1996-2002	USA	AMR	Cohort	56838	Both	Colorectal cancer	8
Le, C; et al.[27] 2013	2010-2011	China	WPR	Cross-sectional survey	9396	Both	DM	5
Leal, J; et al.[64] 2006	2003	Europe	EUR	Cost of illness	NA	Both	CVD, CHD	4
Lee, H; et al.[153] 2002	1999-2000	Canada	AMR	Prospective	166	Both	CKD	4
Lee, HC; et al.[12] 2013	1996-2003	Taiwan	WPR	Cohort	2368	Both	Stroke	6
Legorreta, AP; et al.[57] 1996	1989	USA	AMR	Longitudinal	205	Female	Breast cancer	5
Leigh, JP; et al.[154] 2003	1999	USA	AMR	Modeling	NA	Both	Colorectal cancer, CVD, CKD	NA
Likosky, DS; et al.[42] 2013	1998-1999/ /2008	USA	AMR	Cross-sectional	317043	Both	AMI	5
Lokke, A; et al.[39] 2014	1998-2010	Denmark	EUR	Case-Control	263622	Both	COPD	7
Lopez-Batista, J; et al.[31] 2012	2004	Spain	EUR	Cross-sectional	448	Both	Stroke	5

Table 1. General Characteristics of the Studies Included in this Review (continued)

Lead Author	Period of Surveillance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs	Quality score*
Lou,P; et al.[155] 2012	2008-2009	China	WPR	Cross-sectional	8217	Both	COPD	2
Low, JJ; et al.[18] 2012	2008-2033	Singapore	WPR	Modeling	NA	Female	Cervical cancer	NA
Luo, Z; et al.[58] 2009	1996-2000	USA	AMR	Case-control	17945	Both	Colon cancer	6
Macafee, DA; et al.[17] 2009	1981-2002	UK	EUR	Retrospective	227	Both	Colorectal cancer	4
Mandelblatt, JS; et al.[156] 2006	2000	USA	AMR	Cohort	418	Female	COPD	7
Mapel, DW; et al.[157] 2000	1997	USA	AMR	Case-control	1522	Both	COPD	8
Martin, S; et al.[59] 2007	1995-2003	Germany	EUR	Cohort	3142	Both	DM	2
Marton, P; et al.[158] 2006	2001	USA	AMR	Retrospec-tive-Cohort	49510	Both	COPD	8
Meen, P; et al.[159] 2012	2006-2009	Germany	EUR	Cohort	2255	Both	COPD	5
Menzin, J; et al. 2008	2001-2002	USA	AMR	Cohort	16321	Both	IHD	5
Menzin, J; et al.[160] 2008	2004	USA	AMR	Cohort	8370	Both	COPD	5
Miravitlles, M; et al.[161] 2001	1996-1997	Spain	EUR	Cohort	2414	Both	COPD	6
Miravitlles, M; et al.[54] 2003	1997-1998	Spain	EUR	Cohort	1510	Both	COPD	7
Mittman, N; et al.[90] 2012	2005-2009	Canada	AMR	Cohort	232	Both	Stroke	4
Mohd Nordin, NA; et al.[48] 2012	2005-2009	Malaysia	WPR	Cross-sectional	813	Both	Stroke	6
Morsanutto, A; et al.[162] 2006	2001-2002	Italy	EUR	Retrospective longitudinal	299	Both	DM	3
Nakamura, K; et al.[163] 2008	1990-2001	Japan	WPR	Cohort	4535	Both	DM	4
Nichols, GA; et al.[69] 2010	2000-2008	USA	AMR	Survey	12278	Both	CVD	NA
Nielsen, R; et al.[19] 2009	2003-2004	Iceland/ Norway	EUR	Survey	1415	Both	COPD	6
Nielsen, R; et al.[164] 2011	2005-2006	Norway	EUR	Cohort	286	Both	COPD	6

Table 1. General Characteristics of the Studies Included in this Review (continued)

Lead Author	Period of Surveil-lance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs	Quality score*
Nishimura, S; Zaher, C;[33] 2004	1990-2002	Japan	WPR	Modeling	501	Both	COPD	NA
Norlund, A; et al.[40] 2001	1992-1993	Sweden	EUR	Cross-sectional	1677	Both	DM	4
O'Brien, BD; et al.[165] 2001	1989-1994	Canada	AMR	Survey	593	Both	Colorectal cancer	6
Odden, MC; et al.[71] 2011	NA	USA	AMR	Modeling	NA	Both	CHD	NA
Oglesby, AK; et al.[166] 2006	1989-2003	USA	AMR	Cohort	10780	Both	DM	6
Ohinmaa, A; et al.[65] 2006	2000-2001	Canada	AMR	Survey	2133413	Both	CVD, COPD, DM	4
Oliva, J; et al.[67] 2004	2002	Spain	EUR	Cost of illness	2010365	Both	DM	NA
Perera, PN; et al.[167] 2012	2006	USA	AMR	Retrospective	1254703	Both	COPD	6
Petersen, M; Amer Diabet, A[89] 2008	2007	USA	AMR	Survey/ Modeling	301736	Both	DM	NA
Ramsey, SD; et al.[168] 2003	1984-1994	USA	AMR	Survey	68145	Both	Colorectal cancer	6
Rao, S; et al.[169] 2004	1997-1999	USA	AMR	Cohort	397	Female	Breast cancer	6
Ray, GT; et al.[25] 2000	1995-1996	USA	AMR	Retrospective	2076303	NA	Lung, colon & breast cancer, CVD, IHD, COPD, DM	7
Ray, NF; et al.[92] 1998	1989-1995	USA	AMR	Survey/ Modeling	NA	Both	DM	NA
Riley, GF; et al.[62] 1995	1984-1990	USA	AMR	Retrospective	287013	Both	Lung, breast & colorectal cancer	7
Ringborg, A; et al.[170] 2008	2000-2004	Sweden	EUR	Cohort	37756	Both	DM	4
Rutten-van Molken, MPMH; et al.[66] 1999	1993	Netherlands	EUR	Modeling	NA	Both	COPD	NA
Sasser, AC; et al.[37] 2005	1998-2000	USA	AMR	Cohort	2265	Female	Breast cancer, CVD	8
Schneider, M; et al.[24] 2009	2001-2005	USA	AMR	Survey	1649574	Both	COPD, CKD, cancer, Heart failure, DM	NA

Table 1. General Characteristics of the Studies Included in this Review (continued)

Lead Author	Period of Surveil-lance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs	Quality score*
Seal, BS; et al.[44] 2013	2005-2009	USA	AMR	Longitudinal	5160	Both	Colon Cancer	7
Selke, B; et al.[73] 2003	1999	France	EUR	Cost of illness	69046	Both	Colorectal cancer	6
Sharafk-haneh, A; et al.[171] 2010	1997-2004	USA	AMR	Cohort	59906	Both	COPD	8
Simoni-Wastile, L; et al.[172] 2009	2003-2005	USA	AMR	Cohort	3037	Both	COPD	5
Simpson, AN; et al.[173] 2013	2004-2005	USA	AMR	Case-control	8928	Both	Stroke	7
Sloss, EM; et al.[174] 2004	1995-1998	USA	AMR	Cohort	3149	Both	Stroke	4
Smith, DH; et al.[175] 2004	1996-2001	USA	AMR	Cohort	13796	Both	CKD	8
Soekhlal, RR; et al.[176] 2013	2008-2012	Netherlands	EUR	Retrospective	25657	Both	AMI	6
Song, X; et al.[13] 2011	2004-2009	USA	AMR	Cohort	6675	Both	Colorectal cancer	6
Sorensen, SV; et al.[30] 2012	2000-2007	USA	AMR	Modeling	49674	Both	Breast cancer	NA
Spieler, JF; et al.[177] 2004	1997	France	EUR	Cohort	435	Both	Stroke	6
Taplin, SH; et al.[178] 1995	1990-1991	USA	AMR	Survey	6107	Both	Colorectal & breast cancer	5
Taylor, TN; et al.[91] 1996	1990-1993	USA	AMR	Modeling	NA	NA	Stroke	NA
Tiemann, O; et al.[179] 2008	2005	Europe	EUR	Cross-sectional	3942	Male	AMI	8
Torres, US; et al.[82] 2010	1996-2008	Brazil	AMR	Survey	297108	Both	Colorectal Cancer	6
Toure, K; et al.[180] 2005	1997	Senegal	AFR	Cross-sectional	383	Both	Stroke	NA
Trogdon, JG; et al.[181] 2007	2000-2003	USA	AMR	Cohort	125052	Both	Stroke	3
Tuck, J; et al.[182] 1989	1985-1986	UK	EUR	Cohort	85	NA	Colorectal cancer	NA
Tunceli, O; et al.[183] 2007	1999-2002	USA	AMR	Cohort	512468	Both	DM	8
Unroe, KT; et al.[8] 2011	2000-2007	USA	AMR	Cohort	229543	Both	Heart failure	7

Table 1. General Characteristics of the Studies Included in this Review (continued)

Lead Author	Period of Surveil-lance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs	Quality score*
Van Boven, JFM; et al.[88] 2013	2009	Netherlands	EUR	Cross-sectional	94158	Both	COPD	6
Wan, Y; et al.[32] 2013	2005-2009	USA	AMR	Cohort	278	Female	Breast cancer	7
Ward, A; et al.[10] 2005	1994-1998	Germany	EUR	Prospective	491	Both	Stroke	5
Warren, JL; et al.[38] 2008	1991-2002	USA	AMR	Survey	306709	Both	Lung, colorectal & breast cancer	4
Winter, Y; et al.[184] 2008	2003	Germany	EUR	Cohort	76	Both	Stroke	2
Wright, GE; et al.[185] 2007	1992-1996	USA	AMR	Cohort	6108	Both	Colorectal cancer	8
Yabroff, KR; et al.[78] 2008	1999-2003	USA	AMR	Survey	2342558	Both	Lung, colorectal, cervical & breast cancer	6
Yabroff, KR; et al.[186] 2009	1998-2002	USA	AMR	Cohort	6377	Both	Colorectal cancer	5
Yang, SC; et al.[187] 2013	1998–2010	Taiwan	WPR	Cohort	66535	Both	Lung cancer	6
Yoon, J; et al.[83] 2011	2000-2008	USA	AMR	Cohort	4892300	Both	CVD, CKD, COPD, Stroke	4
Zheng, H; et al.[86] 2010	2004	Australia	WPR	Modeling	NA	Both	CHD	NA
Zhuo, X; et al.[188] 2013	2009-2010	United Kingdom	EUR	Modeling	NA	Both	DM	NA
Zorowitz, R; et al.[189] 2009	2003-2006	USA	AMR	Cohort	3438	Both	Stroke	6

NCD: Non-communicable diseases; NA: Not applicable; RCT: Randomized Control Trials; CVD Cardiovascular disease; CHD: Coronary Heart Disease; CAD: Coronary Artery Disease; IHD: Ischemic Heart Diseases; AMI: Acute myocardial infarction; COPD: Chronic Obstructive Pulmonary Disease; CKD: Chronic Kidney Disease; DM: Diabetes mellitus; AFR: African Region; AMR: Region of the Americas; EMR: Eastern Mediterranean Region; EUR: European Region; SEAR: South-East Asia Region; WPR: Western Pacific Region. *No quality score was applied to the modeling studies

Per patient global healthcare costs and non-healthcare costs of NCDs

Reported healthcare costs associated with NCDs varied across countries and regions, and across the type of NCDs. Reported annual direct costs of NCDs were the highest in the Americas, followed by European and Western Pacific regions; in the region of the Americas, the minimum and maximum

mean reported annual total direct costs for CVD were 6668 USD[7] and 81096 USD[8], respectively, whereas the average annual direct costs for CVD varied from 1643[9] USD to 69440 USD[10], and from 3862 USD[11] to 5693 USD[12] for the European and Western Pacific regions, respectively (**Supplementary Table S1**). A detailed description on the variation of any type of costs of NCDs per world regions is shown in **Supplementary Table S1**.

(i) Direct costs

In 107 studies, mean annual total direct costs and mean directly attributable costs per NCDs patient (**Table 2a**) were estimated. Worldwide, of all the selected NCDs, cancer and CVD (with estimated costs up to 197772 USD[13] and 81096 USD[8], respectively) had the highest reported mean annual total direct costs, whereas DM had the lowest. Average CVD-related direct costs ranged from 1643 USD[9] in Poland to 81096 USD[8] in USA, with CHD having the lowest reported estimates and heart failure imposing the highest costs. Among cancers, the estimated mean annual total direct costs varied: from 4595 USD[14] to 82794 USD[15] for breast cancer; 4964 USD[16] to 161048 USD[15] for lung cancer and 2208[17] USD to 197722 USD[17] for colorectal cancer. Only one study from Singapore reported annual total direct costs for cervical cancer with an average estimate of 8049 USD[18]. COPD annual direct costs varied substantially, with Norway reporting the lowest direct costs (431 USD[19]) and USA reporting the highest (34101 USD[20]). The lowest direct costs for CKD were observed in Germany with an average estimate of 5439 USD[21], whereas mean direct costs for CKD in USA were estimated to be up to 71824 USD[22]. DM average annual direct costs varied from 162 USD [23] in India to 15611 USD in USA[24].

Direct attributable costs for the NCDs were available from only 18 studies. The highest direct attributable costs were observed for cancer (up to 190032 USD[13]), followed by CKD (up to 33585 USD[25]), COPD (up to 22183 USD[24]), CVD (up to 21152 USD[24]) and finally, DM (up to 12246 USD[24]) with the lowest average estimates. Some articles reported inpatient and outpatient costs for NCDs (**Supplemental Table S2)**. These demonstrate that inpatient costs are the main source of direct costs for NCDs. Inpatient costs accounted for 47%-58% of total direct costs of COPD[26] and 63% of total direct costs for DM[27]. Hospital costs represent the main driver of stroke expenditure, accounting for 90%[28] of total direct costs. Hospitalization charges represented the greatest economic burden (55%)[29] for the management of colorectal cancer, followed by medical purchases (24%) and outpatient care (18%).

(ii) Indirect costs

18 studies estimated mean annual indirect costs (Table 2b). Mean annual estimated indirect costs for NCDs patients were highest for cancer and DM, with estimates up to 24740 USD[30] and 23418 USD[31], respectively. Mean annual indirect costs for breast cancer varied extensively, from 2109 USD[32] to 24740 USD[30]. The lowest indirect cost for COPD was reported in Japan, with an average estimate of 326 USD[33], and highest in USA (3393 USD[34]). Mean DM indirect annual costs were estimated at 104 USD in Serbia[35] compared to 7797 USD in China[27]. No study, however, reported annual indirect costs of colorectal cancer, lung cancer, cervical cancer or CKD.

Table 2a. Results of the Included Studies Investigating Annual Direct Costs of NCDs

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	Reported NCDs	Year	Country
Ademi, Z; et al. 2013	Direct costs	Patient/ year	Mean	3862	5508	CVD	2006	Australia
Anis, AH; et al 2000	Direct costs	Patient/ year	Mean	6668	391	CVD	1995	Canada
Di salvo, TG; et al. 1996	Direct costs	Patient/ year	Mean	13907	NA	CVD	1992	USA
Nichols, GA; et al. 2010	Direct costs	Patient/ year	Mean	20512	42247	CVD	2008	USA
Sasser, AC; et al. 2005	Direct costs	Patient/ year	Mean	16313	NA	CVD	2000	USA
Corrao, G; et al. 2014	Direct costs	Patient/ year	Mean	15328	NA	HF	2011	Italy
Ray, GT; et al. 2000	Direct attributable costs	Patient/ year	Mean	10647	NA	HF	1996	USA
Ray, GT; et al. 2000	Direct costs	Patient/ year	Mean	27015	NA	HF	1996	USA
Schneider, KM; et al. 2009	Direct attributable costs	Patient/ year	Mean	21152	NA	HF	2005	USA
Schneider, KM; et al. 2009	Direct costs	Patient/ year	Mean	24517	NA	HF	2005	USA
Unroe, KT; et al. 2011	Direct costs	Patient/ year	Mean	81096	89336	HF	2007	USA
Bottachi, E; et al. 2012	Direct costs	Patient/ year'	Mean	9030	NA	Stroke	2007	Italy
Fernandez De Bodadilla, J; et al. 2008	Direct costs	Patient/ year	Mean	4429	NA	Stroke	2006	Spain
Cleason, L; et al. 2000	Direct costs	Patient/ year	Mean	39527	100233	Stroke	1994	Sweden
Hu, S; et al. 2013	Direct costs	Patient/ year	Mean	4779	NA	Stroke	2011	China

Table 2a. Results of the Included Studies Investigating Annual Direct Costs of NCDs (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	Reported NCDs	Year	Country
Lee, HC; et al. 2013	Direct costs	Patient/ year	Mean	5693	NA	Stroke	2003	Taiwan
Lopez-Batista, J; et al. 2012	Direct costs	Patient/ year	Mean	21520	18535	Stroke	2004	Spain
Simpson, AN; et al. 2013	Direct costs	Patient/ year	Mean	33698	29486	Stroke	2005	USA
Simpson, AN; et al. 2013	Direct attributable costs	Patient/ year	Mean	11164	29486	Stroke	2005	USA
Sloss, EM; et al. 2004	Direct costs	Patient/ year	Mean	39641	NA	Stroke	1998	USA
Spieler, JF; et al. 2004	Direct costs	Patient/ year	Mean	32291	26290	Stroke	1997	France
Taylor, TN; et al. 1996	Direct costs	Patient/ year	Mean	32467	NA	Stroke	1993	USA
Trogdon, JG; et al. 2007	Direct costs	Patient/ year	Mean	7294	NA	Stroke	2003	USA
Winter, Y; et al. 2008	Direct costs	Patient/ year	Mean	7386	9814	Stroke	2003	Germany
Yoon, J; et al. 2011	Direct costs (2000)	Patient/ year	Mean	11251	NA	Stroke	2008	USA
Yoon, J; et al. 2011	Direct costs (2008)	Patient/ year	Mean	8436	NA	Stroke	2008	USA
Ward, A; et a 2005	Direct costs	Patient/ year	Mean	69440	NA	Stroke	1994-1998	Germany
Zorowitz, R; et al. 2009	Direct costs	Patient/ year	Mean	31969	NA	Stroke	2006	USA
Ray, GT; et al. 2000	Direct attributable costs	Patient/ year	Mean	7669	NA	IHD	1996	USA
Ray, GT; et al. 2000	Direct costs	Patient/ year	Mean	17776	NA	IHD	1996	USA
Menzin, J; et al. 2008	Direct costs	Patient/ year	Mean	35005	NA	IHD	2002	USA
Jaworski, R; et al. 2012	Direct costs	Patient/ year	Mean	1643	NA	CHD	2005	Poland
Likosky, DS; et al. 2013	Direct costs	Patient/ year	Mean	40071	NA	AMI	1998-1999	USA

Table 2a. Results of the Included Studies Investigating Annual Direct Costs of NCDs (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	Reported NCDs	Year	Country
Likosky, DS; et al. 2013	Direct costs	Patient/ year	Mean	46667	NA	AMI	2008	USA
Schneider, KM; et al. 2009	Direct attributable costs	Patient/ year	Mean	15796	NA	Cancer	2005	USA
Schneider, KM; et al. 2009	Direct costs	Patient/ year	Mean	19161	NA	Cancer	2005	USA
Baker, MS; et al.1991	Direct costs	Patient/ year	Mean	49032	NA	Breast cancer	1974-1981	USA
Baker, MS; et al. 1991	Lifetime attributable costs	Per patient	Mean	82794	NA	Breast cancer	1974-1981	USA
Broekx, S; et al. 2011	Direct attributable costs	Patient/ year	Mean	2989	NA	Breast cancer	2004	Belgium
Cocquyt, V; et al. 2003	Direct costs	Patient/ year	Mean	4595	NA	Breast cancer	1998	Belgium
Dahlberg, L; et al. 2009	Direct costs	Patient/ year	Mean	60519	NA	Breast cancer	2006	Sweden
Fireman, BH; et al. 1997	Direct costs	Patient/ year	Mean	11953	72	Breast cancer	1987-1991	USA
Fireman, BH; et al. 1997	Direct attributable costs	Per patient	Mean	58608	625	Breast cancer	1987-1991	USA
Gruber, EV; et al. 2012	Direct attributable costs	Patient/ year	Mean	10208	NA	Breast cancer	2009	Germany
Lamerato, L; et al. 2006	Direct costs	Patient/ year	Mean	58973	NA	Breast cancer (recurrence)	2002	USA
Legorreta, AP; et al. 1996	Direct costs	Patient/ year	Mean	42514	NA	Breast cancer	1989	USA
Rao,S; et al. 2004	Direct attributable costs	Patient/ year	Mean	31140	NA	Breast cancer	2004	USA
Rao,S; et al. 2004	Direct costs	Patient/ year	Mean	32118	NA	Breast cancer	2004	USA
Ray, GT; et al. 2000	Direct attributable costs	Patient/ year	Mean	5065	NA	Breast cancer	1996	USA

Table 2a. Results of the Included Studies Investigating Annual Direct Costs of NCDs (continued)

Study	Type of Outcome	Outcome Specified as	Assess-ment Type	Point Estimate	SD for Mean	Reported NCDs	Year	Country
Ray, GT; et al. 2000	Direct costs	Patient/ year	Mean	9899	NA	Breast cancer	1996	USA
Riley, GF; et al. 1995	Direct costs	Patient/ year	Mean	22628	NA	Breast cancer	1984-1990	USA
Sasser, AC; et al. 2005	Direct costs	Patient/ year	Mean	18843	NA	Breast cancer	2000	USA
Sorensen, SV; et al. 2012	Direct costs	Patient/ year	Mean	80572	NA	Breast cancer	2007	USA
Yabroff, KR; et al. 2008	Direct attributable costs	Patient/ year	Mean	17315	NA	Breast cancer	1999-2003	USA
Baker, MS; et al. 1991	Direct costs	Patient/ year	Mean	161048	NA	Lung cancer	1974-1981	USA
Baker, MS; et al. 1991	Lifetime attributable costs	Per patient	Mean	28049	NA	Lung cancer	1974-1981	USA
Chang, S; et al. 2004	Direct costs	Patient/ year	Mean	105876	54132	Lung cancer	1998-2000	USA
Chouaid, C; et al. 2004	Direct costs	Patient/ year	Mean	22003	NA	Lung cancer	1999	France
Fireman, BH; et al. 1997	Direct costs	Patient/ year	Mean	25128	253	Lung cancer	1987-1991	USA
Fireman, BH; et al. 1997	Direct attributable costs	Per patient	Mean	54972	653	Lung cancer	1987-1991	USA
Kang, S; et al. 2012	Direct costs	Patient/ year	Mean	4964	NA	Lung cancer	2008	Australia
Ray, GT; et al. 2000	Direct attributable costs	Patient/ year	Mean	12777	NA	Lung cancer	1996	USA
Ray, GT; et al. 2000	Direct costs	Patient/ year	Mean	24099	NA	Lung cancer	1996	USA
Riley, GF; et al. 1995	Direct costs	Patient/ year	Mean	32724	NA	Lung cancer	1984-1990	USA
Yabroff, KR; et al. 2008	Direct attributable costs	Patient/ year	Mean	68658	NA	Lung cancer	1999-2003	USA
Bouvier, V; et al. 2003	Direct costs	Patient/ year	Mean	26577	NA	Colorectal cancer	1997-1998	France
Brown, ML; et al. 1999	Direct attributable costs	Patient/ year	Mean	13726	NA	Colorectal cancer	1994	USA

Table 2a. Results of the Included Studies Investigating Annual Direct Costs of NCDs (continued)

Study	Type of Outcome	Outcome Specified as	Assess-ment Type	Point Estimate	SD for Mean	Reported NCDs	Year	Country
Brown, ML; et al. 1999	Direct costs	Patient/ year	Mean	27722	NA	Colorectal cancer	1994	USA
Chang, S; et al. 2004	Direct costs	Patient/ year	Mean	66036	26304	Colorectal cancer	1998-2000	USA
Clerc,L; et al. 2008	Direct costs	Patient/ year	Mean	42688	NA	Colorectal cancer	2005	France
Fireman, BH; et al. 1997	Direct costs	Patient/ year	Mean	18189	236	Colorectal cancer	1987-1991	USA
Fireman, BH; et al. 1997	Direct attributable costs	Per patient	Mean	74860	1364	Colorectal cancer	1987-1991	USA
Kerrigan, M; et al. 2005	Direct attributable costs	Patient/ year	Mean	60535	NA	Colorectal cancer	1996-1998	USA
Macafee, DA; et al. 2009	Direct costs (for female)	Patient/ year	Median	2208	NA	Colorectal cancer	1981-2002	UK
Macafee, DA; et al. 2009	Direct costs(for male)	Patient/ year	Median	2856	NA	Colorectal cancer	1981-2002	UK
Luo,Z; et al. 2009	Direct attributable costs	Patient/ year	Mean	39507	NA	Colorectal cancer	2000	USA
Seal, BD; et al. 2013	Direct costs	Patient/ year	Mean	105754	92290	Colorectal cancer	2009	USA
Ray, GT; et al. 2000	Direct attributable costs	Patient/ year	Mean	15588	NA	Colorectal cancer	1996	USA
Ray, GT; et al. 2000	Direct costs	Patient/ year	Mean	22631	NA	Colorectal cancer	1996	USA
Ramsey, SD; et al. 2003	Direct costs	Patient/ year	Mean	6697	NA	Colorectal cancer	1984-1994	USA
Ramsey, SD; et al. 2003	Direct attributable costs	Patient/ year	Mean	2806	NA	Colorectal cancer	1984-1994	USA
Riley, GF; et al. 1995	Direct costs	Patient/ year	Mean	35631	NA	Colorectal cancer	1984-1990	USA
Wright,GE; et al. 2007	Direct costs	Patient/ year	Mean	39399	NA	Colorectal cancer	1996	USA
Yabroff, KR; et al. 2008	Direct attributable costs	Patient/ year	Mean	28090	NA	Colorectal cancer	1999-2003	USA
Yabroff, KR; et al. 2009	Direct attributable costs	Patient/ year	Mean	43611	NA	Colorectal cancer	2002	USA

Table 2a. Results of the Included Studies Investigating Annual Direct Costs of NCDs (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	Reported NCDs	Year	Country
Low, JJ; et al. 2012	Direct costs	Patient/ year	Mean	8049	NA	Cervical cancer	2008-2033	Singapore
Yabroff, KR; et al. 2008	Direct attributable costs	Patient/ year	Mean	22769	NA	Cervical cancer	1999-2003	USA
Song, X; et al. 2011	Direct attributable costs	Patient/ year	Mean	197772	639672	Metastatic Colorectal cancer	2009	USA
Song, X; et al. 2011	Direct costs	Patient/ year	Mean	190032	NA	Metastatic Colorectal cancer	2009	USA
Anis, AH; et al. 2000	Direct costs	Patient/ year	Mean	1777	221	COPD	1994-1995	Canada
Miravitlles, M; et al. 2001	Direct costs	Patient/ year	Mean	1869	NA	COPD	1996-1997	Spain
Blanchette, CM; et al. 2012	Direct costs	Patient/ year	Mean	18305	NA	COPD	2007	USA
Blanchette, CM; et al. 2012	Direct costs	Patient/ year	Mean	13266	NA	COPD	1987	USA
Blanchette, CM; et al. 2008	Direct costs	Patient/ year	Mean	28966	45625	COPD	2004	USA
Chittleborough, CR; et al. 2009	Direct costs	Patient/ year	Mean	551	551	COPD	1997-2002	Australia
Dalal, AA; et al. 2011	Direct costs	Patient/ year	Mean	4856	NA	COPD	2006-2009	USA
Dalal, AA; et al. 2011	Direct costs	Patient/ year	Mean	2574	NA	COPD	2003-2008	USA
Domingo, C; et al. 2006	Direct costs	Patient/ year	Mean	1881	3828	COPD	2006	Spain
D'Souza, AO; et al. 2014	Direct costs	Patient/ Year	Mean	19587	682	COPD	2003-2007	USA
D'Souza, AO; et al. 2014	Direct attributable costs	Patient/ Year	Mean	2323	NA	COPD	2003-2007	USA
Hilleman, DE; et al. 2000	Direct costs	Patient/ year	Median	6429	NA	COPD	1993-1998	USA
Hutchinson, A; et al. 2010	Direct costs	Patient/ year	Median	6656	NA	COPD	2001-2002	Australia
Jansson, SA; et al. 2002	Direct costs	Patient/ year	Mean	692	NA	COPD	1998-1999	UK

Table 2a. Results of the Included Studies Investigating Annual Direct Costs of NCDs (continued)

Study	Type of Outcome	Outcome Specified as	Assess-ment Type	Point Estimate	SD for Mean	Reported NCDs	Year	Country
Nielsen, R; et al. 2011	Direct costs	Patient/ year	Mean	13583.25	NA	COPD	2005-006	Norway
Jensen, MB; et a2013	Direct costs	Patient/ year	Mean	4751	NA	COPD	2004-2006	Denmark
Lokke, A; et al. 2014	Direct costs	Patient/ year	Mean	12321	NA	COPD	1998-2010	Denmark
Lokke, A; et al. 2014	Direct attributable costs	Patient/ year	Mean	7855	NA	COPD	1998-2010	Denmark
Lou, P; et al. 2012	Direct costs	Patient/ year	Mean	14326	NA	COPD	2008-2009	China
Mandelblatt, JS; et al. 2006	Direct costs	Patient/ year	Mean	2491	4215	COPD	2000	USA
Mapel, DW; et al. 2000	Direct attributable costs	Patient/ year	Mean	8512	NA	COPD	1997	USA
Mapel, DW; et al. 2000	Direct costs	Patient/ year	Mean	16949	NA	COPD	1997	USA
Marton, JP; et al. 2006	Direct costs	Patient/ year	Mean	17042	NA	COPD	2001	USA
Meen, P; et al. 2012	Direct costs	Patient/ year	Mean	3826	NA	COPD	2006-2009	Germany
Meen, P; et al. 2012	Direct costs	Patient/ year	Mean	3826	NA	COPD	2006-2009	Germany
Menzin, J; et al. 2008	Direct costs	Patient/ year	Mean	34101	60664	COPD	2004	USA
Miravitlles, M; et al. 2003	Direct costs	Patient/ year	Mean	2374	4908	COPD	2003	Spain
Nielsen, R; et al. 2009	Direct costs	Patient/ year	Mean	431	NA	COPD	2003-2004	Norway
Nielsen, R; et al.2009	Direct costs	Patient/ year	Mean	725	NA	COPD	2003-2004	Iceland
Nielsen, R; et al. 2011	Direct costs	Patient/ year	Mean	13583	NA	COPD	2005-2006	Norway
Nishimura, S; Zaher, C; 2004	Direct costs	Patient/ year	Mean	1311	NA	COPD	1990-2002	Japan
Ray, GT; et al. 2000	Direct attributable costs	Patient/ year	Mean	10176	NA	COPD	1995-1996	USA

Table 2a. Results of the Included Studies Investigating Annual Direct Costs of NCDs (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	Reported NCDs	Year	Country
Ray, GT; et al. 2000	Direct costs	Patient/ year	Mean	25644	NA	COPD	1995-1996	USA
Rutten-Van Molken, MPMH; et al. 1999	Direct costs	Patient/ year	Mean	1413	NA	COPD	1993	Netherlands
Schneider, KM; et al. 2009	Direct attributable costs	Patient/ year	Mean	22183	NA	COPD	2001-2005	USA
Schneider, KM; et al. 2009	Direct costs	Patient/ year	Mean	25547	NA	COPD	2001-2005	USA
Sharafk-haneh, A; et al. 2010	Direct costs	Patient/ year	Mean	6083	NA	COPD	1997-2004	USA
Simoni-Wastila, L; et al. 2009	Direct costs	Patient/ year	Mean	8819	16099	COPD	2003-2005	USA
Van Boven, JFM; et al. 2013	Direct costs	Patient/ year	Mean	1419	NA	COPD	2009	Netherlands
Yoon, J; et al. 2011	Direct costs	Patient/ year	Mean	6616	NA	COPD	2000	USA
Yoon, J; et al. 2011	Direct costs	Patient/ year	Mean	6112	NA	COPD	2008	USA
Schneider, KM; et al. 2009	Direct attrbitutable costs	Patient/ year	Mean	22183	NA	COPD	2005	USA
Schneider, KM; et al. 2009	Direct costs	Patient/ year	Mean	25548	NA	COPD	2005	USA
Baumeister, SE; et al. 2009	Direct costs	Patient/ year	Mean	5439	13350	CKD	2004-2005	Germany
Laliberte, F; et al. 2009	Direct costs	Patient/ year	Mean	18314	52839	CKD	2000-2006	USA
Lamping, DL; et al. 2000	Direct costs	Patient/ year	Mean	71824	NA	CKD	1995-1996	UK
Lee, H; et al. 2002	Direct costs	Patient/ year	Mean	58871	27814	CKD	1999-2000	Canada
Schneider, KM; et al. 2009	Direct attributable costs	Patient/ year	Mean	28462	NA	CKD	2001-2005	USA
Schneider, KM; et al. 2009	Direct costs	Patient/ year	Mean	31826	NA	CKD	2001-2005	USA

Table 2a. Results of the Included Studies Investigating Annual Direct Costs of NCDs (continued)

Study	Type of Outcome	Outcome Specified as	Assess-ment Type	Point Estimate	SD for Mean	Reported NCDs	Year	Country
Yoon, J; et al. 2011	Direct costs	Patient/ year	Mean	23785	NA	CKD	2000	USA
Ray, GT; et al. 2000	Direct attributable costs	Patient/ year	Mean	33585	NA	CKD	1996	USA
Ray, GT; et al. 2000	Direct costs	Patient/ year	Mean	49286	NA	CKD	1996	USA
Yoon, J; et al. 2011	Direct costs	Patient/ year	Mean	17681	NA	CKD	2008	USA
Schneider, KM; et al. 2009	Direct costs	Patient/ year	Mean	31827	NA	CKD	2005	USA
Schneider, KM; et al. 2009	Direct attributable costs	Patient/ year	Mean	28462	NA	CKD	2005	USA
Ballesta, M; et al. 2006	Direct costs	Patient/ year	Mean	3714	NA	DM	1999	Spain
Biorac, N; et al. 2009	Direct costs	Patient/ year	Mean	901	NA	DM	2007	Serbia
Chodick, G; et al. 2005	Direct costs	Patient/ year	Mean	2653	NA	DM	1999	Israel
Chodick, G; et al.2005	Direct costs	Patient/ year	Mean	3010	NA	DM	2000	Israel
Chodick, G; et al. 2005	Direct costs	Patient/ year	Mean	3422	NA	DM	2001	Israel
Costantino ME; et al. 2014	Direct costs	Patient/ year	Mean	9731	NA	DM	2009	USA
Costantino ME; et al. 2014	Direct attributable costs	Patient/ year	Mean	3390	NA	DM	2009	USA
Degli Esposti, L; et al. 2013	Direct costs	Patient/ year	Mean	2291	5164	DM	2009	Italy
Elrayah-Eliadarous, H; et al. 2010	Direct costs	Patient/ year	Mean	215	166	DM	2005	Sudan
Jonsson, B 2002	Direct costs	Patient/ year	Mean	4092	NA	DM	1999	Europe
Kangas, T; et al. 1996	Direct costs	Patient/ year	Mean	7408	NA	DM	1987-1989	Finland
Kirigia, JM; et al. 2009	Direct costs	Patient/ year	Mean	1377	NA	DM	2000	Africa
Kumar, A; et al. 2008	Direct Cost	Patient/ year	Mean	162	NA	DM	2005-2005	India

Table 2a. Results of the Included Studies Investigating Annual Direct Costs of NCDs (continued)

Study	Type of Outcome	Outcome Specified as	Assess-ment Type	Point Estimate	SD for Mean	Reported NCDs	Year	Country
Le, C; et al. 2013	Direct costs	Patient/ year	Mean	952	NA	DM	2010-2011	China
Martin, S; et al. 2007	Direct costs	Patient/ year	Mean	2818	3276	DM	1995-2003	Germany
Morsanutto, A; et al. 2006	Direct costs	Patient/ year	Mean	2452	NA	DM	2001-2002	Italy
Norlund, A; et al. 2001	Direct costs	Patient/ year	Mean	7570	NA	DM	1992-1993	Sweden
Nakamura, K; 2008	Direct costs	Patient/ year	Mean	4416	NA	DM	1990-2001	Japan
Oglesby, AK; et al. 2006	Direct attributable costs	Patient/ year	Mean	2077	NA	DM	1989-2003	USA
Oliva, J; et al. 2004	Direct costs	Patient/ year	Mean	1775	NA	DM	2002	Spain
Petersen, M; Amer Diabet, A 2008	Direct costs	Capita/year	Mean	13196	NA	DM	2007	USA
Petersen, M; Amer Diabet, A 2008	Direct attributable costs	Capita/year	Mean	7471	NA	DM	2007	USA
Ray, NF; et al. 1998	Direct costs	Capita/year	Mean	14617	NA	DM	1997	USA
Ray, NF; et al. 1998	Direct attributable costs	Capita/year	Mean	10743	NA	DM	1997	USA
Ringborg, A; et al. 2008	Direct costs	Patient/ year	Mean	4047	9525	DM	2000-2004	Sweden
Tunceli, O; et al. 2010	Direct attributable costs	Patient/ year	Mean	3373	13198	DM	2006-2009	USA
Tunceli, O; et al. 2010	Direct costs	Patient/ year	Mean	4329	13198	DM	2006-2009	USA
Ray, GT; et al. 2000	Direct attributable costs	Patient/ year	Mean	2444	NA	DM	1996	USA
Ray, GT; et al. 2000	Direct Costs	Patient/ year	Mean	9462	NA	DM	1996	USA
Schneider, KM; et al. 2009	Direct attributable costs	Patient/ year	Mean	12246	NA	DM	2005	USA
Schneider, KM; et al. 2009	Direct costs	Patient/ year	Mean	15611	NA	DM	2005	USA

SD:Standard deviation; NCD: Non-communicable diseases; CVD: Cardiovascular disease; CAD: Coronary Artery Disease ; IHD: Ischemic Heart Disease ; COPD: Chronic Obstructive PulmonaryDisease; CKD: Chronic Kidney Disease ; DM: Diabetes Mellitus

Table 2b. Results of the Included Studies Investigating Annual Indirect Costs of NCDs

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	Reported NCDs	Year	Country
Sasser, AC; et al. 2005	Indirect costs	Patient/year	Mean	6752	NA	CVD	2000	USA
Hu, S; et al. 2013	Indirect costs	Patient/year	Mean	2644	NA	Stroke	2011	China
Lopez-Batista, J; et al. 2012	Indirect costs	Patient/year	Mean	23418	19558	Stroke	2004	Spain
Winter, Y; et al. 2008	Indirect costs	Patient/year	Mean	4018	4634	Stroke	2003	Germany
Jaworski, R; et al. 2012	Indirect costs	Patient/year	Mean	1780	NA	CAD	2005	Poland
Broekx, S; et al. 2011	Indirect costs	Patient/year	Mean	23692	NA	Breast cancer	2004	Belgium
Sasser, AC; et al. 2005	Indirect costs	Patient/year	Mean	11145	NA	Breast cancer	2000	USA
Sorensen, SV; et al. 2012	Indirect costs	Patient/year	Mean	24740	NA	Breast cancer	2007	USA
Wan, Y; et al. 2013	Indirect costs	Patient/year	Mean	2109	NA	Breast cancer	2009	USA
Darkow, T; et al. 2007	Indirect costs	Patient/year	Mean	3393	NA	COPD	2001-2004	USA
Fletcher, MJ; et al. 2011	Indirect costs	Patient/year	Mean	910	NA	COPD	2009	Cross-country
Jansson, SA; et al. 2002	Indirect costs	Patient/year	Mean	970	NA	COPD	1998-1999	UK
Lokke, A; et al. 2014	Indirect costs	Patient/year	Mean	3264	NA	COPD	1998-2010	Denmark
Nishimura, S; Zaher, C; 2004	Indirect costs	Patient/year	Mean	326	NA	COPD	1990-2002	Japan
Ballesta, M; et al. 2006	Indirect costs	Patient/year	Mean	2675	NA	DM	1999	Spain
Biorac, N; et al. 2009	Indirect costs	Patient/year	Mean	104	NA	DM	2007	Serbia
Le, C; et al. 2013	Indirect costs	Patient/year	Mean	7797	NA	DM	2010-2011	China
Kirigia, JM; et al. 2009	Indirect costs	Patient/year	Mean	2958	NA	DM	2000	Africa
Norlund, A; et al. 2001	Indirect costs	Patient/year	Mean	5350	NA	DM	1992-1993	Sweden

SD: Standard deviation; NCD: Non-communicable diseases; CVD: Cardiovascular disease; CAD: Coronary Artery Disease; COPD: Chronic Obstructive Pulmonary Disease; DM: Diabetes Mellitus

(iii) Aggregate costs

Mean annual total costs of NCDs per patient were reported in 17 articles (**Table 2c**). Cancer and stroke led the total costs with average estimates up to 105310 USD[30] and 44937 USD[31] respectively. The mean total cost per patient for breast cancer was estimated at 30000 USD in Belgium[36] and the USA[37], although the mean costs for metastatic breast cancer were three times higher (105310 USD[30]). For lung cancer, mean estimates varied from 4964 USD[16] in Australia to 50495 USD in USA[38] whereas colorectal cancer total costs were 52068 USD[38]. Mean COPD total costs were estimated around 1700 USD in the UK and Japan[33] but exceeded the value of 15500 USD in Denmark[39]. DM total costs were estimated at an average of 12920 USD in Sweden[40] whereas estimated mean total costs in Serbia were 1005 USD[41]. No study reported total costs for cervical cancer or CKD.

Table 2c. Results of the Included Studies Investigating Annual Total Costs of NCDs

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	Reported NCDs	Year	Country
Sasser, AC; et al. 2005	Total costs	Patient/year	Mean	23065	NA	CVD	2000	USA
Hu, S; et al. 2013	Total costs	Patient/year	Mean	7422	NA	Stroke	2011	China
Lopez-Batista, J; et al.2012	Total costs	Patient/year	Mean	44937	18535	Stroke	2004	Spain
Winter, Y; et al. 2008	Total costs	Patient/year	Mean	11396	NA	Stroke	2003	Germany
Jaworski, R; et al. 2012	Total costs	Patient/year	Mean	3423	NA	CHD	2005	Poland
Sorensen, SV; et al. 2012	Total costs	Patient/year	Mean	105310	NA	Metastatic Breast cancer	2007	USA
Broekx, S; et al. 2011	Total attributable costs	Patient/year	Mean	26680	NA	Breast cancer	2004	Belgium
Sasser, AC; et al. 2005	Total costs	Patient/year	Mean	29988	NA	Breast cancer	2000	USA
Warren, JL; et al. 2008	Total costs	Patient/year	Mean	26492	NA	Breast cancer	1991-2002	USA
Kang, S; et al. 2012	Total costs	Patient/year	Mean	4964	NA	Lung cancer	2008	Australia
Warren, JL; et al. 2008	Total costs	Patient/year	Mean	50495	NA	Lung cancer	1991-2002	USA
Warren, JL; et al. 2008	Total costs	Patient/year	Mean	52068	NA	Colorectal cancer	1991-2002	USA

Table 2c. Results of the Included Studies Investigating Annual Total Costs of NCDs (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	Reported NCDs	Year	Country
Jansson, SA; et al. 2002	Total costs	Patient/year	Mean	1662	NA	COPD	1998-1999	UK
Lokke, A; et al. 2014	Total Costs	Patient/year	Mean	15585	NA	COPD	1998-2010	Denmark
Nishimura, S; Zaher, C; 2004	Total costs	Patient/year	Mean	1637	NA	COPD	1990-2002	Japan
Ballesta, M; et al. 2006	Total costs	Patient/year	Mean	6206	NA	DM	1999	Spain
Biorac, N; et al. 2009	Total costs	Patient/year	Mean	1005	NA	DM	2007	Serbia
Le, C; et al. 2013	Total costs	Patient/year	Mean	8749	NA	DM	2010-2011	China
Kirigia, JM; et al. 2009	Total costs	Patient/year	Mean	4336	NA	DM	2000	Africa
Norlund, A; et al. 2001	Total costs	Patient/year	Mean	12920	NA	DM	1992-1993	Sweden

SD: Standard deviation; NCD: Non-communicable diseases; CVD: Cardiovascular disease; IHD: Ischemic Heart Disease, AMI: Acute Myocardial Infarction; COPD: Chronic Obstructive Pulmonary Disease; DM : Diabetes Mellitus

(iv) Costs of NCDs over time

There was an increase in healthcare costs associated with NCDs over time. One study showed that, despite a 19% decline in the hospitalization rate for CHD (acute myocardial infarction) in USA, overall healthcare expenditure per patient increased by 17% from 1998 to 2008 (absolute difference of 6595 USD) and use of outpatient services increased by 65% (absolute difference, 1000 USD)[42] and similarly for heart failure[43]. The average treatment cost of colorectal cancer patients in USA increased by 73% from 2005 to 2009[44], mainly driven by the use of new regimens, higher chances of surgery, and radiation. In USA, COPD-related healthcare costs increased by 5-6% annually[45]. Further, a 29% increase in medical treatment costs for diabetic patients was observed from 1999 to 2001 in Israel (absolute difference, 771 USD)[46].

(v) Costs According to Disease Severity and Comorbidity

Overall healthcare costs secondary to NCDs increased with the severity of the disease, years lived with the condition and co-morbidity[9,36,47-57]. Patients with severe stroke had almost a 40% greater increase in costs compared to mild stroke patients[47]. Among cancer patients, given the same stage of diagnosis, those with one, two or three co-morbidities experienced increased costs of 3737

USD, 4188 USD and 10442 USD respectively[58]. Costs for a diabetic patient tripled between the first and seventh year[59] after diagnosis. An increase in treatment costs of breast cancer by stage was reported, with approximately 52% higher treatment costs for stage II as compared to stage 0[57]. Similarly, a 29859 USD increase was seen with cancer progression from stage I to stage IV[60]. Patients with co-existence of COPD and CVD had 135% higher annual care costs compared with patients without CVD, whereas COPD related total costs were 38% higher[56]. Some studies reported lifetime healthcare costs of NCDs (initial, continuing and terminal care), demonstrating that initial and terminal care are the most costly.[13,15,61-63].

Global healthcare expenditure on the NCDs

Among the reviewed studies, 30 reported healthcare expenditure attributable to specific NCDs (**Table 3a**). CVD accounted for 12% of all healthcare expenditure in the European Union (EU)[64]. CHD healthcare-related costs accounted for 14.2-16.5% of the annual healthcare budget in the American region. In contrast, in the EU, mean healthcare expenditure on CHD was 2.6%; within the EU, Malta has the lowest share (0.6%) and Slovakia the highest (5.9%)[64]. CKD and cancer accounted for 3.2% and 3.4%, of healthcare expenditure respectively[25,65]. In the USA, 1.2% of the healthcare budget was spent exclusively on the treatment of breast cancer[25]. The proportion of national healthcare-related expenditure for COPD ranged from 0.7% in Norway[19], 1-3% in the Netherlands[66] and up to 3.8% in Canada[65]. Again in Canada, 3.8% of healthcare expenditure is attributable to DM[65] whereas in the European Union, DM-related healthcare expenditure was an estimated 7.4%[67] (the Netherlands having the lowest share (1.6%), and Spain the highest)[68].

Table 3a. Healthcare Expenditure and Total Direct Costs Associated with NCDs

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	Reported NCDs	Year	Country
Hodgson, TA; Cohen, AJ 1999	Direct costs	Per year	Mean	195.4 million	CVD	1995	USA
Nichols, GA; et al. 2010	Direct costs	Per year	Mean	400 billion	CVD	2000-2008	USA
Leal, J; et al. 2006	Direct costs	Per year	EUR	151.3 billion	CVD	2003	Europe (cross-country)
*Leigh, JP; et al. 2003	Direct costs	Per year	Mean	15.53 billion	CVD, Cancer, COPD	1999	USA
Odden, MC; et al. 2011	Direct costs	Per year	Mean	130.6 billion	CVD, CHD	2010	USA
Hodgson, TA; Cohen, AJ 1999	Direct costs	Per year	Mean	59.1 million	CHD	1995	USA
Leal, J; et al. 2006	Direct costs	Per year	EUR	32.9 billion	CHD	2003	Europe (cross-country)

In absolute terms, annual CVD hospital costs in the USA reached an estimated 400 billion USD in 2008, doubling the 195 billion USD in 1995[69,70]. In USA, CHD-related hospital costs were estimated at 59.1 million USD in 1995 whereas the CVD-related hospital costs were 130 USD billion in 2010[71]. In the EU, CVD-related hospital costs were estimated at 151 billion USD in 2003, with CHD accounting for 32.9 billion USD[64]. In Australia, annual hospital costs due to CVD were estimated at 164 million USD in 1997[72]. In France and Hungary the annual estimated colorectal cancer health related costs were 565 million USD and 43 million USD respectively[73,74]. In Iran, the minimum annual healthcare-related cost for colorectal cancer was estimated at 39 million USD for the period between 2005 and 2010[75]. For cervical cancer, the estimated costs were 1.83 million USD in Singapore[18], 18.2 million USD in Spain[76], and 12.98 million USD in Malaysia[77]. The estimated total healthcare costs in the USA for lung, colorectal, cervical and breast cancer combined were 5.2 billion[78]. In USA, health-related costs for COPD and CKD in 2005 were an estimated 9.2 billion USD[24]. COPD costs accounted for 232 million USD in Iceland[19] whereas both COPD and DM accounted for 162 million USD in Australia[79]. DM hospital-related costs varied from 9.7 billion USA in the African Region[80], to 41.1 billion USD[68] in Europe and to 160 billion USD in USA[81] .

A data series of hospital expenditure on CVD was only available in the USA. This showed a two fold increase in healthcare share from 1995 to 2008, with estimated health costs of 195 billion USD in 1995 to 400 billion USD in 2008[69,70]. An increase in healthcare expenditure was also seen for colorectal cancer in Brazil, from 18.54 million USD in 1996 to 37.64 million USD in 2008[82]. Yoon, J.J et al showed a sharp increase in healthcare spending on most chronic diseases from 2000 to 2008, with CKD having the highest increase by more than 1.62 billion USD[83].

Table 3a. Healthcare Expenditure and Total Direct Costs Associated with NCDs (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	Reported NCDs	Year	Country
Caro, JJ; et al. 2006	Hospitalization costs	Per year	Mean	20.5 million	Stroke	1990-1995	France
Dewey, HM; et al. 2003	Hospitalization costs	Per year	Mean	164 million	Stroke	1997	Australia
Kolominsky-Rabas, PL; et al. 2006	Direct costs	Per year	Mean	10.6 billion	Stroke	2004	Germany
*Simpson, A.N; et al. 2013	Direct costs	Per year	Mean	133.41 million	CVD, Stroke	2004	USA
Beaulieu, N; et al. 2009	Medication costs	Per year	Mean	31.8 billion	Lung cancer	2009	Worldwide
Beaulieu, N; et al. 2009	Nonmedical costs	Per year	Mean	12.0 billion	Lung cancer	2009	Worldwide
Beaulieu, N; et al. 2009	Medication costs	Per year	Mean	20.2 billion	Colorectal cancer	2009	Worldwide

Table 3a. Healthcare Expenditure and Total Direct Costs Associated with NCDs (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	Reported NCDs	Year	Country
Beaulieu, N; et al. 2009	Nonmedical costs	Per year	Mean	7.9 billion	Colorectal cancer	2009	Worldwide
Selke, B; et al. 2003	Direct costs	Per year	Mean	564.59 million	Colorectal Cancer	1999	France
Boncz, I; et al. 2010	Direct costs	Per year	Mean	43.4 million	Colorectal cancer	2001	Hungary
Davari, M; et al. 2012	Direct costs	Per period of surveillance	Minimum	39.3 million	Colorectal cancer	2005 −2010	Iran
Torres, US; et al. 2010	Direct costs	Per year	Mean	18.54 million	Colorectal cancer	1996	Brazil
Torres, US; et al. 2010	Direct costs	Per year	Mean	37.64 million	Colorectal cancer	2008	Brazil
Beaulieu, N; et al. 2009	Medication costs	Per year	Mean	14.2 billion	Breast cancer	2009	Worldwide
Beaulieu, N; et al. 2009	Nonmedical costs	Per year	Mean	8.3 billion	Breast cancer	2009	Worldwide
Boncz, I; et al. 2010	Direct costs	Per year	Mean	37.4 million	Breast cancer	2001	Hungary
Beaulieu, N; et al. 2009	Medication costs	Per year	Mean	865.4 million	Cervical cancer	2009	Worldwide
Beaulieu, N; et al. 2009	Nonmedical costs	Per year	Mean	703.6 million	Cervical cancer	2009	Worldwide
Boncz, I; et al. 2010	Direct costs	Per year	Mean	4.5 million	Cervical cancer	2001	Hungary
Aljunid, S; et al. 2010	Direct costs	Per year	Mean	12.98 million	Cervical cancer	2007-2008	Malysia
Gil, A; et al. 2007	Hospitalization costs	Per year	Mean	18.19 million	Cervical cancer	1999-2002	Spain
Low, JJ; et al. 2012	Direct cost	Per year	Mean	45.7 million	Cervical cancer	2008-2033	Singapore
Yabroff, KR; et al. 2008	Aggregated costs	Per 5 years	Mean	26 billion	Lung, colorectal, cervical & breast cancer	1999-2003	USA
Yabroff, KR; et al. 2008	Aggregated costs	Per year	Mean	5,2 billion	Lung, colorectal, cervical & breast cancer	1999-2003	USA
Nielsen, R; et al. 2009	Direct costs	Per year	Mean	232.3 million	COPD	2003-2004	Iceland

Table 3a. Healthcare Expenditure and Total Direct Costs Associated with NCDs (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	Reported NCDs	Year	Country
Schneider, KM; et al. 2009	Direct costs	Per year	Mean	9.2 billion	COPD, CKD	2001-2005	USA
Chittleborough, CR; et al. 2009	Direct costs	Per year	Mean	162 million	COPD, DM	2001-2002	Australia
Chodick, G; et al. 2005	Direct costs	Per year	Mean	1159.2 million	DM	2001	Israel
Dewey, HM; et al. 2001	Direct costs	Per year	Mean	82.12 million	CVD, Stroke	1997	Australia
Hogan, P; et al. 2003	Direct attributable costs	Per year	Mean	119 billion	DM	2002	USA
Hogan, P; et al. 2003	Direct costs	Per year	Mean	160 billion	DM	2002	USA
Jonsson, B 2002	Direct costs	Per year	Mean	41.1 billion	DM	1999	Europe
Kirigia, JM; et al. 2009	Direct costs	Per year	Mean	9.7 billion	DM	2000	Africa
Oliva, J; et al. 2004	Direct costs	Per year	Mean	3.25 billion	DM	2002	Spain
Oliva, J; et al. 2004	Hospitalization costs	Per year	Mean	1096 million	DM	1999	Spain
Oliva, J; et al. 2004	Hospitalization costs	Per year	Mean	1198 million	DM	2002	Spain
Petersen, M; Amer Diabet, A 2008	Direct costs	Per year	Mean	230.5 million	DM	2007	USA
Petersen, M; Amer Diabet, A 2008	Direct attributable costs	Per year	Mean	130.3 billion	DM	2007	USA
Ray, NF; et al1998	Direct costs	Per year	Mean	112.8 billion	DM	1989-1995	USA
Ray, NF; et al. 1998	Direct attributable costs	Per year	Mean	64 billion	DM	1997	USA

* Studies limited in a certain number of population, not for the entire country.

SD: standard deviation; NCD: Non-communicable diseases; CVD: Cardiovascular disease; CHD: Coronary Heart Disease; COPD: Chronic Obstructive Pulmonary Disease; CKD: Chronic Kidney Disease; DM: Diabetes Mellitu

Table 3b. Annual Income Losses (indirect costs) Associated with NCDs

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	Reported NCDs	Year	Country
Hogan, P; et al. 2003	Indirect attributable costs	Per year	Mean	52 billion	DM	2002	USA
Kirigia, JM; et al. 2009	Indirect costs	Per year	Mean	20.8 billion	DM	2000	Africa
Leal, J; et al. 2006	Indirect costs	Per year	Mean	92921 million	CVD	2003	Europe (cross-country)
Dewey, HM; et al. 2001	Indirect costs	Per year	Mean	51.71 million	CVD, Stroke	1997	Australia
Leal, J; et al. 2006	Indirect costs	Per year	Mean	31835 million	CHD	2003	Europe (cross-country)
Selke, B; et al. 2003	Indirect costs	Per year	Mean	679.12 million	Colorectal Cancer	1999	France
Petersen, M; Amer Diabet, A 2008	Indirect attributable costs	Per year	Mean	65.2 billion	DM	2007	USA
Ray, NF; et al. 1998	Indirect attributable costs	Per year	Mean	78.3 billion	DM	1997	USA
Klever-Deichert, G; et al. 1999	Indirect costs	Per year	Mean	71 billion	CVD	1996	Germany
Nichols, GA; et al. 2010	Indirect costs	Per year	Mean	600 billion	CVD	2000-2008	USA
Zheng, H; et al. 2010	Indirect costs	Per year	Mean	2.21 billion	CHD	2004	Australia
Beaulieu, N; et al. 2009	Productivity loss	Per year	Mean	13.7 billion	Lung cancer	2009	Worldwide
Beaulieu, N; et al. 2009	Productivity loss	Per year	Mean	8.2 billion	Colorectal cancer	2009	Worldwide
Beaulieu, N; et al. 2009	Productivity loss	Per year	Mean	1.7 billion	Cervical cancer	2009	Worldwide
Beaulieu, N; et al. 2009	Productivity loss	Per year	Mean	8.4 billion	Breast cancer	2009	Worldwide
Aljunid, S; et al. 2010	Indirect costs	Per year	Mean	4.11 million	Cervical cancer	2007-2008	Malaysia
Van Boven, JFM; et al. 2013	Indirect costs	Per year	Mean	388.6 million	COPD	2009	Netherlands

NCD: Non-communicable diseases; CVD: Cardiovascular disease; CHD: Coronary Heart Disease; COPD: Chronic Obstructive Pulmonary Disease

Impact of the NCDs on national income

In general, NCDs have a large impact on national income mainly due to loss of productivity as a result of absenteeism and inability to work in 13 studies (**Table 3b**). There was a 463 billion USD increase in economic loss in USA due to CVD for the period 1993-2008[69]. In the EU, estimated economic loss in 2003 was 92.9 billion USD for CVD, of which CHD accounted for 31.84 billion USD. Estimated loss in national income from CHD-related productivity loss in 1996 was 71 billion USD in Germany[84]. Economic loss from stroke in 1997 was 51.7 million USD in Australia[85] whereas CHD-related productivity loss was 2.2 billion USD in 2004[86]. Worldwide, economic loss from colorectal, lung, breast and cervical cancer at 2009 year were 13.7, 8.2, 1.7 and 8.4 billion USD respectively [87]. In Malaysia, estimated income losses from cervical cancer-related productivity loss were 4.1 million USD. In the Netherlands, estimated losses in national income from COPD were 388 million USD[77,88]. National income losses from DM were estimated at 20.8 billion in the African region in 2000 and at 65.2 billion in 2007 in USA[80,89].

Table 3c. Healthcare Expenditure and National Income Losses (direct and indirect costs) Associated with NCDs

Study	Type of Outcome	Outcome Specified as	Assess-ment Type	Point Estimate	Reported NCDs	Year	Country
Klever-Deichert, G; et al. 1999	Direct + indirect costs	Lifetime	Mean	108.9 billion	CVD	1999	Germany
Nichols, GA; et al. 2010	Direct + indirect costs	Per year	Mean	1 trillion	CVD	2000-2008	USA
Dewey, HM; et al. 2003	Direct + indirect costs	Lifetime	Mean	1.3 billion	Stroke	1997	Australia
Dewey, HM; et al. 2001	Direct + indirect costs	Per year	Mean	868.8 million	CVD, Stroke	1997	Australia
Mittman, N; et al. 2012	Direct + indirect costs	Per year	Mean	3.47 billion	CVD, Stroke	2005-2009	Canada
Taylor, TN; et al. 1996	Direct + indirect costs	Lifetime	Mean	72.37 billion	CVD, Stroke	1990-1993	USA
Leal, J; et al. 2006	Direct + indirect costs	Per year	Mean	244249 million	CVD	2003	Europe (cross-country)
Leal, J; et al. 2006	Direct + indirect costs	Per year	Mean	64.7 billion	CHD	2003	Europe (cross-country)
Beaulieu, N; et al. 2009	Direct + indirect costs	Per year	Mean	57.4 billion	Lung cancer	2009	Worldwide
Warren, JL; et al. 2008	Direct + indirect costs	Per year	Mean	2.5 billion	Lung cancer	1991-2002	USA

Table 3c. Healthcare Expenditure and National Income Losses (direct and indirect costs) Associated with NCDs

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	Reported NCDs	Year	Country
Beaulieu, N; et al. 2009	Direct + indirect costs	Per year	Mean	36.3 billion	Colorectal cancer	2009	Worldwide
Selke, B; et al. 2003	Direct + indirect costs	Per year	Mean	1.24 billion	Colorectal Cancer	1999	France
Warren, JL; et al. 2008	Direct + indirect costs	Per year	Mean	2.5 billion	Colorectal cancer	1991-2002	USA
Beaulieu, N; et al. 2009	Direct + indirect costs	Per year	Mean	3.2 billion	Cervical cancer	2009	Worldwide
Aljunid, S; et al. 2010	Direct + indirect costs	Per year	Mean	17.08 million	Cervical cancer	2007-2008	Malaysia
Beaulieu, N; et al. 2009	Direct + indirect costs	Per year	Mean	30.9 billion	Breast cancer	2009	Worldwide
Jansson, SA; et al. 2002	Direct + indirect costs	Per year	Mean	1.1 billion	COPD	1998-1999	Sweden
Nishimura, S; Zaher, C; 2004	Direct + indirect costs	Per year	Mean	9.1 billion	COPD	1990-2002	Japan
Van Boven, JFM; et al. 2013	Direct + indirect costs	Per year	Mean	133.7 million	COPD	2009	Netherlands
Hogan, P; et al. 2003	Total attributable costs (Direct + indirect)	Per year	Mean	171 billion	DM	2002	USA
Kirigia, JM; et al. 2009	Direct + indirect costs	Per year	Mean	30.4 billion	DM	2000	Africa
Le, C; et al. 2013	Direct + indirect costs	Per year	Mean	46.7 million	DM	2010-2011	China
Petersen, M; Amer Diabet, A 2008	Direct + indirect costs	Per year	Mean	195.5 billion	DM	2007	USA
Ray, NF; et al. 1998	Direct + indirect costs	Per year	Mean	142.5 billion	DM	1997	USA

SD: standard deviation; NCD: Non-communicable diseases; CVD: Cardiovascular disease; COPD: Chronic Obstructive Pulmonary Disease, DM: Diabetes Mellitus

Combined impact of NCDs on national income and healthcare expenditure

19 studies reported the impact of NCDs on both healthcare expenditure and national income (**Table 3c**). The total estimated cost of CVD in Germany in 1999 was 108.9 billion USD whereas for the entire EU, the estimate was 244.3 billion USD for the year 2003, with CHD accounting for 26% of this cost[64,84]. Stroke costs were up to 1.3 billion USD in Australia, 3.47 billion USD in Canada and 72.4 billion USD in USA[72,90,91]. Worldwide, colorectal, lung, breast and cervical cancer made

up 41% (127.8 billion USD) of the 310.15 billion USD aggregate cost of new cancer cases in 2009, with lung cancer posing the highest economic burden (57.4 billion USD) [87]. In the USA, colorectal cancer and lung cancer total costs were 2.5 billion USD[38] each whereas in France, the total colorectal cancer costs were estimated at 1.24 billion USD[73]. In Malaysia, the total estimated cervical cancer costs were 17.1 million[77]. Total COPD costs varied from 133.7 million in the Netherlands to 1.1 billion USD in Sweden and 9.1 billion USD in Japan[33,49,88]. Total estimated costs of DM increased from 142.5 billion USD in 1997, to 171 billion USD in 2002 and to 195.5 billion USD in 2007[81,89,92]. DM imposed 30.4 billion USD in costs on the African region and 46.7 billion USD in costs in China[27,80].

DISCUSSION

This systematic review summarizes 153 studies published worldwide that investigate the impact of six major NCDs (CHD, stroke, COPD, major cancers, type 2 diabetes and CKD) at the macro-economic level (i.e. health-related costs, healthcare budgets and national income). The studies suggest a steady global increase in healthcare expenditure on NCDs over the years. Additionally, NCDs undermine national economic development, with estimated losses in national income in excess of 600 billion USD[69].

In most countries, the highest expenditure was attributable to CVD. Between 12% and 16.5% of the overall healthcare budget is spent on this one condition alone; the proportion spent on the other NCDs ranges from 0.7% to 7.4%. In the USA and Brazil hospital expenditure on major NCDs doubled in a decade to an estimated 200 billion USD. An increasing share of healthcare expenditure on major NCDs has been reported previously and especially so in Germany where an increase from 27-51% of total health expenditure was reported[93]. Similarly, in the USA, CHD-related healthcare costs were five times higher in 2008 than in 1996. Yet, little is known about what drives current and future NCDs-related healthcare costs. Interesting insights come from Australia; these show that the introduction of new technologies and changes in treatment practices (volume of treatment services) are more likely to drive healthcare costs as compared to ageing or other factors[94]. In the overall projected increase in health expenditure in Australia up to 2032, volume of treatment services had the largest contribution (AUD 81.3 billion), followed by population ageing (AUD 37.8 billion) and population growth (AUD 34.4 billion). Also, Aaron, H et al. in a summary of the current evidence reported that most of the anticipated increase in total health care spending in USA is attributed to growth of age-specific health care spending and some will be caused by population ageing [95]. However, although health care spending at a time point in time may be influenced by ageing and in particular by the remaining life expectancy (and increases in longevity), there is limited data available projecting how the health care spending curve will evolve as life expectancy increases [95]. More research is warranted in this respect.

Further, NCDs have a large impact on national income, with estimated losses ranging from 4.1 million USD due to cervical cancer in Malaysia, to 71 billion USD in Germany and 600 billion USD in the USA due to CHD. Large-scale productivity losses mainly due to absenteeism and an

inability to work, caused by the debilitating physical and mental impact of NCDs, have a direct detrimental impact on national income. A 2005 WHO report indicated that estimated losses in national income due to CVD, stroke and diabetes were 3 billion USD in Brazil, 9 billion USD in India, 11 billion USD in Russia and 18 billion USD in China[96]. Bradley et al. demonstrated that, with the same level of colorectal cancer risk factors in USA, estimated economic losses due to colorectal cancer would raise from 24.2 billion USD in 2011 to 339 billion USD in 2020[97]. A macro-economic simulation presented at the World Economic Forum in 2011 showed that over the next two decades, NCDs would lead to a staggering 47 USD trillion cumulative output losses globally, representing 75% of global GDP in 2010[98].

While the current study is the most detailed systematic review on the topic, it is limited by the fact that the evidence on economic burden of NCDs in LMIC is generally scarce. Most of the evidence in this review originates from high-income countries. Limited research capacity, inadequate financial investment, healthcare system development, a lack of electronic health records, and language restrictions may contribute to this shortage[99]. Using a systematic search in Pubmed and Embase, 14 review articles[100] [101-113] (including four systematic reviews[100-102,107]) evaluating the economic impact of different NCDs on healthcare expenditure were found. The majority of these reviews were not performed systematically and previous systematic reviews [100-102,107] have been published on the costs of specific NCDs. Valtorta and colleagues [101] investigated the financial consequences of cancers, stroke, and heart failure but did not include diabetes, CVD, COPD, or CKD. Yabroff and colleagues [100] used only MEDLINE, were focused on recent articles in English language, and tackled only colorectal cancer.

Findings of this systematic review generally concur with and further extend the previous reviews. This systematic review evaluates economic consequences of six major NCDs using a global perspective in a single comprehensive investigation. Two reviewers, working in tandem, screened and selected the studies, while references of the included studies were additionally screened for any missing evidence. This approach ensured that we included most of the relevant articles in our review. Similar to previous reviews, however, we found substantial methodological limitations. Age range or stage of disease at diagnosis or other patients characteristics that may influence care and costs, were frequently not reported. Furthermore, many studies did not clearly state the method used to estimate costs and among the others, different approaches were used to calculate the same type of costs; e.g. direct attributable costs to diabetes were calculated by 1) including the direct costs of the events undergoing investigation; 2) comparing the cost of diabetes patients to those with no diabetes history; or 3) comparing previous resource use to resources use after the event. It may be argued that the studies using the first approach may not include all the costs associated with the disease. For example diabetes patients are more likely to have fractures than those without diabetes [114]. Moreover, several methodological concerns of the studies reviewed were observed, related to sample selection and representativeness, case definition, the nature of costs included (e.g. all-cause or event-related) and the analysis costs of data over time. Also, there are differences among countries with regard to health care/welfare system which may partly explain the large variation of health care spending across countries and world regions. In many countries, private spending

accounts for three quarters of national health expenditure whereas in some others, there is a large burden of health care into the public purse [115,116]. Although, it would be of interest to compare health care spending and the impact of NCDs on national income based on the organization of health care/ welfare system, this becomes challenging because of continuing pattern changes and shifts in health care/ welfare system in past several decades [116]. Hence, comparisons across studies were difficult and a meaningful quantitative pooling of the existing data remains unfeasible. Therefore, future studies, especially those involving economic burden assessment using a standardized approach and based in LMI settings, are warranted.

In spite of data limitations, the estimates reported here show that NCDs pose a significant financial burden on healthcare budgets and nations' welfare that is likely to increase over time. Further work is necessary to standardize the methods to consistently assess the economic impact of NCDs worldwide and to involve hitherto under-addressed LMI populations across the globe.

Supplementary Material can be found online:
http://link.springer.com/article/10.1007%2Fs10654-014-9984-2

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2.2 The Global Impact of Non-communicable Diseases on Macro-Economic Productivity: a Systematic Review

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ABSTRACT

Non-communicable diseases (NCDs) have large economic impact at multiple levels. To systematically review the literature investigating the economic impact of NCDs (including coronary heart disease (CHD), stroke, type 2 diabetes mellitus (DM), cancer (lung, colon, cervical and breast), chronic obstructive pulmonary disease (COPD) and chronic kidney disease (CKD)) on macro-economic productivity. Systematic search, up to November 6th 2014, of medical databases (Medline, Embase and Google Scholar) without language restrictions. To identify additional publications, we searched the reference lists of retrieved studies and contacted authors in the field. Randomized controlled trials (RCTs), cohort, case-control, cross-sectional, ecological studies and modelling studies carried out in adults (>18 years old) were included. Two independent reviewers performed all abstract and full text selection. Disagreements were resolved through consensus or consulting a third reviewer. Two independent reviewers extracted data using a predesigned data collection form. Main outcome measure was the impact of the selected NCDs on productivity, measured in DALYs, productivity costs, and labor market participation, including unemployment, return to work and sick leave. From 4542 references, 126 studies met the inclusion criteria, many of which focused on the impact of more than one NCD on productivity. Breast cancer was the most common (n=45), followed by stroke (n=31), COPD (n= 24), colon cancer (n=24), DM (n=22), lung cancer (n=16), CVD (n=15), cervical cancer (n=7) and CKD (n=2). Four studies were from the WHO African Region, 52 from the European Region, 53 from the Region of the Americas and 16 from the Western Pacific Region, one from the Eastern Mediterranean Region and none from South East Asia. We found large regional differences in DALYs attributable to NCDs but especially for cervical and lung cancer. Productivity losses in the USA ranged from 88 million US dollars (USD) for COPD to 20.9 billion USD for colon cancer. CHD costs the Australian

economy 13.2 billion USD per year. People with DM, COPD and survivors of breast and especially lung cancer are at a higher risk of reduced labor market participation. Overall NCDs generate a large impact on macro-economic productivity in most WHO regions irrespective of continent and income. The absolute global impact in terms of dollars and DALYs remains an elusive challenge due to the wide heterogeneity in the included studies as well as limited information from low- and middle-income countries.

INTRODUCTION

Non-communicable diseases (NCDs), such as coronary heart disease (CHD), stroke, chronic obstructive pulmonary disease (COPD), cancer, type 2 diabetes and chronic kidney disease (CKD) currently constitute the number one cause of morbidity and mortality worldwide, claiming 36 million lives each year (accounting for 63% of all adult deaths) [1]. Infectious disease prevention and control, economic growth, improvements in medical and scientific knowledge, and health and social systems development have all contributed to increased life expectancy, improved quality of life and increased likelihood of living to age 60 years and beyond. While these are notable achievements, together with lifestyle-related shifts, these epidemiological and socio-demographic changes also mean that the burden of NCDs will grow [2].

Productivity is a measure of the efficiency of a person, business or country in converting inputs into useful outputs. The productive age span of a person is from adulthood to retirement and ranges from 18 years to around 65 years of age depending on, amongst other things, profession and country. The measurement of productivity greatly relies on the output and the economic or social system context. The focus in this report is macro-economic productivity loss in the productive age range due to NCDs. Key macro-economic measures related to the labor market include: (un-) employment, (loss in) hours worked (including full or part-time work status change), presenteeism (defined as impaired performance while at work), absenteeism, disability adjusted life years (DALYs) and productivity costs/losses. Key macro-economic outcomes are reduction in the able workforce, NCD-related health and welfare expenditure and loss of income earned by the productive workforce. While both the burden of NCDs and the socio-economic contexts vary greatly, the impact of the former on macro-economic outcomes across the global regions remains unclear.

We aimed to systematically identify and summarize the literature investigating the impact of six NCDs (CHD, stroke, COPD cancer, type 2 diabetes and CKD) on macro-economic productivity and to determine directions for future research.

METHODS

Search Strategy and Inclusion Criteria

We systematically searched the electronic medical databases (Medline, Embase and Google Scholar) up to November 6th, 2014 (date of last search) to identify relevant articles evaluating the

macro-economic consequences of the six selected NCDs, specifically the impact on economic productivity of working age citizens. The complete search strategy is available in **Appendix 1**. We defined the major NCDs of interest as coronary heart disease (CHD), stroke, chronic obstructive lung disease (COPD), type 2 diabetes mellitus (DM), cancer (lung, colon, breast and cervical) and chronic kidney disease (CKD). The step-wise inclusion and exclusion procedure is outlined in **Figure 1**. Eligible study design included randomized controlled trials (RCTs), cohort, case-control, cross-sectional, systematic reviews, meta-analysis, ecological studies and modeling studies. We included studies that estimated the impact of at least one of the NCDs defined above on at least one of the following measures of macro-economic productivity: DALYs, economic costs related to reduced work productivity, absenteeism, presenteeism, (un) employment, (non-) return to work (RTW) after sickness absence and medical/sick leave. DALY is also considered as essentially it is an economic measure of human productive capacity for the affected individual and when taken together (e.g. all those in a company, society etc.) forms an economic measure also on the group level. Only studies involving adults (>18 years old) were included, without any restriction on language or date.

Study Selection

Two independent reviewers screened the titles and abstracts of the initially identified studies to determine if they satisfied the selection criteria. Any disagreements were resolved through discussion and consensus, or by consultation with a third reviewer. In order to ensure that all retrieved full texts (of the selected abstracts) satisfied the inclusion criteria appropriately, they were further assessed by two independent reviewers. We further screened the reference lists of all retrieved studies to retrieve relevant articles. Systematic reviews were not included in the data extraction but a supplementary scan of their reference lists was performed to identify any additional studies.

Data Extraction

A data collection form (DCF) was prepared to extract the relevant information from the included full texts, including study design, World Health Organization (WHO) region, participants, NCD-related exposure and macro-economic outcome characteristics. When evaluating economic costs, US dollars (USD) was used as outcome measure. If a study reported costs in another currency, the corresponding exchange rate to USD as reported by the study itself was used. However, if an exchange rate was not provided, we calculated USD applying the conversion rate for the indicated study time-period.

Quality Evaluation

To evaluate the quality of the included non-randomized studies, we applied the Newcastle-Ottawa Scale (NOS) [3]. The NOS scale assesses the quality of articles in three domains: selection, comparability and exposure. 'Selection' assesses four items and a maximum of one star can be awarded for each item. 'Comparability' awards a maximum of two stars to the one item within the category. Finally, 'exposure' includes four items for which one star can be awarded. A quality score

is made for each study by summing the number of stars awarded, and thus the NOS scale can have maximum of nine stars. We used this scale to assess the quality of case-control and cohort studies. For cross-sectional and descriptive studies, we used an adapted version of NOS scale (**Appendix 2**).

Statistical Methods

We aimed to pool the results using a random effects model. If pooled, results would be expressed as pooled relative risks with 95% confidence intervals. Pooling possibility was conditional on the level of heterogeneity between studies.

Table 1. General Characteristics of the Studies Included in this Review

Lead Author	Period of Surveillance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs
Adepoju OE; et al. 2013[71]	2007-2012	USA	RA	Retrospective	376	Both	DM
Ahn E; et al.200931	1993 - 2002	South Korea	WPR	Cross-sectional	1,594	Female	Breast Cancer
Alavinia SM, Burdorf A. 200869	2004	10 EU countries	ER	Cross-sectional	11,462	Both	CVD, Stroke, DM
Alexopoulos EC, Burdorf A. 200154	1993 - 1995	The Netherlands	ER	Prospective cohort	326	Male	COPD
Anesetti-Rothermel A, Sambamoorthi A. 201110	2007	USA	RA	Cross-sectional	12,860	Both	COPD, CVD, Stroke, DM
Angeleri F; et al. 199380	NR	Italy	ER	Prospective study	180	Both	Stroke
Arrossi S; et al. 200723	2002 - 2004	Argentina	RA	Cross-sectional	120	Female	Cervical Cancer
Bains M; et al. 201244	2008 - 2009	UK	ER	Prospective cohort	50	Female	Colon Cancer
Balak F; et al. 200834	2001 - 2007	The Netherlands	ER	Retrospective cohort	72	Female	Breast Cancer
Bastida E; Pagan JA 200281	1994-1999	USA	RA	Population based	1,021	Both	DM
Black-Schaffer RM, Osberg JS. 199082	1984 – 1986	USA	RA	Prospective study	79	Both	Stroke
Bogousslavsky J, Regli F. 198783	NR	Switzerland	ER	Prospective study	41	Both	Stroke
Boles M; et al. 200484	2001	USA	RA	Cross-sectional	2,264	Both	DM
Bouknight RR; et al. 200637	2001-2002	USA	RA	Prospective study	416	Female	Breast Cancer

Table 1. General Characteristics of the Studies Included in this Review (continued)

Lead Author	Period of Surveillance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs
Bradley C, Bednarek H 200285	1999	USA	RA	Cross-sectional	184	Both	Breast Cancer, Colon Cancer, Lung Cancer
Bradley C; et al. 2002a86	1992	USA	RA	Retrospective study	5,974	Female	Breast cancer
Bradley C; et al. 2002b87	1992	USA	RA	Cross-sectional	5,728	Female	Breast cancer
Bradley C; et al. 200588	2001-2002	USA	RA	Prospective study	817	Female	Breast Cancer
Bradley C; et al. 200689	2001-2002	USA	RA	Prospective study	239	Female	Breast Cancer
Bradley C; Dahman B. 201333	2007 - 2011	USA	RA	Cross-sectional	828	Both	Breast Cancer
Bradley CJ; et al. 201140	2005	USA	RA	Modelling study	NR	Both	Colon Cancer
Bradshaw D; et al. 200766	2000 - 2000	South Africa	AR	Modelling	NR	Both	DM
Broekx S; et al. 201190	1997 - 2004	Belgium	ER	Cost-of-Illness analysis	20,439	Female	Breast Cancer
Burton WN; et al. 200491	2002	USA	RA	Survey	16,651	Both	DM
Carlsen K; et al. 201445	2001 - 2009	Denmark	ER	Epidemiological	4,343	Both	Colon Cancer
Carlsen K; et al. 201429	2001-2011	Denmark	ER	Cross-sectional and propective	14750	Female	Breast Cancer
Catalá-López F; et al. 201413	2008	Spain	ER	Cross-sectional	37,563,454	Both	Stroke
Choi SK; et al. 200742	2001 - 2003	South Korea	WPR	Prospective cohort	305	Male	Colon Cancer
Collins JJ; et al. 200592	2002	USA	RA	Survey	7,797	Both	DM
Costilla R; et al. 201322	2006	New Zealand	WPR	Modelling	NR	Both	Breast Cancer, Colon Cancer, Lung Cancer, Cervical Cancer
Da Costa DiBonaventura M; et al. 201253	2009	USA	RA	Cross-sectional	20,024	Both	COPD
Dall TM; et al. 200968	2007 - 2007	USA	RA	Modelling	NR	NR	DM
Darkow T; et al. 200763	2001 - 2004	USA	RA	Case-control	4,045	Both	COPD

Table 1. General Characteristics of the Studies Included in this Review (continued)

Lead Author	Period of Surveillance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs
De Backer G; et al. 200693	1994 – 1998	Belgium	ER	Prospective cohort	15,740	Both	DM
Eaker S; et al. 201194	1993 - 2003	Sweden	ER	Cross-sectional	28,566	Female	Breast Cancer
Earle CC; et al. 201046	2003 - 2005	USA	RA	Prospective cohort	2,422	Both	Lung Cancer, Colon Cancer
Ekwueme D; et al. 201426	1970 - 2008	USA	RA	Retrospective Cohort	53,368	Female	Breast Cancer
Etyang AO; et al. 20146	2007-2012	Kenya	AR	Prospective surveillance	18,712	Both	CVD, Stroke, DM
Fantoni SQ; et al. 201038	2004-2005	France	ER	Cross-sectional	379	Female	Breast cancer
Fernandez de Larrea-Baz N; et al. 200995	2000	Spain	ER	Ecological	40,376,294	Both	Breast Cancer, Colon Cancer, Lung Cancer
Ferro J; Crespo M. 199496	1985 - 1992	Portugal	ER	Prospective cohort	215	Both	Stroke
Fu AZ; et al. 200997	2004-2006	USA	RA	Survey	46,617	Both	DM
Gabriele W, Renate S. 200918	2001 - 2004	Germany	ER	Prospective cohort	70	Both	Stroke
Genova-Maleras R; et al. 20124	2008	Spain	ER	Modelling	NR	Both	CVD, Stroke, COPD, Lung Cancer, Colon Cancer, Breast Cancer, DM
Gordon L; et al. 200847	2003 - 2004	Australia	WPR	Prospective cohort	975	Both	Colon Cancer
Hackett M; et al. 201219	2008 - 2010	Australia	WPR	Prospective cohort	441	Both	Stroke
Halpern MT; et al. 200398	2000	USA	RA	Economical evaluation	447	Both	COPD
Hansen JA; 2008 et al.99	NR	USA	RA	Cross-sectional	203	Female	Breast Cancer
Hauglann B; et al. 201230	1992 - 1996	Norway	ER	National Registry cohort	3,096	Female	Breast Cancer
Hauglann BK; et al. 201449	1992-1996	Norway	ER	Case-control	1480	Both	Colon Cancer
Helanterä I; et al. 201265	2007	Finland	ER	Cross-sectional	2,637	Both	CKD
Herquelot E; et al. 2011100	1989-2007	France	ER	Prospective cohort	20,625	Both	DM

Table 1. General Characteristics of the Studies Included in this Review (continued)

Lead Author	Period of Surveillance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs
Holden L; et al. 2011[52]	2004 - 2006	Australia	WPR	Cross-sectional	78,430	Both	CVD, COPD, DM
Hoyer M; et al. 2013[101]	2007 - 2008	Sweden	ER	Prospective cohort	651	Female	Breast Cancer
Jansson SA; et al. 2002[59]	1999	Sweden	ER	Economic Evaluation	212	Both	COPD
Kabadi GS; et al. 2013[17]	2005-2006	Tanzania	AR	Prospective surveillance Study	16	Both	Stroke
Kang H-Y; et al. 2011[16]	2008	South Korea	WPR	Economic Evaluation		Both	Stroke
Kappelle JL; et al.1994[102]	1977 - 1992	USA	RA	Prospective study	296	Both	Stroke
Katzene llenbogen JM; et al. 2011[14]	1997-2002	Western Australia	WPR	Modelling, ecologocial	68,661	Both	Stroke
Kessler, RC; et al. 2001[70]	1995-1996	USA	RA	Survey	2,074	Both	DM
Klarenbach S; et al. 2002[64]	1988 - 1994	USA	RA	Cross-sectional	5,558	Both	CVD, COPD, DM, CKD
Kotila M; et al. 1984[103]	1978-1980	Finland	ER	Prospective	255	Both	Stroke
Kremer AM; et al. 2006[55]	2000 - 2001	Australia	ER	Cross-sectional	826	Both	COPD
Kruse, M; et. Al. 2009[104]	1980 - 2003	Denmark	ER	Cohort	2,212	Both	CHD
Lauzier S; et al. 2008[35]	2003	Canada	RA	Prospective cohort	962	Female	Breast Cancer
Lavigne JEl; et al. 2003[67]	1999 - 1999	USA	RA	Cross-sectional	472	Both	DM
Leigh JP; et al. 2002[105]	1996	USA	RA	Ecological study	2,395,650	Both	COPD
Leng CM. 2008[106]	2004 - 2005	Singapore	WPR	Retrospective Cohort	29	NR	Stroke
Lenneman, J; et al. 2011[107]	2005-2009	USA	RA	Survey	577,186	Both	DM
Lindgren P; et al. 2008[108]	1994	Sweden	ER	Cross-sectional	393	Both	Stroke
Lokke, A; et al. (1) 2014[62]	1998-2010	Denmark	ER	Case-control	262,622	Both	COPD
Lokke, A; et al. (2) 2014[61]	1998-2010	Denmark	ER	Case-control	1,269,162	Both	COPD

Table 1. General Characteristics of the Studies Included in this Review (continued)

Lead Author	Period of Surveillance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs
Lopez-Bastida J; et al. 2012[15]	2004	Canary Islands, Spain	ER	Cross-sectional	448	Both	Stroke
Mahmoudlou A; 2014[39]	2008	Iran	EMR	Cross-sectional	72,992,154	Both	Colon Cancer
Maunsell E; et al. 2004[32]	1999 - 2000	Canada	RA	Cross-sectional	57,307	Female	Breast Cancer
Mayfield, JA; et al.1999[109]	1987	USA	RA	Survey	35,000	Both	DM
McBurney CR; et al. 2004[110]	1999-2000	USA	RA	Cross-sectional survey	89	Both	CVD
Molina R; et al. 2008[111]	2004 – 2005	Spain	ER	Cross-sectional	347	Both	Breast Cancer, Colorectal Cancer, Lung Cancer
Molina Villaverde R et al. 2008[112]	NR	Spain	ER	Cohort	96	Female	Breast Cancer
Moran AI; et al. 2008[5]	2000 - 2029	China	WPR	Ecological and Modelling	1,270,000,000	Both	CVD
Nair K; et al. 2012[113]	2000 - 2007	USA	RA	Economic Evaluation	853,496	Both	COPD
Neau JP; et al. 1998[114]	1990 – 1994	France	ER	Retrospective	67	Both	Stroke
Niemi ML; et al. 1988[115]	1978 – 1980	Finland	ER	Retrospective case-series	46	Both	Stroke
Nishimura S, Zaher C. 2004[58]	1990 - 2002	Japan	WPR	Modelling study	1,848,000	Both	COPD
Noeres D; et al. 2013[28]	2002 - 2010	Germany	ER	Prospective cohort	874	Female	Breast Cancer
Nowak D; et al. 2004[60]	2001	Germany	ER	Cross-sectional	814	Both	COPD
O'Brien A; et al. 2010[116]	NR	USA	RA	Cross-sectional	98	Both	Stroke
Ohguri T et al; 2009[117]	2000-2005	Japan	WPR	Cross-sectional	43	Both	Lung Cancer, Colon Cancer
Orbon K; et al. 2005[56]	1998 - 2000	The Netherlands	ER	Cross-sectional	2,010	Both	COPD
Osler M; et al. 2014[12]	2001-2009	Denmark	ER	Cohort	21,926	Both	CVD
Park JH; et al. 2008[48]	2001 - 2006	South Korea	WPR	Cross-sectional	2,538	Both	Lung Cancer, Colon Cancer, Breast Cancer, Cervical Cancer

Table 1. General Characteristics of the Studies Included in this Review (continued)

Lead Author	Period of Surveillance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs
Park JH; et al. 2009[118]	2001 - 2006	South Korea	WPR	Prospective study	1,602	Both	Lung Cancer, Colon Cancer, Breast Cancer, Cervical Cancer
Peters G; et al. 2013[119]	NR	Nigeria	AR	Cross-sectional	110	Both	Stroke
Peuckmann V; et al. 2009[120]	1989 – 1999	Denmark	ER	Cross-sectional	1,316	Female	Breast Cancer
Quinn AC; et al. 2014[20]	1998-2008	UK	ER	Prospective Cohort	214	Both	Stroke
Robinson N; et al. 1989[121]	1985 – 1989	UK	ER	Cross-sectional	2,104	Both	DM
Roelen CAM; et al. 2009[122]	2001 - 2005	The Netherlands	ER	Ecological	259	Female	Breast Cancer
Roelen CAM; et al. 2011[50]	2004 - 2006	The Netherlands	ER	Retrospective Cohort	300,024	Both	Lung Cancer, Breast Cancer
Saeki S, Toyonaga T. 2010[123]	2006 - 2007	Japan	WPR	Prospective cohort	325	Both	Stroke
Sasser AC; et al. 2005[8]	1998 - 2000	USA	RA	Economic Evaluation	38,012	Female	Breast cancer, CVD
Satariano WA; et al. 1996[27]	1984-1985 1987-1988	USA	RA	Cross-sectional	1,011	Female	Breast Cancer
Short PF; et al. 2005[124]	1997-1999	USA	RA	Cross-sectional	1,433	Both	Breast Cancer
Short PF; et al. 2008[11]	2002	USA	RA	Cross-sectional	6,635	Both	CVD, Stroke, COPD, DM
Sin DD; et al. 2002[125]	1988 - 1994	USA	RA	Cross-sectional	12,436	Both	COPD
Sjovall K; et al. 2012[36]	2004-2005	Sweden	ER	Ecological study	14,984	Both	Breast Cancer, Colon Cancer, Lung Cancer
Spelten ER; et al. 2003[126]	NR	The Netherlands	ER	Prospective cohort	235	Female	Breast Cancer
Stewart DE; et al. 2001[127]	NR	Canada	RA	Cross-sectional	378	Female	Breast Cancer
Strassels SA; et al. 2001[128]	1987 – 1988	USA	RA	Cross-sectional	238	Both	COPD
Syse A; et al. 2008[51]	1953-2001	Norway	ER	Cross-sectional population based	1,116,300	Both	Breast Cancer, Lung Cancer, Colorectal Cancer
Taskila-Brandt T; et al. 2004[24]	1987-1988 1992-1993	Finland	ER	Cross-sectional population based	5,098	Both	Cervical Cancer, Breast Cancer, Colon Cancer Lung Cancer

Table 1. General Characteristics of the Studies Included in this Review (continued)

Lead Author	Period of Surveillance	Location	WHO region	Study Design	Number in Analysis	Gender	Reported NCDs
Taskila T; et al. 2007129	1997-2001	Finland	ER	Cross-sectional	394	Female	Breast Cancer
Teasell RW; et al. 2000130	1986-1996	Canada	RA	Retrospective cohort	563	Both	Stroke
Tevaarwerk AJ; et al. 201343	2006 - 2008	USA and Peru	RA	Cross-sectional	530	Both	Breast Cancer, Lung Cancer, Colon Cancer
Timperi AW; et al. 2012131	2006 - 2011	USA	RA	Prospective cohort	2,013	Female	Breast Cancer
Torp S; et al. 201225	1999 - 2004	Norway	ER	Prospective Registry	9,646	Both	Cervical Cancer, Breast Cancer, Colon Cancer, Lung Cancer
Traebert J; et al. 201321	2008	Brazil	RA	Modelling, Ecological	NR	Both	Cervical Cancer, Breast Cancer, Colon Cancer, Lung Cancer
van Boven JFM; et al. 201357	2009	The Netherlands	ER	Economic Evaluation	45,137	Both	COPD
Van der Wouden JC et al. 1992132	1978-1980	The Netherlands	ER	Cross-sectional	313	Female	Breast Cancer
Vestling M; et al. 2003133	NR	Sweden	ER	Retrospective study	120	Both	Stroke
Wang PS; et al. 2003134	NR	USA	RA	Cross-sectional	199	Both	CVD, COPD, Diabetes
Ward ME; et al. 2002135	1993 – 1994	USA	RA	Cross-sectional	2,529	Both	COPD
Wozniak; et al. 1999136	NR	USA	RA	Retrospective study	203	Both	Stroke
Yaldo A; et al. 201441	2006-2009	USA	RA	Case-Control	330	Both	Colon Cancer
Yabroff KR; et al. 2004137	2000	USA	RA	Cross-sectional	496	Both	Breast Cancer, Colon cancer
Zhao Z; Winget M. 20117	2003 - 2006	USA	RA	Retrospective Cohort	10,487	Both	CVD (CHD)
Zheng H; et al. 20109	2004	Australia	WPR	Economic Evaluation	NR	Both	CVD (CHD)

Abbreviations: AR: African Region, COPD: Chronic Obstructive Pulmonary Disease, CKD: Chronic Kidney Disease, CVD: Cardiovascular Disease, DM: Diabetes Mellitus, EMR; Eastern Mediterranean Region, ER: European Region, NCD: No-communicable Diseases, NR: Not Reported, RA: Region of the Americas, USA: United States of America, WHO: World Health Organization, WPR: Western Pacific Region.

RESULTS

General Characteristics of the Included Studies

From 4542 references initially identified, a total of 126 unique studies met the inclusion criteria **(Figure 1** and **Table 1)**. All eligible studies were published between 1984 and 2014.

Of the 126 studies identified, 52 were from the WHO European Region, 53 from the Region of the Americas (of which all but two were from Canada or the United States of America [USA]), 16 from the Western Pacific Region, four were from the WHO African Region and one from the Eastern Mediterranean Region. We found no studies from South East Asia. The majority of the identified studies were observational in design, analyzed prospectively as well as cross-sectional. Two studies reported cross-sectional data from an RCT and six were modeling studies. National or hospital-based disease registries were often used to select patients, which were in some cases linked to national socio-economic databases to extract corresponding employment data. The control group, if used, was often a sample from the general population and sometimes sought within the same environment of the patients (e.g. same company). Many studies focused on the impact of more than one NCD on productivity. Most studies used one measure of productivity. Of all the published studies including cancers, cervical cancer was included in seven studies, breast cancer in 45, colon cancer in 24 and lung cancer in 16. Stroke was included in a total of 31 studies, COPD in 24, DM in 22 and CHD was included in 15 studies. Relevant data on CKD was included in only two of the studies and two of the studies mention NCDs in general.

Measures of Productivity

Measures of productivity impact in the available studies included DALYs, absenteeism, presenteeism, labor market (non-) participation, RTW, change in hours worked and medical/sickness leave. Most studies focused on the direct impact on the patient but a minority also examined the impact on caregivers/spouses. Outcomes were quantified using risks, proportions, odds, dollars, years and days. In some studies, time-to-event data was analyzed using Cox proportional-hazards regression. Adjusting for education, age and employment status was most frequently applied, although the measurement of education and employment was not consistently defined, measured or validated. A small minority of studies reported differences in impact according to ethnicity. Pooling of outcomes was not possible due to substantial heterogeneity across and within NCD groups (I^2> 70%).

Impact of Cardiovascular Disease on Productivity

Of all DALYs on a population level in Spain **(Table 2a)**, 4.2% were attributable to CHD [4] with an estimated age-standardized rate of 4.7 per 1000 persons per year. In China, DALYs attributable to CHD were estimated to be 8,042,000 for the year 2000 and predicted to more than double in 2030, rising up to 16,356,000 [5]. In the same study, the estimated DALY in 2000 was 16.1 per 1000 persons and predicted to be 20.4 in 2030 (estimate not accounted for age). A study from Kenya estimated the DALY to be 68 per 100,000 person-years of observation [6]. CHD-related productivity

loss in the USA was estimated to be 8539 USD per person per year (PP/PY), at 10175 USD PP/PY [7] for absenteeism and 2698 USD PP/PY for indirect work-related loss [8]. Total absenteeism-related costs in Australia were estimated at 5.69 billion USD, mortality-related costs at 23 million USD and costs related to lower employment at 7.5 billion USD [9]. An estimated 4.7 working days PP/PY were lost in the USA owing to CHD [10]. Also in the USA, the odds of experiencing limited amount of paid work due to illness were significantly higher for those with CHD compared to the control group, with an odds ratio (OR) of 2.91 for women (95% CI 2.34 – 3.61) and 2.34 for men (95% CI 1.84 – 2.98) [11]. In Denmark workforce participation increased with increasing time from 37% after 30 days to 65% after 5 years of diagnosis[12]. In a study conducted in 10 European Union (EU) countries, no difference was found for the risk of non-participation in the labor force between those with and without self-reported CHD with an OR of 0.96 (95% CI 0.66 – 1.40).

Impact of Stroke on Productivity

Stroke accounted for 3.5% of all DALYs reported in Spain **(Table2b)** with a rate of 3.8 per 1000 people [4]. Another study from Spain reports a total count of DALYs of 418,052 with a higher number of male than for female (220,005 vs. 198,046) [13] . A study from Kenya reports a rate of 166 DALYs per 100,000 person-years observed [6]. In Western Australia, the average annual stroke-attributable DALY count is an estimated 26315 for men and 30918 for women [14]. In Spain, costs after diagnosis increased over time for caregivers but declined for patients (14732 USD in caregivers compared to 2696 USD among patients after one year and 15621 USD to 1362 USD after two years) [15]. Modeled productivity losses in South Korea were higher for a severe stroke among men (537724 USD) than women (171157 USD) [16]. A prospective surveillance study from Tanzania report a mean costs of productivity loss to be 213 USD [17]. Inconclusive evidence of the impact of stroke on RTW was reported. Estimates ranged from 26.7% to 75% in studies reporting RTW in stroke patients after one year of the event [18,19]. In Nigeria, 55% returned to work at a mean of 19.5 months after stroke. A report from the United Kingdom (UK) found that 47% were unemployed one year after stroke [20]. Increased odds to report limited ability for paid work were found among men (3.86) and women (2.26) after stroke [11].

Impact of Cervical Cancer on Productivity

There are strong regional differences in the percentage of DALYs attributable to cervical cancer **(Table 2c)** among women, from 1.6% (absolute DALYs, 1061 per year) in New Zealand to 13.4% (2516 per year) in Brazil [21,22]. Cervical cancer patients in Argentina reported negative outcomes after one year; 45% of patients reported reduced labor market participation, 28% experienced work interruption and 5% changed work [23]. Compared to the general population, the relative risk (RR) for cervical cancer survivors in labor force participation was 0.77 (95% CI 0.67 – 0.90), 2 to 3 years after diagnosis in Finland [24]. In Norway however, no differences were found 5 years from diagnosis with an OR of 0.92 (0.63 – 1.34) [25].

Impact of Breast Cancer on Productivity

Of all the DALYs attributable to cancers among women, 27.3% (17840 per year) in New Zealand **(Table 2d)** and 13.4% (6280 per year) in Brazil are attributable to breast cancer [21,22]. Total mortality-related lifetime productivity loss costs in the USA were estimated to be 5.5 billion USD [26]. This was differentially distributed between the two ethnic groups reported, with 71% (or 3.9 billion USD) of the costs attributable to white women and 24% (or 1.3 billion) attributable to black women. Differential RTW and sick absence rates are also observed comparing black and white women in the USA; the percentage of white women returning to work three months after diagnosis was 74.2% compared to 59.6% of black women; the proportion reporting sick leave was 25.8% of white women compared to 40.4% of black women [27]. One year after primary surgery in Germany, nearly three times as many cancer survivors had left their job as compared to women in the control group. [28] Various studies suggest higher unemployment among breast cancer survivors, reported by around half after one year, 72% after two years [29], 43% after six years and 18% after nine years [27,28,30-32]. In contrast, in a study assessing unemployment among the spouses of breast cancer patients, no differences were found [33]. Differences between countries in average time to RTW were also found, from 11.4 months in the Netherlands [34] and 7.4 months in Canada [35] to only three months in Sweden [36]. Percentage of RTW after one year ranged from 54.3% in a cross-sectional study from France to 82% in a prospective study from the USA [37,38].

Impact of Cancer on Productivity

In New Zealand, of all the DALYs attributable to cancers, 12.9% (8431 per year) among women and 13.5% (8316 per year) among men are attributable to colon cancer **(Table 2e)** [22]. In Brazil, these proportions are 9.3% among women and 7.5% among men [21]. In Spain, 2.1% of DALY's overall are attributable to colon cancer [4]. In Iran the total burden of colorectal cancer in 2008 was 52,534 DALYs and higher for men than for women [39]. In the USA, annual productivity losses were calculated to be 20.9 billion USD [40], while costs due to absenteeism after 1 year of diagnosis was 4,245 USD per patient compared to the general population [41]. Although the DALY and dollar costs of colon cancer are undoubtedly large, the evidence for micro-level labor market indicators including risk and proportions of RTW, sickness absence and employment following diagnosis and treatment is however inconclusive [25,42-49]. In New Zealand, of all cancer-attributable DALYs, 14.4% (9334 per year) among women and 15.9% (9806 per year) among men are attributable to lung cancer **(Table 2f)** [22]. In Brazil, lung cancer results in an estimated 10832 DALYs per year, 9.8% of all cancer-related DALYs among women and 24.5% among men [21]. In Spain, 3.4% of all DALYs are attributable to lung cancer [4]. Most of the first year of disease (275 days) is spent in sickness absence in Sweden [36] and between 33-79% of lung cancer patients in the USA were unemployed 15 months after diagnosis [43,46]. Average time to re-enter the labor market was 484 days for full-time work and 377 for part-time work in the Netherlands [50]. The odds of re-entry into the labor market were significantly lower for lung cancer than the general population [24,25,51].

Impact of COPD on Productivity

COPD patients have a higher chance of working fewer hours, of absenteeism and of poorer work performance (presenteeism) **(Table 2g).** [11,52,53]. A COPD patient loses around 8.5 workdays per year due to disease [10,54]. Between 39-50% of people stopped working due to the onset of COPD in the Netherlands [55,56]. COPD-related productivity losses cost the US economy around 88 million USD or around 482966 working days per year [57]. Modeled annual costs of COPD, estimated at 1.47 billion USD [58], are higher in Japan than the USA. The productivity loss costs PP/PY were somewhat comparable between Germany, Sweden and the Netherlands (566, 749 and938 USD respectively) [57,59,60], but differed four-fold to estimated costs in Denmark (2816 to 3819 USD)[61,62] and more than 10-fold to what was estimated (9815 USD) in the USA [63]. In the USA, 8.5 work days are lost PP/PY on average[10], while COPD patients take an estimated 8.6 days of sickness absence in the Netherlands during a two year follow-up period [54]. Also in the Netherlands, 39% of COPD patients left the labor force due to disease onset [55].

Impact of Chronic Kidney Disease on Productivity

Only two studies **(Table 2h)** examined the impact of CKD on productivity. One found that renal dysfunction was independently associated with labor force non-participation, with an odds ratio of 7.94 (95% confidence interval, 1.60 to 39.43) [64]. The second study, evaluating labor market participation in CKD patients specifically after dialysis or transplantation, found that 35% of these CKD patients were unemployed [65].

Impact of Diabetes Mellitus on Productivity

In Spain, nearly 2% of all mortality-related DALYs are attributable to DM [4]. In South Africa, 162877 DALYs annually are attributable to DM **(Table 2i)** [4,66]. A study from Kenya reports a rate of 364 DALYs per 100,000 observed person-years [6]. An estimated 7.2 days are lost PP/PY due to DM in the USA[10] and DM patients have an increased risk of absenteeism, presenteeism and inability to work [4,10,11,52,64,67-69]. Productivity days lost per year due to diabetes ranged from 3.6 to 7.3 [10,70]. In the USA, proportion of productivity loss was large due to premature mortality (49%) and presenteeism (44%) compared to absenteeisim (4%) and total productivity related costs were estimated to be 1,962,314 USD [71].The odds of non-participation of the labor force for diabetes patients compared to the general population were slightly higher with borderline significance in the EU, an OR of 1.38 (95% CI 0.99 – 1.93) [69].

Table 2a: Results of the Included Studies Investigating the Impact of CVD on Productivity

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
McBurney CR; et al. 2004	Return to work	Return to work at a mean of 7.5 months	Percent	76.4	NR	NR	4
	Presenteeism	Perceived work performance	Mean	3.6	0.52	NR	
Moran Al; et al. 2008	DALYs	Observed period 2000	Count	8042000	NR	NR	NA
		Observed period 2000	Rate	16.1	NR	NR	
		Predicted 2010	Count	10730000	NR	NR	
		Predicted 2010	Rate	16.5	NR	NR	
		Predicted 2020	Count	13422000	NR	NR	
		Predicted 2020	Rate	18.2	NR	NR	
		Predicted 2030	Count	16356000	NR	NR	
		Predicted 2030	Rate	20.4	NR	NR	
Osler M; et al. 2014	Labor market participation	Workforce participation 30 days after diagnosis (among patients who were part of the workforce at time of diagnosis)	Percent	37.2	NR	NR	5
		Workforce participation 1 year after diagnosis (among patients who were part of the workforce at time of diagnosis)	Percent	40.1	NR	NR	
		Workforce participation 2 years after diagnosis (among patients who were part of the workforce at time of diagnosis)	Percent	45.0	NR	NR	
Osler M; et al. 2014 (continued)	Labor market participation	Workforce participation 5 years after diagnosis (among patients who were part of the workforce at time of diagnosis)	Percent	65.2	NR	NR	
Sasser AC; et al. 2005	Productivity Loss Costs	Attributable annual indirect work-loss costs per patient	USD	2698	NR	NR	8
Short PF; et al. 2008	Unemployment	Limited amount of paid work possible due to illness Female	OR	2.91	NR	2.34 - 3.61	5

Table 2a: Results of the Included Studies Investigating the Impact of CVD on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
		Limited amount of paid work possible due to illness Male	OR	2.34		1.84 - 2.98	
Wang PS; et al. 2003	Absenteeism	Annual Excess in Days	Mean	8.8	7.0 (SE)	NR	4
	Presenteeism	Annual Excess in Days	Mean	8.9	11.8 (SE)	NR	
	Absenteeism and Presenteeism combined	Annual Excess in Days	Mean	16.3	12.7 (SE)	NR	
Zhao Z, Winget M. 2011	Productivity Loss Costs	Short term one year productivity costs/per person	USD	8539	NR	NR	6
		Absenteeism one year productivity costs/per person	USD	10175	NR	NR	
Zheng H; et al. 2010	Productivity Loss Costs	Absenteeism related total	USD	568500000	NR	NR	NA
		Mortality related	USD	23565000	NR	NR	
		Due to lower employment	USD	750000000	NR	NR	

Table 2b: Results of the Included Studies on the Impact of Stroke on Productivity

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Alavinia SM, Burdorf A. 2008	Unemployment	Non participation in the labour force	OR	1.110	NR	(0.530 - 2.320)	4
Anesetti-Rothermel A, Sambamoorthi A; et al. 2011	Sick Leave	Work days in last year lost due to illness	Mean	17.960	5.83 (SE)	--	6
Angeleri F; et al. 1993	Return to work	Return to work 12 to 196 months (mean 37.5) in hemiplegic patients	Percent	20.64	NR	NR	6
Black-Schaffer RM; et al. 1990	Return to Work	Return to work at 6 to 25 months post-rehabilitation	Percent	49	NR	NR	3
		Time return to work in months from rehabilitation	Mean	3.1	2.12	NR	
		Return to prior job at 6 to 25 months post-rehabilitation	Percent	43	NR	NR	
Bogousslavsky; et al. 1987	Return to work	Return to work 6 to 96 months (mean 46)	Count	19	NR	NR	3
Catalá-López F; et al. 2014	DALYs	Total	Count	418,052	NR	NR	4
		Male	Count	220,005	NR	NR	
		Female	Count	198,046	NR	NR	
Etyang AO; et al. 2014	DALYs	Rate per 100,000 person year of observation	Rate	166	NR	NR	5
Ferro J; Crespo M. 1994	Unemployment	Inactive at end of follow-up (mean 33.4 months, range 1 - 228 months)	Percent	27	NR	NR	4
Gabriele W, Renate S. 2009	Return to Work	Return to work after one year of those employed	Percent	26.7	NR	NR	4
Genova-Maleras R; et al. 2012	DALYs	Rate per 1000 age standardised	Rate	3.8	NR	NR	NA

Table 2b: Results of the Included Studies on the Impact of Stroke on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
		Percentage of all causes of mortality	Percent	3.5	NR	NR	
Hackett M; et al. 2012	Return to Work	Return to work one year after event	Percent	75	NR	NR	2
Kabadi GS; et al. 2014	Return to Work	Average months off work in six month follow up period	Mean	6	NR	NR	4
	Costs	Mean productivity losses due to stroke	USD	213	NR	NR	
Kang H-Y; et al. 2011	Productivity Loss Costs	Male, Total modelled costs per severe stroke per year	USD	537724	NR	NR	NA
		Female, Total modelled costs per severe stroke per year	USD	171157	NR	NR	
Kappelle JL; et al. 1994	Unemployment	Unemployment at 0.02 to 16 years after event (mean 6 years)	Percent	58	NR	NR	5
Katzenellenbogen JM; et al. 2012	DALYs	Male	Count	26315	NR	NR	NA
Klarenbach S; et al. 2002	Unemployment	Non-participation in Labour Force	OR	2.21	NR	(0.7 – 7)	6
Kotila M; et al. 1984	Return to Work	Return to work after 12 months	Percent	59	NR	NR	4
Leng C; et al. 2008	Return to Work	Return to work in 1 year	Percent	55.0	NR	NR	NA
Lindgren P; et al. 2008	Productivity Loss Costs	Indirect costs during one ear	USD	17844	NR	(12275 – 23864)	4
Lopez-Bastida et al. 2012	Productivity Loss Costs	Indirect per person, 1 year after stroke	USD	2696	6462	NR	5
		Indirect per person, 2 year after stroke	USD	1393	4754	NR	
		Indirect per person, 3 year after stroke	USD	1362	4931	NR	
		Caregivers cost per person per year, 1 year after stroke	USD	14732	14616	NR	
		Caregivers cost per person per year, 2 year after stroke	USD	15621	14693	NR	
		Caregivers cost per person per year, 3 year after stroke	USD	13759	15470	NR	
Neau JP; et al. 1998	Return to Work	Return to work in same position as prior to stroke	Percent	54	NR	NR	3 6
		Return to work after 0 to 40 month (mean 7.8)	Percent	73	NR	NR	

Table 2b: Results of the Included Studies on the Impact of Stroke on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Niemi M; et al. 1988	Return to Work	Return to work after 4 years	Percent	54	NR	NR	
O'Brien A; et al. 2010	Return to Work	Return after 6 to 18 months	Percent	56.0	NR	NR	1
Peters G; et al. 2013	Return to Work	Return to work after 3 to 104 months (mean 19.5)	Percent	55	NR	NR	3
Quinn AC; et al. 2014	Return to Work	unemployment at 1 year follow up	Percent	47	NR	NR	3
Roelen CAM; et al. 2011	Return to Work	Return to work after 3 to 104 months (mean 19.5)	Percent	55.0	NR	NR	6
Saeki S, Toyonaga T. 2010	Return to Work	Return to work at 18 months	Percent	55.0	NR	NR	6
Short PF; et al. 2008	Unemployment	Limited amount of paid work possible due to illness Female	OR	2.26	NR	1.56- 2.26	5
		Limited amount of paid work possible due to illness Male	OR	3.86	NR	2.55-3.60	
Teasell RW; et al. 2000	Return to Work	Return to work at 3 months	Percent	20	NR	NR	3
		Return to work full-time at 3 months	Percent	6	NR	NR	
Vestling M; et al.2003	Return to work	Return to work mean of 2.7 years	Percent	41	NR	NR	3
		Time to return to work in months	Mean	11.9	9	NR	
		Return to work with reduced work hours	Percent	21	NR	NR	
Wozniak MA; et al. 1999	Return to Work	Return to work after 1 year	Percent	53	NR	NR	6
		Return to work after 2 year	Percent	44	NR	NR	

Table 2c. Results of the Included Studies on the Impact of Cervical Cancer on Productivity

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Arrossi S; et al. 2007	Return to Work	Reduced in hours worked (patients)	Percent	45	NR	NR	4
		Change of work (pat.)	Percent	5	NR	NR	
		Starting paid work (pat.)	Percent	14	NR	NR	
		Increased in hours worked (pat.)	Percent	11	NR	NR	
		Odds of work interruption (pat.)	OR	4	NR	NR	
		Odds of reduction in hours worked (pat.)	OR	1	NR	NR	
		Odds of starting paid work (pat.)	OR	2	NR	NR	
		Odds of increase in hours worked (pat.)	OR	1	NR	NR	
		Work interruption (caregivers)	Percent	3	NR	NR	
		Reduction in hours worked (caregivers)	Percent	61	NR	NR	
		Change of work (caregivers)	Percent	2	NR	NR	
		Starting paid work (caregivers)	Percent	5	NR	NR	
Costilla R; et al. 2013	DALYs	Female	Count	1016	NR	NR	NA
		Percentage of all cancers, female	Percent	1.6	NR	NR	
		Rate per 10000 people (age standardized)	Rate	84	NR	NR	
Park JH; et al. 2008	Labour market participation	Time until job loss between patients and controls Cox PH	HR	1.32	NR	0.95 - 1.82	7
Park JH; et al. 2009	Labour market participation	Time until job loss between patients and controls Cox PH	HR	1.68	NR	1.40-2.01	5
		Time until re-employment between patients and controls Cox PH	HR	0.67	NR	0.46-0.97	
Taskila-Brandt T; et al. 2004	Labor market participation	Employment status cancer survivors 2-3 years post-diagnosis compared to general population (58 vs75%)	RR	0.77	NR	0.67-0.90	6
Torp S; et al. 2012	Labor market participation	Employment in 5 years from diagnosis	OR	0.92	NR	0.63- 1.34	9
Traebert J; et al. 2013	DALY	Rate per 10000 people (age standardized)	Rate	118.7	NR	NR	NA

Table 2d. Results of the Included Studies on the Impact of Breast Cancer on Productivity

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Ahn E; et al. 2009	Labour market drop-out	Not working current for cancer survivors vs. the general population (adjusted)	OR	1.680	1.350	2.100	3
		OR of not working for cancer survivors of currently not working compared with their employment status at the time of diagnosis	OR	1.630	1.510	1.760	
	Unemployment	Adjusted OR for not working at the time of diagnosis vs. the general population	OR	1.210	0.960	1.530	
Balak F; et al. 2008	Sick Leave	Months to fully return to work	Mean	11.4	NR	NR	3
		Months to return to partial work	Mean	9.5	NR	NR	
Bouknight RR; et al. 2006	Return to Work	Return to work in 12 months after diagnosis	Percent	82	NR	NR	5
		Return to work in 18 months after diagnosis	Percent	83	NR	NR	
Bradley C, Bednarek H. 2002	Unemployment	Unemployed 5-7 years after diagnosis for cancer survivors	Percent	54.8	NR	NR	5
		Unemployed 5-7 years after diagnosis for cancer survivors	Percent	45.4	NR	NR	
Bradley C; et al. 2002a	Labor market participation	Probability of working of breast cancer patients compared to controls at mean of 7 years	Percent	-7	4	NR	8
Bradley C; et al. 2002b	Labor market participation	Probability of working of breast cancer patients compared to controls at mean of 7.15 years	Percent	-10	4	NR	5
Bradley C; et al. 2005	Employment	Probability of being employed for patients compared to controls at 6 months	Percent	-25	NR	NR	7
		Reduced weekly hours of work for patients compared to controls after 6 months	Percent	-18	NR	NR	
Bradley C; et al. 2006	Absenteeism	Days absent from work evaluated at 6 months after diagnosis	Mean	44.5	55.2	NR	7
Bradley C, Dahman B. 2013	Labor market participation	Probability of stopping work at 2 months post diagnosis (husbands of female patients)	OR	2.642	NR	(0.848 - 8.225)	5
Broekx S; et al. 2011	Productivity	Indirect costs work per patient per year (attributable)	USD	5248	NR	NR	3

Table 2d. Results of the Included Studies on the Impact of Breast Cancer on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
		Indirect costs housekeeping per patient per year (attributable)	USD	2034	NR	NR	
		Indirect costs mortality per patient per year (attributable)	USD	14203	NR	NR	
		Sick leave days per year	USD	47.2	NR	NR	
		Total indirect costs per patient per year (attributable)	USD	21485	NR	NR	
Carlsen K; et al. 2014	Unemployment	% of working women two years after treatment	Percent	72	NR	NR	5
Costilla R; et al. 2013	DALYs	DALYs % of all cancers	Percent	27.2	NR	NR	NA
		Rate per 10000 people (age standardized)	Rate	1065	NR	NR	
		DALYs	Count	17840	NR	NR	
Eaker S; et al. 2011	Sick leave	Percentage difference of sickness absence comparing patients 5 years after diagnosis with women without breast cancer	Percent	10.100	NR	NR	7
Ekwueme D; et al. 2014	Productivity loss	Mortality-related total lifetime productivity loss (whites)	USD	3920400000	NR	NR	4
		Mortality-related total lifetime productivity loss (blacks)	USD	1323200000	NR	NR	
		Mortality-related total lifetime productivity loss/per death (all)	USD	1100000	NR	NR	
		Mortality-related total lifetime productivity loss/per death (whites)	USD	1090000	NR	NR	
		Mortality-related total lifetime productivity loss/per death (blacks)	USD	1110000	NR	NR	

Table 2d. Results of the Included Studies on the Impact of Breast Cancer on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
		Mortality-related total lifetime productivity loss (all)	USD	5488600000	NR	NR	
Fantoni SQ; et al. 2010	Return to work	Return to work 12 months after starting treatment	Percent	54.3	NR	NR	5
		Return to work after 3 years after starting treatment	Percent	82.1	NR	NR	
	Sick leave	Duration of sick leave 36 months after starting treatment in months	Mean	1.8	NR	9.2-12.1	
Fernandez de Larrea-Baz, N; et al 2009	DALYs	Rate per 10000 people, age standardized, male	Rate	2	NR	NR	4
Genova-Maleras R; et al. 2012	DALYs	Rate per 1000 people, age standardized	Rate	1.6	NR	NR	NA
		Percentage of all causes of mortality	Percent	1.4	NR	NR	
Hansen JA; et al. 2008	Presenteeism	Average score difference on work limitation scale between cases and non-cancer controls	Mean	2.9	NR	NR	5
Hauglann B; et al. 2012	Unemployment	Unemployment at 9 years in females	Percent	18	NR	NR	9
Hoyer M; et al. 2013	Unemployment	Unemployment at follow up	Percent	26	NR	NR	4
Lauzier S; et al. 2008	Sick Leave	Percent taking sick leave for one week or more	Percent	90.7	NR	NR	6
		Weeks of absence due to breast cancer	Count	32.3	NR	NR	
Maunsell E; et al. 2004	Unemployment	Unemployment among disease free survivors	Risk Ratio	1.35	NR	1.08 - 1.7	7
	Unemployment	Unemployment among survivors with new breast cancer event	Risk Ratio	2.24	NR	1.57 - 3.18	
	Unemployment	Unemployment among all survivors (3 years after diagnosis)	Risk Ratios	1.46	NR	1.18 - 1.81	
	Productivity loss	Survivors reporting part-time working compared to controls (3 years after diagnosis)	Percent	4	NR	NR	

Table 2d. Results of the Included Studies on the Impact of Breast Cancer on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
	Productivity loss	Change in working hours among survivors - change over time compared to controls (3 years after diagnosis)	Mean	-2.6	NR	NR	
Molina R; et al. 2008	Return to work	Return to work at mean time since diagnosis(32.5 months)	Percent	56	NR	NR	5
Molina Villaverde R; et al. 2008	Return to work	Return to work by end of treatment	Percent	56	NR	NR	NA
Noeres D; et al. 2013	Unemployment	6 years after diagnosis	Percent	43.2	NR	NR	5
		1 year after diagnosis	Percent	49.8	NR	NR	
Park JH; et al. 2008	Labour market participation	Time until job loss (months)	Mean	36	NR		7
		Time until 25% of patients were re-employment (months)	Mean	30	NR		
Park JH; et al. 2009	Labour market participation	Cox proportional analysis comparing time until job loss between patients and controls	HR	1.83	NR	1.60-2.10	5
		Cox proportional analysis comparing time until re-employment between patients and controls	HR	0.61	NR	0.46-0.82	
Peuckmann V; et al. 2009	Labor market participation	Age-standardized prevalence of employment at 5 to 15 years post primary surgery	Percent	49	NR	NR	4
		Age standardized risk ratio (SRR) of employment at 5 to 15 years post primary surgery	SRR	1.02	NR	0.95-1.10	
		Age-standardized prevalence of sick leave at 5 to 15 years post primary surgery	Percent	12	NR	NR	
		Age standardized risk ratio (SRR) of sick leave at 5 to 15 years post primary surgery	SRR	1.28	NR	0.88-1.85	
Roelen CAM; et al. 2011	Return to work	Time to return to full-time work (days)	Count	349.0	NR	329- 369	6
		Time to return to part-time work (days)	Count	271.0	NR	246- 296	
Roelen CAM; et al. 2009	Return to Work	Return to work at 2 years	Percent	89.4	NR	NR	4
	Sick Leave	Days of absence due to breast cancer	Count	349	NR	NR	

Table 2d. Results of the Included Studies on the Impact of Breast Cancer on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Sasser AC; et al. 2005	Productivity loss costs	Attributable annual indirect work-loss costs per female patient	USD	5944.0	NR	NR	8
Satariano WA; et al.1996	Return to work	3 months after diagnosis (white women)	Percent	74.2	NR	NR	3
	Return to work	3 months after diagnosis (black women)	Percent	59.6	NR	NR	
	Sick leave	3 months after diagnosis (white women)	Percent	25.8	NR	NR	
	Sick leave	3 months after diagnosis (black women)	Percent	40.4	NR	NR	
Short PF; et al. 2005	Unemployment	The chances of quitting work/ unemployment 1 to 5 years after diagnosis	OR	0.44	NR	0.20-0.95	5
Sjovall K; et al. 2012	Sick leave	Days sick leave taken before return to work	Count	90	NR	NR	5
Spelten ER; et al. 2003	Return to work	Time to return to work after diagnosis analyzed using Cox PH	HR	0.45	NR	0.24-0.86	4
Stewart DE; et al. 2001	Unemployment	Unemployment assessed at least at 2 years after diagnosis, mean of 9 years	Percent	41	NR	NR	3
Syse A; et al. 2008	Labor market participation	Employment probability in the year 2001 of cancer survivors compared to general population	OR	0.74	NR	0.65 – 0.84	6
Taskila-Brandt T; et al. 2004	Labor market participation	Employment status of cancer survivors 2-3 years post-diagnosis compared to general population (61 vs 65%)	RR	0.95	NR	0.92-0.98	6
Taskila T; et al. 2007	Work ability	Current work ability assessed between 0 and 10 by questionnaire (reference group 8.37)	Mean	8.23	NR	NR	8
Tevaarwerk AJ; et al. 2013	Unemployment	Unemployment	Percent	19.4	NR	NR	6
Timperi AW; et al 2012	Unemployment	6 months post diagnosis	Percent	52.0	NR	NR	4
Torp S; et al. 2012	Labor market participation	Employment five years from diagnosis	OR	0.74	NR	0.63 - 0.87	9

Table 2d. Results of the Included Studies on the Impact of Breast Cancer on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Traebert J; et al. 2013	DALYs	Percentage of all cancers, female	Percent	21.9	NR	NR	NA
		Rate per 10000 people, age standardized, male	Rate	3.2	NR	NR	
		Percentage of all cancers, male	Percent	0.3	NR	NR	
		Total	Count	6032.3	NR	NR	
		Rate per 10000 people, age standardized, female	Rate	195	NR	NR	
Van der Wouden JC; et al 1992	Labor market participation	Changes in employment status at least 5 years cancer free	Percent	-7	NR	NR	3
		Maintained employment status after diagnosis	Percent	16	NR	NR	
Yabroff KR; et al. 2004	Labor market participation	Job in past 12 months, compared to control group (45.9% with a p-value < 0.001 for difference)	Percent	36.9	NR	31.0-42.8	6

Table 2e. Results of the Included Studies on the Impact of Colon Cancer on Productivity

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Bains M; et al. 2012	Unemployment	6 months after surgery	Percent	61	NR	NR	2
Bradley CJ; et al. 2011	Productivity loss	Annual productivity losses total 2020 modelled (millions)	USD	21780	NR	NR	NA
		Annual productivity losses total 2005 (millions)	USD	20920	NR	NR	
Bradley C, Bednarek H; 2002	Unemployment	Unemployed 5-7 years after diagnosis cancer survivors	Percent	54.8	NR	NR	5
		Unemployed 5-7 years after diagnosis spouse of cancer survivors	Percent	53	NR	NR	
Carlsen K; et al. 2014	Return to Work	Return to work after 1 year after diagnosis	Percent	69	NR	NR	8
Choi K; et al. 2007	Unemployment	Lost job at 24 months in males	Percent	46	NR	NR	7
Costilla R; et al. 2013	DALYs	Female	Count	8431	NR	NR	NA
Fernandez de Larrea-Baz, N; et al 2009	DALYs	Rate per 10000 people, age standardized, female	Rate	212	NR	NR	4
		Rate per 10000 people, age standardized, male	Rate	284	NR	NR	
		Rate per 10000 people, age standardized, total	Count	99833	NR	NR	
Genova-Maleras R; et al. 2012	DALYs	Rate per 1000 people, age standardized	Rate	2.3	NR	NR	NA
		Percentage of all causes of mortality	Percent	2.1	NR	NR	
Gordon L; et al. 2008	Return to work	Working one year after diagnosis (%)	Percent	65	NR	NR	5
Hauglann BK; et al. 2014	Return to work	% of employed that were on sick-leave at some point after one year of diagnosis	Percent	85			9
		Sickness absence for CRC localized, the OR is for 3 years after diagnosis	Odds Ratio	2.61	1.36	4.95	

Table 2e. Results of the Included Studies on the Impact of Colon Cancer on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
		Sickness absence for CRC regional, the OR is for 3 years after diagnosis	Odds Ratio	1.09	0.56	2.11	
		Sickness absence for CRC distant, the OR is for 3 years after diagnosis	Odds Ratio	2.30	0.57	0.927	
Mahmoudlou A; 2014	DALYs	total burden of colorectal cancer according to DALY in Iran in 2008	Count	52534	NR	NR	8
Mahmoudlou A; 2014 (continued)	DALYs	DALYs for men in 2008	Count	29928	NR	NR	
		DALYs for women in 2008	Count	22606	NR	NR	
Molina R; et al. 2008	Return to work	Return to work at mean time since diagnosis(32.5 months)	Percent	55	NR	NR	5
Ohguri T; et al. 2009	Sick leave	Attendance rate after return to work of employees with disease compared to controls (p-value 0.67)	Percent	86	NR	NR	4
Park JH; et al. 2008	Return to Work	Time until re-employment (patients after job loss) Cox PH analysis	HR	0.96	NR	0.7 - 1.32	7
	Unemployment	Cox PH analysis time until job loss	HR	1.04	NR	0.91 - 1.2	
Park JH; et al. 2009	Labour market participation	Cox PH analysis comparing time until job loss between patients and controls	HR	1.69	NR	1.50-1.90	5
		Cox PH analysis comparing time until re-employment between patients and controls	HR	0.57	NR	0.43-0.75	
Sjovall K; et al. 2012	Sick Leave	Days sick leave	Count	115	NR	NR	5
Syse A; et al. 2008	Employment	Employment probability in year 2001 of cancer survivors compared to general population - men	OR	0.67	NR	0.58 – 0.78	6

Table 2e. Results of the Included Studies on the Impact of Colon Cancer on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Taskila-Brandt T; et al. 2004	Labor market participation	Employment status of cancer survivors 2-3 years post-diagnosis compared to general population (53 vs 59%)	RR	0.90	NR	0.81-0.99	6
Tevaarwerk AJ; et al. 2013	Unemployment	Unemployment	Percent	24.1	NR	NR	6
Torp S; et al. 2012	Labour market participation	Employment in five years from diagnosis (females)	OR	0.84	NR	(0.53 - 1.35)	9
		Employment in five years from diagnosis (male)	OR	0.7	NR	(0.43 - 1.15)	
Traebert J; et al. 2013	DALYs	Rate per 10000 people, age standardized, female	Rate	82.6	NR	NR	NA
		Percentage of all cancers, female	Percent	9.3	NR	NR	
		Rate per 10000 people, age standardized, male	Rate	73.1	NR	NR	
		Percentage of all cancers, male	Percent	7.5	NR	NR	
		Total	Count	4867.2	NR	NR	
Yabroff KR; et al. 2004	Labor market participation	Job in past 12 months, compared to control group (45.9% with a p-value < 0.001 for difference)	Percent	22.4	NR	15.6-29.3	6
	Sick leave	Days lost from wok due to health problems in past 12 months compared to control group (5.7% with a p-value < 0.001 for difference)	Mean	10.0	NR	3.4-16.7	
	Presenteeism	Limited in work due to health issues compared to control group (17.6% with a p-value of <0.001 for difference)	Percent	32.4	NR	24.2-40.6	
Yaldo A; et al. 2014	Absenteeism	Mean higher absenteeism costs after 1 year of diagnosis compared to controls	USD	4245	NR	NR	7

Table 2f. Results of the Included Studies on the Impact of Lung Cancer on Productivity

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Bains M; et al. 2012	Unemployment	6 months after surgery	Percent	61	NR	NR	2
Bradley C, Bednarek H; 2002	Unemployment	Unemployed 5-7 years after diagnosis cancer survivor	Percent	62.2	NR	NR	5
		Unemployed 5-7 years after diagnosis spouse of cancer survivor		51.3	NR	NR	
Costilla R; et al. 2013	DALYs	Female	Count	9334	NR	NR	NA
		% of all cancers (female)	Percent	14.3	NR	NR	
		Rate per 10000 people (age standardised, female)	Rate	849	NR	NR	
		Male	Count	9806	NR	NR	
		% of all cancers (male)	Percent	15.9	NR	NR	
		Rate per 10000 people (age standardised, male)	Rate	775	NR	NR	
Earle CC; et al. 2010	Unemployment	Unemployment at 15 months	Percent	79	NR	NR	4
Fernandez de Larrea-Baz, N; et al 2009	DALYs	Rate per 10000 people (age standardised, female)	Rate	98	NR	NR	4
Genova-Maleras R; et al. 2012	DALYs	Percentage of all causes of mortality	Percent	3.4	NR	NR	NA
		Rate per 1000 people, age standardized	Rate	3.8	NR	NR	
Molina R; et al. 2008	Return to work	Return to work at mean time since diagnosis(32.5 months)	Percent	15	NR	NR	5
Ohguri T; et al. 2009	Sick leave	Attendance rate after return to work of employees with disease compared to controls (p-value 0.59)	Percent	75	NR	NR	4
Park JH; et al. 2008	Labour market participation	Time until job loss	Cox PH	1.31	NR	1.12 - 1.53	7
		Time until re-employment (patients after job loss)	Cox PH	0.79	NR	0.55 - 1.16	

Table 2f. Results of the Included Studies on the Impact of Lung Cancer on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Park JH; et al. 2009	Labour market participation	Cox proportional analysis comparing time until job loss between patients and controls	HR	2.22	NR	1.93-2.65	5
		Cox proportional analysis comparing time until re-employment between patients and controls	HR	0.45	NR	0.32-0.64	
Roelen CAM; et al. 2011	Return to work	Time to return to full-time work (days)	Count	484.0	NR	(307-447)	6
Syse A; et al. 2008	Employment	Employment probability in year 2001 of cancer survivors compared to general population - men	OR	0.37	NR	0.31 – 0.45	6
		Employment probability in year 2001 of cancer survivors compared to general population - women	OR	0.58	NR	0.48 – 0.71	
Sjovall K; et al. 2012	Sick leave	Days	Count	275	NR	NR	5
Taskila-Brandt T; et al. 2004	Labor market participation	Employment status of cancer survivors 2-3 years post-diagnosis compared to general population (19 vs 43%)	RR	0.45	NR	0.34-0.59	6
Tevaarwerk AJ; et al. 2013	Unemployment	Unemployment	Percent	33	NR		6
Torp S; et al. 2012	Unemployment	Employment in five years from diagnosis (male)	OR	0.39	NR	0.18 - 0.83	9
Traebert J; et al. 2013	DALYs	Rate per 10000 people, age standardized, female	Rate	87.6	NR	NR	NA

Table 2g: Results of the Included Studies on the Impact of COPD on Productivity

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Alexopoulos EC; et al. 2001	Sick leave	Days of sick leave during 2 year follow up attributable to COPD	Mean	8.53	NR	NR	2
Anesetti-Rothermel A, Sambamoorthi A; et al. 2011	Sick Leave	Work days in last year lost due to illness	Mean	8.600	0.76 (SE)	NR	6
Da Costa DiBonaventura M; et al. 2012	Productivity loss	Percentage reporting absenteeism (difference between cases of COPD and controls)	Percent	4.190	NR	NR	7
		Absenteeism hours (over last 7 days) (difference between COPD cases and controls)	Mean	1.250	NR	NR	
		Percentage reporting presenteeism (difference between cases of COPD and controls)	Percent	16.550	NR	NR	
		Estimated number of hours of presenteeism in last 7 days (difference between COPD cases and controls)	Mean	4.780	NR	NR	
		Percentage of those reporting work impairment (difference between cases of COPDand controls)	Percent	17.280	NR	NR	
		Percentage reporting absenteeism (difference between cases of COPD and controls)	Percent	2.330	NR	NR	
		Absenteeism hours (over last 7 days) (difference between cases of COPD and controls)	Mean	0.330	NR	NR	
Da Costa DiBonaventura M; et al. 2012 (continued)		Estimated number of hours of presenteeism in last 7 days (difference between cases of COPD and controls)	Mean	2.070	NR	NR	
		Percentage of those reporting work impairment (difference between cases of COPD and controls)	Percent	11.530	NR	NR	
Darkow T; et al. 2007	Productivity Loss	Indirect per person per year	USD	9815	NR	(8384-11246)	6
Genova-Maleras R; et al. 2012	DALYs	Rate per 1000 age standardised	Rate	2.6	NR	NR	2
		Percentage of all causes of mortality	Percent	2.3	NR	NR	

Table 2g: Results of the Included Studies on the Impact of COPD on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Halpern MT; et al. 2003	Productivity loss	Costs due to work loss up from 45 years up to age of retirement per patient per day	USD	100.55	NR	NR	6
		Days lost per patient of working age per year	Mean	18.7	NR	NR	
		Days lost per caregiver of working age per year	Mean	1.7	NR	NR	
	Unemployment	Unemployment due to condition	Percent	34	NR	NR	
Holden L; et al. 2011	Productivity Loss	Absenteeism (no. of full/part days missed from work in last 4 weeks)	IRR	1.57	NR	1.33 -1.86	3
		Presenteeism (self-rated score of overall performance in last 4 weeks)	IRR	1.22	NR	1.04 - 1.43	
Jansson SA; et al. 2002	Productivity Loss	Indirect per person per year	USD	749	NR	NR	6
Kremer AM; et al. 2006	Unemployment	Percentage of who stopped work (among people in work) because of the onset of COPD	Percent	39	NR	NR	5
Leigh JP; et al. 2002	Productivity loss	Total indirect costs in 1996 in billions of dollars	USD	2,1400	NR	NR	3
Lokke, A; et al. (1) 2014	Unemployment	% receiving income from employment	Percent	16.7	NR	NR	7
	Productivity loss	indirect costs per patient before the diagnosis	USD	4266	NR	NR	
		indirect costs per patient after diagnosis	USD	2816	NR	NR	
Lokke, A; et al. (2) 2014	Productivity loss	indirect costs per patient before the diagnosis	USD	5912	NR	NR	9
		indirect costs per patient after diagnosis	USD	3819	NR	NR	
	Unemployment	% of spouses receiving income from employment	Percent	36.9	NR	NR	
Nair K; et al. 2012	Productivity Loss	Short term one year productivity costs/per person	USD	527	NR	NR	9
		Absenteeism one year productivity costs/per person	USD	55	NR	NR	
		Total costs	USD		NR	NR	
Nishimura S, Zaher C. 2004	Productivity Loss	Modelled total annual costs per year in country (millions)	USD	1471	NR	NR	2
Nowak D; et al. 2004	Productivity loss	early retirement (per patient/ year) (all COPD stages)	USD	566	NR	NR	3
		early retirement (per patient/year) (light COPD)	USD	489	NR	NR	

Table 2g: Results of the Included Studies on the Impact of COPD on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
		early retirement (per patient/ year) (medium COPD)	USD	567	NR	NR	
		early retirement (per patient/year) (severe COPD)	USD	1,064	NR	NR	
		disability (per patient/year) (all COPD stages)	USD	398	NR	NR	
		disability (per patient/year) (light COPD)	USD	459	NR	NR	
		disability (per patient/year) (medium COPD)	USD	249	NR	NR	
		disability (per patient/year) (severe COPD)	USD	340	NR	NR	
Orbon K; et al. 2005	Unemployment	Unemployment	Percent	53.8	NR	NR	4
Sin DD; et al. 2002	Employment	Adjusted probability of being in work force for those with self-reported COPD compared to those without self-reported COPD	Percent	-3.9	NR	-1.3 - - 6.4	4
	Productivity loss	Total loss productivity cost in 1994 in billions	USD	9.9	NR	NR	
Short PF; et al. 2008	Unemployment	Limited amount of paid work possible due to illness (female)	OR	2.63	NR	2.03- 3.42	5
Strassels SA; et al.2001	Productivity loss	Number of lost work days COPD related	Mean	1.0	NR	<0.1– 2.0	5
		Number of restricted activity days COPD related	Mean	15.9	NR	10.3-21.5	
van Boven JFM; et al. 2013	Productivity Loss	Costs total per patient a year (2009)	USD	938	NR	NR	6
		Costs in total (2009)	USD	88340000	NR	NR	
	Absenteeism	Days total per patient (2009)	Count	10.7	NR	NR	
		Days total (2009)	Count	482966	NR	NR	
Wang PS; et al. 2003	Absenteeism	Annual Excess in Days	Mean	19.4	8.9 (SE)	NR	4
	Presenteeism	Annual Excess in Days	Mean	27.5	15.6 (SE)	NR	
	Absenteeism & Presenteeism combined	Annual Excess in Days	Mean	42.9	17.0 (SE)	NR	
Ward MW; et al. 2002	Unemployment	Inability to work attributable to COPD	Percent	10.6	NR	NR	6

Table 2h: Results of the Included Studies on the Impact of Chronic Kidney Disease on Productivity

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Helantera I; et al. 2012	Unemployment	Unemployed in 2007 for patients with dialysis or after kidney transplant	Percent	35	NR	NR	6
Klarenbach S; et al. 2002	Unemployment	Non-participation in Labour Force	OR	7.94	NR	1.6 – 39.43	6

Table 2i. Results of the Included Studies on the Impact of Type 2 Diabetes Mellitus on Productivity

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Adepoju OE; et al. 2013	Absenteeism	Absenteeism Days total	Count	11664	NR	NR	9
		Absenteeism Costs total	USD	85314	NR	NR	
		Proportion of total productivity losses attributable to absenteeism	Percent	4	NR	NR	
		Days of reduced time at work as a sum of Inpatient and ambulatory visits	Count	7864	NR	NR	
		Costs of reduced time at work as sum of Inpatient and ambulatory visits	USD	866744	NR	NR	
		Proportion of total productivity losses attributable to reduced time at work	Percent	3	NR	NR	
	Presenteeism	Presenteeism Days total	Count	7864	NR	NR	
		Presenteeism Costs total	USD	866744	NR	NR	

Table 2i. Results of the Included Studies on the Impact of Type 2 Diabetes Mellitus on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
		Proportion of total productivity losses attributable to presenteeism	Percent	44	NR	NR	
	Productivity loss	Costs of premature mortality costs as a product of YLL and income	USD	953373	NR	NR	
		Proportion of total productivity losses attributable premature mortality	Percent	49	NR	NR	
		Total productivity related loss	Count	20064	NR	NR	
		Total productivity related costs loss	USD	1962314	NR	NR	
Alavinia SM, Burdorf A. 2008	Unemployment	Non participation in the labor force	OR	1.380	NR	0.990 - 1.930	4
Anesetti-Rothermel A, Sambamoorthi A; et al. 2011	Sick Leave	Work days in last year lost due to illness	Mean	7.250	1.18 (SE)	NR	6
Bastida, E; Pagan, JA 2002	Productivity loss	Unemployment due to diabetes In females	Maximum likelihood	-0.073	0.198	NR	NA
		Unemployment due to diabetes In males	Maximum likelihood	-1.047	0.447	NR	
Boles, M; et al. 2004	Productivity loss	Lost earnings per diabetic person/week	USD	67	NR	NR	4
	Absenteeism	Absenteeism	OR	2.285	NR	1.167 - 4.474	
		Absenteeism	Least squares regression coefficient	3.254	7.286	NR	

Table 2i. Results of the Included Studies on the Impact of Type 2 Diabetes Mellitus on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
	Presenteeism	Presenteeism	OR	1.271	NR	0.724 - 2.230	
		Presenteeism	Least squares regression coefficient	4.308	4.369	NR	
Bradshaw D; et al. 2007	DALYs	Total	Count	162877	NR	NR	3
Burton, WN; et al. 2004	Presenteeism	Time management (work the required no. of hours; start work on time)	OR	1.401	NR	1.14 - 1.73	5
		Physical work activites (e.g. repeat the same hand motions; use work equipment)	OR	1.415	NR	1.15 - 1.75	
		Mental/interpersonal activities (concentration; teamwork)	OR	1.233	NR	1.02 - 1.50	
		Overall output (complete required amount of work; worked to capability)	OR	1.158	NR	0.95 - 1.42	
Collins, JJ; et al. 2005	Productivity loss	Impairment score (WIS)	Count	17.8	NR	15.9, 19.6	7
		Absent hours per patient/month	Count	1.3	NR	0.6, 1.9	
		Work Impairment	Linear regression coefficient	-2.4	NR	NR	
		Absence	Logistic regression coefficient	1.2 (not significant)	NR	NR	
Dall TM; et al. 2009	Productivity loss	Absenteeism	USD	2470	NR	NR	1
De Backer G; et al. 2006	Sick Leave	Univariate analysis of high one year incidence rate of sick leave in diabetes compared to controls (25.3%) in men (p-value <0.001)	Percent	36.9	NR	NR	8
		Univariate analysis of long absences (defined as more than 7 days) in diabetes compared to controls (19.3%) in men, (p-value 0.002)	Percent	25.3	NR	NR	

Table 2i. Results of the Included Studies on the Impact of Type 2 Diabetes Mellitus on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
		Univariate analysis for repetitive absences in diabetes compared to controls (14.5%) in men (p-value <0.001)	Percent	21.2	NR	NR	
		Adjusted analysis of high one year incidence rate of sick leave in diabetes compared to controls in men	OR	1.51	NR	1.22 – 1.88	
		Adjusted analysis of long absences in diabetes compared to controls in men	OR	1.11	NR	0.87-1.41	
		Adjusted analysis for repetitive absences in diabetes compared to controls in men	OR	1.54	NR	1.20 – 1.98	
		Univariate analysis of high one year incidence rate of sick leave in diabetes compared to controls (25.1%) in women (p-value <0.04)	Percent	33.9	NR	NR	
		Univariate analysis of long absences (defined as more than 7 days) in diabetes compared to controls (25.2%) in women, (p-value 0.04)	Percent	33.9	NR	NR	
De Backer G; et al. 2006 (continued)	Sick Leave (continued)	Univariate analysis for repetitive absences in diabetes compared to controls (24.0%) in women (p-value 0.002)	Percent	36.7	NR	NR	
		Adjusted analysis of high one year incidence rate of sick leave in diabetes compared to controls in women	OR	1.38	NR	0.89 – 2.14	
		Adjusted analysis of long absences in diabetes compared to controls in women	OR	1.45	NR	0.94-2.23	
		Adjusted analysis for repetitive absences in diabetes compared to controls in men	OR	1.71	NR	1.12 – 2.62	

Table 2i. Results of the Included Studies on the Impact of Type 2 Diabetes Mellitus on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Etyang AO; et al. 2014	DALYs	Rate per 100,000 PY of observation	Rate	364	NR	NR	5
Fu, AZ; et al. 2009	Productivity loss	Work loss days due to diabetes/year	Count	6.7	NR	NR	8
		Bed days due to diabetes/year	Count	13	NR	NR	
Genova-Maleras R; et al. 2012	DALYs	Rate per 1000 age standardised	Rate	2.2	NR	NR	2
		Percentage of all causes of mortality	Percent	1.9	NR	NR	
Herquelot, E; et al. 2011	Presenteeism	Work disability due to diabetes	Incidence rate per 1,000 person-years	7.9	NR	NR	7
		Work disability due to diabetes	HR	1.7	NR	1.0- 2.9	
Holden L; et al. 2011	Productivity Loss	Absenteeism, number of full/part days missed from work in last 4 weeks	IRR	1.17	NR	1.09 - 1.26	3
Lenneman J, et al. 2011	Productivity loss	Productivity impairment	Unstandardized linear regression coefficient	1.816	NR	0.717- 2.820	4
Klarenbach, S; et al. 2002	Unemployment	Non-participation in Labour Force	OR	2.17	NR	1.2 - 3.93	6
Kessler, RC; et al. 2001	Productivity loss	Impairment days	Count	3.6	0.8	NR	2
		Any work impairment	OR	1.1	NR	0.6 - 1.9	
		Impairment days	Unstandardized linear regression coefficient	-0.3	0.5	NR	
Lavigne JEl; et al. 2003	Productivity loss	Work while feeling unwell	Percent	0.54	NR	NR	4
Mayfield, JA; et al.1999	Productivity loss	Work disability due to diabetes	Probit model estimates	1.46	0.228	NR	8
		Work disability due to diabetes	Percent	25.6	NR	NR	

Table 2i. Results of the Included Studies on the Impact of Type 2 Diabetes Mellitus on Productivity (continued)

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
		Work loss days due to diabetes	Linear regression	0.67	0.318	NR	
		Work loss days due to diabetes per year	Count	5.65	NR	NR	
		Lost earnings per diabetic person/year	USD	3099	NR	NR	
Robinson N; et al. 1989	Unemployment	Rate of unemployed in those economically active for males (controls 7.8%)	Percent	21.9	NR	NR	7
		Rate of unemployed in those economically active for females (controls 5.1%)	Percent	11.5	NR	NR	
		Rate of unemployed in those economically active for females (controls 7.0% with a p-value of <0.001 for difference)	Percent	18			
Short PF; et al. 2008	Unemployment	Limited amount of paid work possible due to illness Female	OR	1.54	NR	1.23 - 1.92	5
		Limited amount of paid work possible due to illness Male	OR	2.02	NR	1.57 - 2.6	
Wang PS; et al. 2003	Absenteeism	Annual Excess in Days	Mean	6.4	6.0 (SE)	NR	4

Table 2j. Results of the Included Studies Investigating the Impact of Noncommunicable Diseases on Productivity

Study	Type of Outcome	Outcome Specified as	Assessment Type	Point Estimate	SD for Mean	95% CI	Quality Score
Torp S; et al. 2012	Unemployment	Unemployment at follow up	Percent	25.6	NR	NR	9
Earle CC; et al. 2010	Unemployment	Unemployment at 15 months	Percent	69	NR	NR	4

Abbreviations: Cox PH: Cox Proportional Hazard Regression, DALY's: Disability Adjusted Life Years, IRR: Incidence Risk Ratio, NCD: No-communicable Diseases, NA: Not Applicable NR: Not Reported, OR: Odds Ratio, RR: Relative Risk, SD: Standard Deviation, USD: United States of America dollars.

DISCUSSION

This systematic review identified 126 studies investigating the impact of NCDs on productivity. Most studies (96%)were from the Western world (North America, Europe or Asia Pacific), with limited evidence available from Brazil, South Africa, Kenya, Tanzania, Iran, Japan, South Korea and Argentina. Macro-economic productivity losses were measured in percentage and absolute numbers of DALYs and annual productivity loss costs (in USD). Studies also estimated productivity losses using labor market indicators including unemployment, RTW, absenteeism, presenteeism, sickness absence and loss in working hours. There is a clear scarcity in literature concerning the effect of CKD on productivity, with only two studies both reporting a substantial impact on productivity [64,65].

Diversity in the Macroeconomic Measures and Outcomes

There were considerable global differences in the NCD-attributable DALY burden, especially the differential impact of each NCD comparing high-income countries (HIC) and low- and middle-income countries (LMIC). Lung and colon cancer account for nearly 30% of all cancer-attributable DALYs in men in New Zealand whereas in Brazil, lung cancer alone accounts for nearly 25%. Among women in HIC, breast cancer seems to impose a large productivity burden whereas cervical cancer impacts more dramatically in LMIC [4,21,22]. Although DALYs are a reliable measure and capture both years of life lost and years spent in ill-health, we found inconsistent application in the identified studies; some estimated proportions within specific disease groups or of the overall DALY burden in a country; others estimated absolute DALY numbers.

Diversity in the Macro-economic Impact of the Cardiopulmonary Diseases

Absolute costs (measured in USD) were estimated for COPD, CHD, and stroke events [15,57,7,9,58,71]. These studies mainly came from HIC, although two studies, one from Kenya and one from Tanzania, were also retrieved. In Australia, absenteeism and lower employment due to CHD cost 13.2 billion USD annually, as well as an additional 23 million USD in mortality-related costs[9]. Evidence suggests that COPD costs around 88 million USD or nearly 500,000 working days per year in the US compared to 1.47 billion (modeled) in Japan. While annual COPD-related productivity costs were comparable in Germany, Sweden and the Netherlands (between 566-938 USD), costs differed four-fold (2816-3819 USD) in Denmark, 10-fold (9815 USD) in the USA[57,59-63]. In the USA, nearly half of the annual 1.96m USD productivity losses due to DM are attributable to mortality, with 44% attributable to presenteeism and just 4% to absenteeism In South Korea, modeled productivity losses for a stroke were 68% higher among men compared to women [16]. Around half of all stroke survivors in unemployed after one year [20]. In Tanzania, productivity losses after six months following stroke were 213 USD on average although these losses were most acutely experienced by those in higher skill roles [17]. Interestingly, indirect productivity losses were higher among caregivers than stroke patients themselves and costs increased for caregivers but declined for patients after one and two years following a stroke in Spain. COPD patients

experience reduced working hours, unemployment, absenteeism and presenteeism [10,11,52-56]. DM patients also have an increased risk of reduced labor market participantion [10,11,52,64]. By contrast, other than for absenteeism[10] the evidence for the risk of reduced labor market participation due to CVD is inconclusive. In Kenya, 68/100,000 person year observed are attributable to CVD compared to 166/100,000 for stroke and 364 /100,000 for DM [6]. Although evidence is limited, the higher productivity impact associated with diseases with a large morbidity was perhaps to be expected; chronic diseases such as COPD and DM affect people during their productive years and cannot really be 'cured', only managed. The extent to which employers or societies support and enable NCD populations to remain members of the productive workforce will also differentially distribute the impact. The extent to which secondary or tertiary prevention is possible will also affect productivity estimates, specifically so for labor market indicators such as RTW, change in work status or unemployment

Diversity in the Macroeconomic Impact of Cancer

Lung cancer survival is associated with reduced labor market participation through sickness absence, extended RTW [36,50] and unemployment [25,43,46]. Total mortality-related lifetime productivity loss due to breast cancer were an estimated 5.5 billion USD in the USA [26] and annual productivity losses due to colon cancer costs the US economy 20.9 billion USD [40].We found inconclusive evidence of risk of reduced labor market participation (RTW, sickness absence and unemployment) following colon cancer diagnosis and treatment [25,42-46,48]. The evidence for breast cancer-related labor market drop-out shows higher unemployment among survivors one, two, six and nine years after diagnosis [29-32]. Evidence from the USA also suggests ethnicity-patterned differences in sick leave and unemployment [27]. Along with possible socio-economic differences associated with these outcomes [72], pathophysiological differences may also play a role. African-American women have lower incidence of breast cancer but higher mortality and are also diagnosed in later stages and with more aggressive types of tumors [73]. However, we are cautious in over interpretation of this finding as few studies included ethnicity. Geographic differences in average months to RTW were observed from 11.4 in the Netherlands [34] to 7.4 in Canada [35] to just three months in Sweden [36].

Although evidence is limited, the higher productivity impact associated with diseases with a large morbidity was perhaps to be expected; chronic diseases such as COPD and DM affect people during their productive years and cannot really be 'cured', only managed. It is surprising that half of all productivity losses in the USA attributable to DM are due to mortality rather than absenteeism and presenteeism. The extent to which employers or societies support and enable NCD populations to remain members of the productive workforce will also differentially distribute the impact both within societies but also comparing more affluent to less affluent countries. The extent to which secondary or tertiary prevention is possible will also affect productivity estimates, specifically so for labor market indicators such as RTW, change in work status or unemployment.

Comparison with the Previous Work

Findings of this systematic review generally concur with and further extend the previous reviews. This study is a comprehensive systematic review tackling work-related burden of six major NCDs using a global perspective and without language limitation. Two reviewers included and assessed the studies and references of the included studies were tracked for any missing evidence. These approaches ensured that we included most of the relevant articles in our review. Similar to previous reviews, we found that, due to a great amount of variation in the studies included, comparability and pooling the studies were not possible. Most of the previous reviews were performed non-systematically and previous systematic reviews have included studies only in English. Previous studies were mainly focused on the impact of cancers [74-78] on work-related outcomes (mainly RTW) and often included a mix of cancers without specifying the type of cancer. Van Muijen and colleagues [78] reviewed only cohort studies of cancer-related work outcomes and were focused on English language. Steiner and colleagues [76] reviewed English publications published up until 2003, Breton and colleagues were focused only on diabetes and Krisch and colleagues focused on COPD in Germany[79].

Strengths and Limitations of the Current Work

In this systematic review we evaluated the literature concerning the impact on productivity of six top NCDs. These six were selected based on their dominance in the global burden of disease and together make a huge contribution to mortality and morbidity worldwide. Several important issues are out of scope for this work but do merit future research. First, we did not look into the underlying mechanisms of what forces people with NCDs in and out of the labor force, specifically in terms of co-morbidities (certain NCDs cluster in the same populations) and financial/social means available at an individual and collective level. How these mechanisms interact will also be different according to the level of economic and social development. For example, children in LMIC are more likely to be forced into the labor market due to the onset of NCDs in parents compared to children in HIC and the productive output of this child cannot replace the loss due to drop out by the parents. These related topics should be addressed separately to better understand how to modify and target these outcomes more specifically. Second, we observed wide heterogeneity in all domains within the studies selected, including study design, methods and sources used to measure productivity, adjustment for confounders and analyses. Third, no identified studies quantified the differential productivity impact by national economic development and labor market structure across countries. How these inter-country macro-economic differences might mitigate or magnify productivity losses associated with NCDs is worth further exploration. Fourth, we identified a crucial gap of relevant information from LMICs – limiting the relevance of our review most acutely in these settings. This lack of evidence could reflect differences in disease burden, in research capacity, in welfare systems and in epidemiological surveillance. The burden of NCDs is growing rapidly in LMIC; countries that often lack capacity in these key areas of support, prevention and knowledge generation. Further evaluation, therefore, of the macro-economic impact in the LMIC countries is urgently needed. Also, many NCDs affect people cumulatively over time; people may suffer DM, may experience absenteeism/presenteeism as a result, may reduce work as DM worsens and may finally drop out of the workforce due a stroke or CHD, which is related to

the DM. Given NCDs are shifting more and more into chronic conditions, as our understanding of treatment and natural history improve, it would be of great interest to investigate the effects over the life course rather than using short time horizons such as a year. This is no mean feat, but could be crucial for developing a better understanding of the economic impact of NCDs on a regional, national and international level. Also out of scope for this review but of interest for future work are the productivity-related impact of behavioural risk factors that contribute to the development of NCDs.

Conclusions

In summary, available studies indicate that the six main NCDs generate a large impact on macro-economic productivity in the WHO regions. However, this evidence is heterogeneous, of varying quality and not evenly geographically distributed. Data from LMI countries in economic and epidemiological transition are virtually absent. Further work to reliably quantify the absolute global impact of NCDs on macro-economic productivity and DALYs is urgently required.

Supplementary Material can be found online: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4457808/

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Chapter 3

Nutrition and Cardiometabolic Risk Factors

3.1 Polyunsaturated Fatty Acids and Serum C-reactive Protein: the Rotterdam Study

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ABSTRACT

We aimed to investigate whether total and individual (n-3, n-6 and n-3/n-6 ratio) polyunsaturated fatty acids (PUFAs) intake are prospectively associated with serum C-reactive protein (CRP), a marker of inflammation. We included 4,707 participants (1,943 men and 2,764 women) of the Rotterdam Study, a prospective follow-up study among subjects aged 55 years and older in the Netherlands. At baseline (1989-1993), dietary intake of PUFAs was assessed by a validated food frequency questionnaire. CRP was measured at baseline and at the third center visit (1997-1999). Regression coefficients (βs) and 95% CI were obtained by using linear generalized estimating equations. Dietary intake of butter and margarines explained most of the variance of PUFAs intake. After adjusting for possible confounding factors, higher intake of total PUFAs was associated with lower levels of CRP (β=-0.08, 95%CI=-0.15, -0.01; 4th quartile vs. 1st quartile). Similarly, intake of n-6 PUFAs was inversely related to CRP (β=-0.09, 95%CI=-0.16, -0.01 4th quartile vs. 1st quartile). No consistent trends were observed regarding n-3 PUFAs or n-3/n-6 PUFAs ratio and CRP. This study suggests that high intakes of total PUFAs are associated with lower levels of CRP reflecting diminished chronic systemic inflammation, which was mainly driven by n-6 PUFAs.

INTRODUCTION

Several dietary guidelines on cardiovascular disease and other chronic diseases encourage high consumption of both classes of polyunsaturated fatty acids (PUFAs), n-3 and n-6, as a replacement for other types of fatty acids such as saturated fatty acids[1,2]. Despite the consistency of favorable recommendations regarding dietary PUFAs, there are concerns that high amount of n-6 PUFAs in the diet may contribute to increase inflammation[3,4]. N-6 PUFAs trigger the formation of arachidonic acid, and therefore the synthesis of pro-inflammatory eicosanoids[5]. Elevated production of pro-inflammatory eicosanoids could increase levels of biomarkers of chronic systemic inflammation such as C-Reactive Protein (CRP) which has been associated with cardiometabolic outcomes and other age related disorders[6-8]. In addition, n-6 PUFAs compete with n-3 PUFAs for the same enzymes in fatty acids elongation and desaturation, and therefore could reduce the formation of anti-inflammatory eicosanoids that are derived from long chain n-3 PUFAs such as resolvins and neuroprotectins[9]. Thus far, only a few human studies have investigated the role that dietary intake of n-6 PUFAs may play on inflammation with contradictory results[10-13]. Also, there is little evidence to support a detrimental role of n-6 PUFAs on cardiovascular diseases[14,15]. In contrast, several studies found n-3 and n-6 PUFAs intake combined to be associated with lower inflammatory profile and cardiovascular outcomes[16,17]. Moreover, in the western diet, the consumption of plant derived n-3 PUFAs has almost doubled during the last decades, moving from 1 to 1.9 g/day[18]. Thus far, there is little controversy on the beneficial role of marine-derived n-3 PUFAs supplementation on chronic systemic inflammation[19], but, very little is known about the role of plant derived n-3 PUFAs on inflammation and particularly on CRP[20].

In this study we investigated the association between total PUFAs and individual PUFAs intake (n-3, n-6 and n-3/n-6 PUFAs ratio) and serum CRP in the Rotterdam Study, a prospective cohort of individuals 55 years old and above.

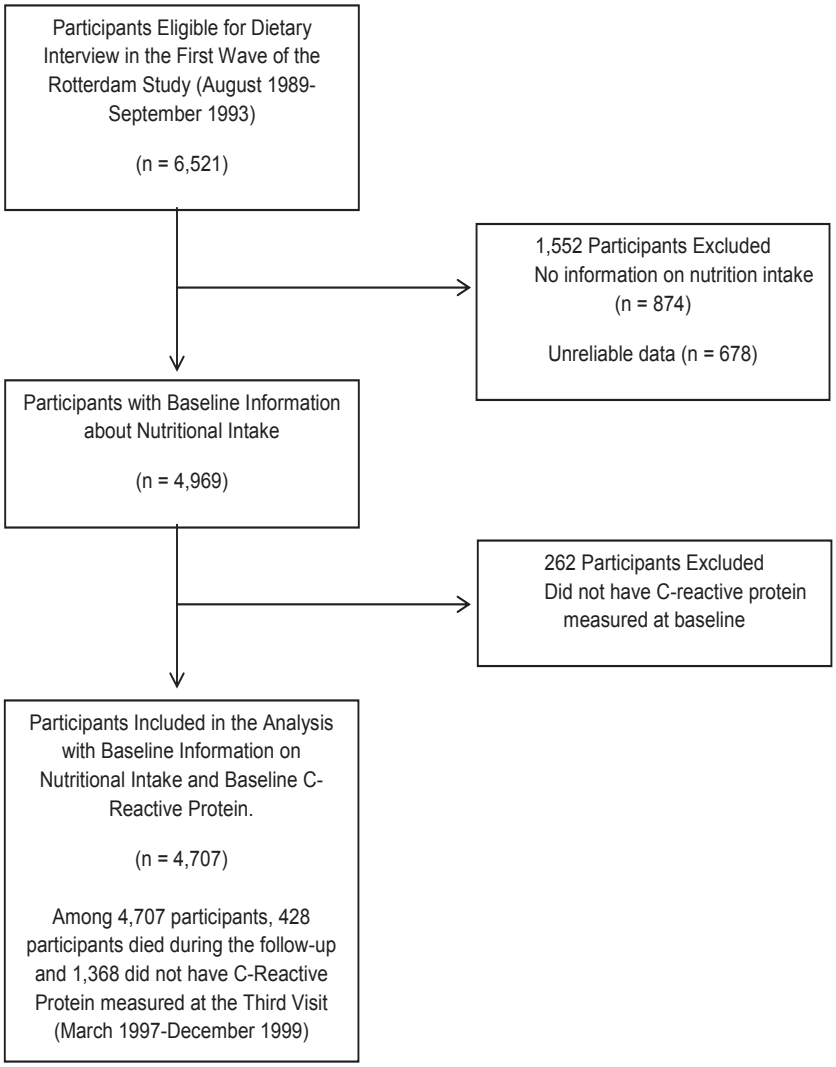
METHODS

Study population

The study was performed within the framework of the Rotterdam Study (RS), a population-based cohort among persons ≥55 years and older in the Ommoord district of Rotterdam, the Netherlands. The rationale and design of the RS is described elsewhere[21] . The baseline examination took place in 1989-1993 (RS-I). Trained research assistants collected data on current health status, use of medication, medical history, lifestyle and risk indicators for chronic diseases during an extensive home interview. Subsequently, the participants visited the study center for detailed clinical examinations and assessment of diet. Follow up visits were held every 2-3 years[21]. The study was approved by the Medical Ethical Committee of Erasmus University, and all participants gave informed consent. The present study used data from the baseline examination of the original cohort (RS-I). Among the participants there were 4,707 persons with available measurements for the

analyses (**Figure 1**). Among them, 1,796 participants died or did not have serum CRP measured (because of logistic reasons or no consent) at the third round visit (March 1997- December 1999). Our subset included 537 participants who reported use of anti-inflammatory drugs at baseline and/or during the follow up. A sensitivity analysis was performed after removing these subjects.

Figure 1 Flow Chart of Participants in the Study, The Rotterdam Study, 1989-1993



Assessment of diet

At baseline (1989-1993), participants indicated on a checklist at home all foods and beverages that they consumed at least twice a month during the preceding year. The completed checklist formed the basis for an interview at the study center by a trained dietician. A computerized validated 170-item semi-quantitative food frequency questionnaire (FFQ) was used[22]. These dietary data were

converted into total energy intake and fatty acid intakes per day using the Dutch Food Composition Table of 1993[23]. In a validation study including 80 participants of the RS, the FFQ was compared with fifteen 24-h food records collected over a 1-y period and with 24-h urinary urea excretion during 4 nonconsecutive days[22]. After adjustment for age, sex, energy, and within-person variation, the partial Pearson correlation coefficient was 0.62 for polyunsaturated fat[22].

Serum CRP

High-sensitivity CRP was measured in non-fasting frozen serum of study participants at baseline (1989-1993) and at the third center visit (1997-1999). A rate near-infrared particle immunoassay (Immage Imunnochemistry System, Beckman Coulter, Fullerton, CA, USA) was used. 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.

Assessment of covariates

The information on current health status, medical history, medication use, smoking, and socioeconomic status were assessed at the home interview at baseline. Education was defined as low (primary education), intermediate (secondary general or vocational education), or high (higher vocational education or university). Household income was categorized in low (<1700 euro/month), middle (1700-3000 euro/month) or high (≥3000 euro/month). Participants were asked whether they were currently smoke cigarettes, cigars, or pipe. Blood pressure was measured in the sitting position at the right upper arm with a random-zero-sphygmomanometer. Cardiovascular disease was defined as a history of myocardial infarction, coronary artery bypass, or percutaneous transluminal coronary angioplasty. Type 2 diabetes mellitus was diagnosed if a random serum glucose level was ≥11 mmol/L or if a person used glucose lowering drugs. Development of chronic diseases until the third round of the study was defined as the presence of cardiovascular disease, diabetes mellitus and cancer at the baseline and until the end of the third round visits (December 1999). Serum cholesterol was determined by an automated enzymatic procedure in a non-fasting blood sample. Anti-inflammatory drug use was defined as use of anti-inflammatory and anti-rheumatic drug, immunosuppressive and immune-modulating agent and bile and liver therapy at baseline and third round and dichotomized: Yes or No. The Dutch Healthy Diet (DHD)-index was used to take into account overall dietary quality. The DHD-index represents compliance to the Dutch Guidelines for a Healthy Diet as assessed from the FFQ at baseline[24] . To avoid over adjustment, the PUFAs component was removed from the DHD-index. Therefore, the following components were included within the DHD-index in this study: intake of vegetable, fruit, dietary fiber, fish, trans fat, saturated fat, alcohol and sodium. At the third visit to research center, the total weekly duration of physical activity was assessed by an adapted version of the Zutphen Physical Activity Questionnaire and the Longitudinal Aging Study Amsterdam Physical Activity Questionnaire[25,26]. Body mass index was calculated as weight divided by height squared (kg/m^2).

Statistical analyses

Continuous variables are reported as mean ± SD unless stated otherwise and categorical variables were presented as percentages. Pearson correlations were used to assess the association between PUFAs intake and their main dietary food sources. We used natural log-transformed values of serum CRP concentrations to better approximate normal distribution. To account for systematic measurement error and gender differences in dietary fatty acids intake, dietary fat intake was adjusted for total energy intake and gender by using the residual method in the analysis[27]. Dietary PUFAs intake was analyzed continuously and after categorization into quartiles. We fitted linear regression models using generalized estimating equations (GEE) with exchangeable correlation structure adjusting for the within-subject correlations due to the repeated measurements of CRP in the same individual (partial *Pearson* correlation=0.55 and intra-class correlation=0.67 for natural log transformed CRP)[28]. Regression coefficients (βs) and 95% Confidence Intervals on the basis of robust standard errors (95% CIs) were obtained. First, we calculated age and gender adjusted coefficients for the following exposures: total PUFAs, n-6, n-3 and n-3/n-6 PUFAs ratio. Subsequently, we adjusted for potential confounders based on previous findings[29,30], or when the covariate changed the estimate by more than 10%. Hence, final multivariable models were adjusted for: education level, household income, energy adjusted dietary cholesterol intake, physical activity, body mass index, smoking, anti-inflammatory drug use, DHD-index, prevalent chronic diseases at 3rd round, total serum cholesterol, HDL-cholesterol, systolic blood pressure, and n-3 or n-6 PUFAs for the analysis concerning n-6 and n-3 PUFAs respectively. Variables that were studied but did not alter the estimates by 10% or more included: dietary protein intake, hormone replacement therapy, statin use, and diastolic blood pressure. All analyses were also performed in men and women separately and the results presented are only for the multivariable models. In case of stratification, the intake of each fatty acids was divided into sex-specific quartiles. Before stratification, an interaction term between quartiles of exposure of interest and gender was added in the fully adjusted model and tested. We stratified by dietary fish intake (fish eaters vs. non fish eaters) and margarine and butter intake (stratified by median intake) to examine if dietary sources of n-3 PUFAs may play a role on the association between n-3 PUFAs and CRP. Moreover, to look if inclusion of subjects with no information on the second measure of CRP (1,719 subjects) could affect our results, we rerun all the analyses excluding them. To adjust for potential bias associated with missing data we used multiple imputation procedure (N= 5 imputations) (**Web Table S1**). Rubin's method was used for the pooled regression coefficients (β) and 95% CIs. All analyses were done using SPSS statistical software (SPSS, version 21.0; SPSS Inc, Chicago, Illinois**).**

Table 1 Characteristics of Study Participants, The Rotterdam Study, 1989-1997

	Male (*n*=1,943)			Female (*n*=2,764)		
Characteristic	**Mean (SD)**	**No.**	**%**	**Mean (SD)**	**No.**	**%**
Age, years	67.15 (7.29)			67.86 (8.00)		
Total PUFAs (g/day)	18.07 (8.41)			13.60 (6.83)		
N-6 PUFAs (g/day)	14.68 (7.85)			11.10 (6.32)		
N-3 PUFAs (g/day)	1.20 (0.54)			0.98 (0.48		
Dietary protein intake (g/day)	88.77 (20.69)			76.72 (17.04)		
Dietary cholesterol intake (g/day)	257.86 (88.95)			217.14 (72.15)		
Total energy intake (kcal/day)	2247.54 (506.34)			1791.46 (406.18)		
Education status						
Low		721	37.2		1704	61.7
Intermediate		916	47.1		941	34.0
High		306	15.7		119	4.3
Household income (%, N)						
Low (<1700 euro/month)		185	9.5		654	23.7
Middle (1700-3000 euro/month)		951	49		1385	50.1
High (≥3000 euro/month)		807	41.5		725	26.2
Physical activity-overall (min/week)	2436.37 (1137.78)			2652.64 (1094.57)		
Body mass index[a]	25.76 (2.91)			26.68 (4.00)		
Never/former smokers	71.1	1381	71.1		2238	81.0
Current smokers	28.9	562	28.9		526	19.0
Anti-inflammatory drugs	7.9	154	7.9		383	13.9
Lipid reducing drugs	2.7	53	2.7		72	2.6
Total DHD-index	45.50 (9.86)			50.28 (9.88)		
Diseases at 3rd visit						
Prevalent chronic disease		909	46.8		836	30.2
CHD		596	30.7		401	14.5

Diabetes		329	16.9		415	15.0
Cancer		197	10.1		157	5.7
Total cholesterol (mmol/l)	6.36 (1.13)			6.89 (1.19)		
HDL-cholesterol (mmol/l)	1.22 (0.33)			1.46 (0.36)		
Systolic blood pressure (mmHg)	138.36 (21.82)			138.36 (74.61)		
Diastolic blood pressure (mmHg)	74.61 (11.29)			73.35 (11.16)		
HRT	0.02	6	0.02		75	2.7
CRP, 1st round (mg/dl)[b]	0.187 (6.71)			0.176 (5.30)		
CRP, 2nd round (mg/dl)[b]	0.235 (11.48)			0.241 (9.02)		
Vegetable oils (g/day)	2.21 (4.31)			1.81 (3.27)		
Butter, margarines and hard frying fats (g/day)**	35.18 (18.85)			26.46 (15.11)		
Fish (g/day)	15.04 (19.20)			13.98 (17.61)		
Poultry(g/day)	12.61 (14.57)			12.49 (13.74)		
Red and processed meat (g/day)	111.96 (50.05)			85.56 (41.52)		
Sweet desserts and confectionary (g/day)	118.85 (81.00)			95.69 (70.55)		
Chips (g/day)	0.44 (3.75)			0.28 (2.28)		
Wholegrain (g/day)	148.95 (89.58)			114.80 (62.85)		
Total dairy[c] (g/day)	382.80 (273.35)			395.10 (242.65)		

CHD: Coronary Heart Disease; CRP, C-reactive protein; DHD-index, The Dutch Healthy Diet-index; HRT, hormone replacement therapy; No., number; %, percentage; PUFAs, polyunsaturated fatty acids; SD, standard deviation.

[a] kg/m^2

[b] median (range)

[c] including cheese

RESULTS

Characteristics of the study population and dietary intake of nutrients are shown in **Table 1**. Intake of total PUFAs, n-6 and n-3 PUFAs were lower in women than in men (13.60 g/day ± 6.83 g/day vs. 18.07 g/day ± 8.41 g/day for total PUFA , 11.10 g/day ± 6.32 g/day vs.14.68 g/day ± 7.85 g/day for n-6 PUFAs and 0.98 g/day ± 0.48 g/day vs. 1.20 g/day ± 0.54 g/day for n-6 PUFA) (**Table 1**). Specific dietary food intakes that significantly contributed to n-6 PUFAs and n-3 PUFAs intake are shown in table 2. Pearson (*r*) correlations of 0.31, 0.26 and 0.20 were found between n-6 PUFAs intake and butter (including margarines and hard frying fats), vegetable oils and wholegrain respectively (**Table 2**). The correlations of n-3 PUFAs with the specific dietary foods intake (**Table 2**), showed the highest correlations for butter (including margarines and hard frying fats), fish and red and processed meat (r=0.44, 0.33 and 0.24 respectively). The overall variance explained by these food items for n-6 PUFAs and n-3 PUFAs was 22% and 37% respectively, with butter and margarines explaining most of the variance for both n-6 and n-3 PUFAs (**Table 2**).

Table 2 Correlations Coefficients between n-6 and n-3 Polyunsaturated Fatty Acids and their Dietary Food Sources, The Rotterdam Study, 1989-1993[a].

Food Source	**n-6 PUFAs (g/day) Pearson Correlation *(r)***	**n-3 PUFAs (g/day) elation *(r)***
Vegetable oils (g/day)	0.26	0.06
Butter, margarines and hard frying fats (g/day)	0.31	0.44
Fish (g/day)	0.03	0.33
Poultry(g/day)	0.08	0.12
Red and processed meat (g/day)	0.15	0.24
Sweet desserts and confectionary (g/day)	0.14	0.13
Chips (g/day)	NS[c]	0.03
Wholegrain (g/day)	0.20	0.15
Total dairy (g/day)[b]	NS	0.05

NS, not significant (P<0.05); PUFAs: polyunsaturated fatty acids

[a] Overall variance explained by these food items: 22% for n-6 PUFAs; 37% for n-3 PUFAs.

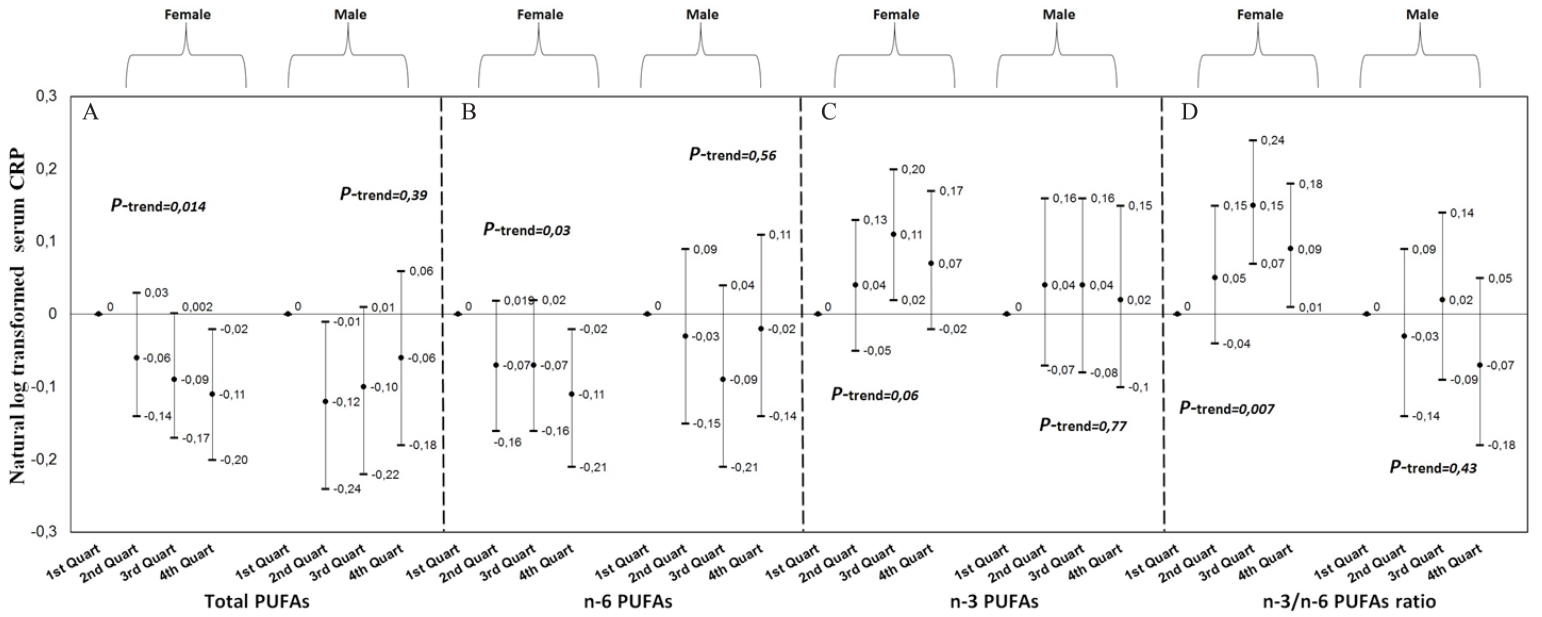
[b] including cheese

[c] does not significantly contribute to n-3 or n-6 PUFAs (p-value >0.05), otherwise, the p-value<0.05

Dietary intake of PUFAs and serum CRP

We observed an inverse association between total PUFAs intake and serum CRP after adjustment for age and gender (comparing extreme quartiles, β=-0.12, 95%CI=-0.19, -0.04; *P* for trend=0.003) (**Table 3**). The overall results changed only slightly after multivariable adjustment (**Table 3**). Also, higher n-6 PUFAs intake was associated with lower levels of CRP (comparing extreme quartiles:

Figure 2 Dietary intake of Polyunsaturated Fatty Acids and CRP by Gender, The Rotterdam Study, 1989-1997. The figure shows A) Total quarterly polyunsaturated fatty acids (PUFAs) intake; B) quarterly n-6 PUFAs intake; C) quarterly n-3 PUFAs intake and D) quarterly n-3/n-6 PUFAs intake. In all the analyses, serum C-Reactive Protein (CRP) is natural log-transformed. Tests for trend were carried out by entering the categorical variables as continuous variables in multivariable linear models fitted in Generalized Estimated Equations. *P*-trend: Figure A, women = 0.014, men = 0.33; Figure B, women = 0.03, men = 0.56; Figure C, women= 0.06, men = 0.77; Figure D, women = 0.007, men = 0.43. $P_{Interaction}$: Total PUFAs x Gender, *P*=0.19; n-6 PUFAs x Gender, *P*=0.37; n-3 PUFAs x Gender, *P*=0.20; n-3/n-6 PUFAs ratio x Gender, *P*=0.029.● Female ◆ Male



multivariable β= -0.09, 95%CI=-0.16, -0.01; *P* for trend=0.033) (**Table 3**). Intake of n-3 PUFAs was associated with an increase in serum CRP in the age and gender adjusted model (comparing extreme quartiles: β= 0.08, 95%CI= 0.006, 0.16; P for trend=0.01) (**Table 3**). Nevertheless, after adjustment for other covariates, no consistent relationship was found (**Table 3**; *P* for trend=0.16). Similarly, no linear trend was observed between n-3/n-6 PUFAs ratio and serum CRP in the multivariable model (*P* for trend=0.23) (**Table 3**).

Table 3 Dietary Intake of Polyunsaturated Fatty Acids and Serum C-Reactive Protein, The Rotterdam Study, 1989-1997

Quartile, by PUFAs Type	CRP First Round, mg/DL (Median)	CRP Third Round, mg/DL (Median)	Model 1[b]		Model 2[c]	
			β	95% CI	β	95% CI
Total PUFAs						
Continuous[c]			-0.06	-0.1, -0.02	-0.03	-0.07, 0.01
Quartile 1	0.195	0.261	0	Referent	0	Referent
Quartile 2	0.176	0.230	-0.11	-0.19, -0.04	-0.009	-0.16, -0.02
Quartile 3	0.174	0.234	-0.12	-0.20, -0.05	-0.10	-0.17, -0.02
Quartile 4	0.173	0.233	-0.12	-0.19, -0.04	-0.08	-0.15, -0.01
P value[d]				0.003		0.039
n-6 PUFAs						
Continuous			-0.06	-0.1, -0.02	-0.03	-0.08, 0.01
Quartile 1	0.198	0.253	0	Referent	0	Referent
Quartile 2	0.178	0.240	-0.07	-0.15, 0.004	-0.07	-0.14, -0.004
Quartile 3	0.174	0.240	-0.09	-0.16, -0.01	-0.07	-0.14, 0.005
Quartile 4	0.172	0.227	-0.12	-0.20, -0.04	-0.09	-0.16, -0.01
P value				0.002		0.033
n-3 PUFAs						
Continuous			0.39	-0.20, 0.99	0.17	-0.43, 0.76
Quartile 1	0.169	0.207	0	Referent	0	Referent
Quartile 2	0.179	0.238	0.09	0.01, 0.16	0.05	-0.03, 0.12
Quartile 3	0.193	0.262	0.15	0.08, 0.23	0.08	0.01, 0.16
Quartile 4	0.178	0.246	0.08	0.006, 0.16	0.05	-0.03, 0.12
P value				0.01		0.16

n-3/n-6 PUFAs ratio						
Continuous[e]			0.06	-0.05, 0.17	0.00	-0.10, 0.11
Quartile 1	0.165	0.221	0	Referent	0	Referent
Quartile 2	0.183	0.230	0.06	-0.01, 0.14	0.02	-0.06, 0.09
Quartile 3	0.193	0.256	0.14	0.07, 0.21	0.09	0.02, 0.16
Quartile 4	0.178	0.246	0.06	-0.02, 0.13	0.02	-0.05, 0.09
P value				0.05		0.23

β, regression coefficients; CI, confidence intervals; CRP, C-reactive protein; PUFAs: polyunsaturated fatty acids
[a]In all the analyses, serum CRP is natural log-transformed.
[b]Model 1:adjusted for age (continuous) and gender
[c]Model 2: Model 1 + education level (low, intermediate, high), household income (low, middle, high), smoking status (current vs. former/never) physical activity (continuous), body mass index (continuous), dietary cholesterol intake (continuous), total serum cholesterol (continuous), HDL-cholesterol (continuous), anti-inflammatory drugs (yes vs. no), Dutch Healthy Diet-index excluding PUFAs component (continuous), systolic blood pressure (continuous) and presence of chronic disease (yes vs. no). The analysis on n-3 and n-6 PUFAs were additionally adjusted for each other (continuo
[c]Reported per 10 units increase in energy adjusted dietary fatty acids intake
[d]Tests for trend were carried out by entering the categorical variables as continuous variables in multivariable linear models fitted in Generalized Estimated Equations.
[e]2 subjects were removed because of extreme negative values

Dietary intake of PUFAs and serum CRP by gender

A linear negative trend between total PUFAs and CRP was observed only in women (*P* for trend=0.014 in women vs. 0.39 in men) (**Figure 2 A**). Also, high intake of n-6 PUFAs was associated with lower levels of CRP in females (comparing extreme quartiles: multivariable β=-0.11, 95%CI=-0.21, -0.02, *P* for trend=0.03) but not in males (**Figure 2 B**). Among females, 3rd quartile of n-3 PUFAs was positively associated with CRP (compared to the 1st quartile, 3rd quartile: β=0.11, 95%CI=0.02, 0.20) but not in males (**Figure 2 C**). Nevertheless, no linear trend was observed neither in men, nor in women (**Figure 2 C**). In contrast, high dietary n-3/n-6 PUFAs ratio was related to higher serum CRP in females but not in males (comparing extreme quartiles: multivariable β=0.09, 95%CI=0.01, 0.18; *P* for trend=0.007;$P_{Interaction}$=0.029 for gender x n-3/n-6 PUFA interaction; **Figure 2 D**).

Additional analysis

When stratified by dietary fish intake, the positive association between n-3 PUFAs or n-3/n-6 PUFAs ratio and CRP was observed only among participants who did not consume fish or among participants with high butter consumption (higher than median) (**Web Figure S1**). No association was observed between n-3 PUFAs or n-3/n-6 PUFAs ratio and CRP among fish-eater participants or among participants with butter consumption lower than median (**Web Figure S2**).

Exclusion of subjects who reported use of anti-inflammatory drugs at baseline and during the follow-up from our analyses or restriction of all analyses on participants with available information of both measures of CRP (2,911 participants) did not substantially affect our results (results presented stratified by gender) (**Web Figure S3-S4**).

DISCUSSION

Overall we observed an inverse association for total PUFAs intake and serum CRP irrespective of gender, other dietary factors, lifestyle and cardiovascular risk factors. These associations seemed to occur more prominently in women.

Our findings are in accordance with previous studies reporting decreased CRP with total PUFAs intake[10,16]. Pischon and his colleagues showed that a combination of both n-3 and n-6 PUFAs is related to a lower inflammatory profile[16]. Also, a recent prospective study showed that total PUFAs intake are inversely associated with plasma CRP[10].

Both n-3 and n-6 PUFAs inhibit the activities of delta 6 and delta 5 desaturase and the activity of cyclooxygenase, which are all involved in fatty acid regulation that influences pro- and anti-inflammatory mediators[31-33]. Therefore, high intake of both n-3 and n-6 PUFAs could lead to a reduction in inflammation. Also, PUFAs can modify the activity of transcription factors, such as peroxisome proliferator activated receptors and nuclear factor kappa B. Peroxisome proliferator activated receptors can interfere with the activation on nuclear factor kappa B, by inhibiting signaling molecules, and therefore impeding the production of pro-inflammatory cytokines[34].

Concerns have been raised that high consumption of n-6 PUFAs in diet may increase the risk of chronic diseases by enhancing inflammation[3,4]. These concerns comes from the fact that n-6 PUFAs intake has been generally assumed to be associated with production of eicosanoids which have pro-inflammatory properties[35]. However, recent studies suggest that arachidonic acid, a metabolite of n-6 PUFAs, serves as a precursor for a group of anti-inflammatory mediators as well[36]. Also, it has been reported that some arachidonic acid -derived eicosanoids have both pro and anti-inflammatory roles. For instance, it decreases the production of 4-series leukotrienes[11], and promotes the formation of lipoxins[11] that have been found to have anti-inflammatory properties[12].

Thus far, few studies have been published that directly explore whether dietary n-6 PUFAs affect serum CRP. A recent systematic review of randomized clinical on dietary n-6 PUFAs intake and inflammation concluded that there is no evidence that dietary intake of n-6 PUFAs increases inflammatory markers[37]. However the studies included in the systematic review had not enough statistical power which was limited by their small number of participants and their internal and extern validity was questioned (the largest study had 60 subjects that completed the experiment and some of the studies were conducted in metabolic wards with 6 to 9 participants). Moreover, Ferruci and colleagues observed that higher levels of plasma arachidonic acid concentrations were associated with lower interleukin-6 and higher transforming growth factor beta levels[38]. Interleukin-6 is a marker of inflammation and regulates the production of CRP whereas transforming growth factor beta has potent anti-inflammatory activities.

A few studies focused on total n-3 PUFAs and CRP, have shown inverse or no associations so far[13,39-41]. Different from our study, most of these studies were of cross-sectional design and could not address the causality. Moreover, the studies showing inverse associations between dietary n-3 PUFAs intake and CRP, come mainly from Japan, where the consumption of fish intake, and

therefore long-chain n-3 PUFAs is high[39-41]. One of these studies showed that the associations were stronger when eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) intakes were high[41]. Also, the only prospective study conducted in middle-age populations, but based on only one measure of serum CRP available at follow up, concluded that the inverse association found between total n-3 PUFAs and CRP was mainly driven by long chain n-3 PUFAs[10]

Previously, it has been suggested that the inconsistent findings of n-3 PUFAs from epidemiological studies might be due to different sources of PUFAs[42]. Also, it has been shown that plant-derived n-3 PUFAs can have different roles on health (including immunity and inflammation) as compared to marine-derived n-3 PUFAs[20,43,44]. The n-3 fatty acids are present in the diet as alpha linolenic acid (ALA), EPA and DHA. ALA is found in some vegetable oils, meat and margarine products whereas the richest dietary source of EPA and DHA is fatty fish. ALA is de-saturated into EPA, which is converted later into DHA, but this conversion is limited in humans[45]. EPA and DHA increases an alternative family eicosanoids which frequently have lower potency than those produced from arachidonic acid [5].

In our study, the main source of n-3 PUFAs was butter and margarine. Also, a strong correlation between n-3 PUFAs and red and processed meat was seen. In a large European study by Saadatian-Elahi et al, a positive correlation between processed meat and ALA, EPA and DHA was reported, but not between red meat and ALA[46]. In the Netherlands, red and processed meat generally may contain limited levels of EPA and DHA. The difference between our results and those from Saadatian-Elahi may be explained by different food practices related to meat consumption (e.g. addition of sauces and oils). Therefore we postulate that the correlation between meat intake and n-3 fatty acids we found is probably explained by ALA. Moreover, 39% of the study participants did not consume fish, which may imply a higher ALA intake as compared to EPA and DHA, and thus may further add to explaining the results of our study. In addition, the association between n-3 PUFAs or n-3/n-6 PUFAs ratio and CRP was observed mainly among participants with lower fish intake and higher butter consumption. This may suggest that dietary sources of n-3 PUFAs might be important to take into account when evaluating their role on inflammation and other health outcomes.

We found potential gender differences in the association between dietary PUFAs intake and CRP, which is in line with previous studies[16,40]. It has been suggested that the observed differences between men and women may be mainly due to differences in dietary intake. In our study, we corrected for gender differences in dietary intake, therefore, the differences observed in men and women are not likely to be due to differences in dietary intake. Previous studies have reported gender differences in the conversion of ALA to EPA and DHA[47]. It has been shown that women have more ALA availability than men because of lower partitioning of women toward beta oxidation[48,49]. In addition, there might be gender differences in the activity of desaturation-elongation pathway for the conversion of EPA to DHA[50]. Also, this may be explained by the action of estrogen in the conversion of ALA to EPA and DHA because DHA synthesis is almost threefold greater in women using oral contraceptives than those without it[49].

Our study is unique among previous investigations because of its prospective design, large population-based study group and adjustment for a broad range of confounders. Also, to our knowledge, this is the first prospective study to use measures of CRP in two time points.

First, dietary fat intake was assessed at baseline, and there may have been changes in fat consumption over time. Second, the FFQ can be limited by errors in reporting and recall and by incomplete assessment of all sources of fat intake, which may introduce misclassification in dietary intakes and would bias results toward the null. However, the data are suitable for ranking people and the FFQ we used was validated and showed a relatively high correlation with the intake of fatty acids. Third, we were not able to investigate associations with specific n-3 PUFAs or serum levels of fatty acids. Furthermore, although CRP has been associated with cardiovascular disease in many observational studies[6], the nature of the association remains under discussion since Mendelian randomization studies did not establish a relation with cardiovascular disease and causality might not be present[51]. Hence, if PUFAs intake contribute to lower inflammation and to reducing subsequent cardiometabolic risk needs to be further established. Also, we did not have repeated measurements of other inflammatory markers. Investigation of for example interleukin-6 or TNF-alpha could have strengthen our results and further clarify the association between fatty acids intake and inflammation. Finally, physical activity was measured in the third round of the Rotterdam Study. Therefore, we cannot fully exclude residual confounding by physical activity levels.

In conclusion, we found that high intake of PUFAs (mainly n-6 PUFAs) are associated with lower levels of CRP which might reflect diminished chronic systemic inflammation. The current study may provide additional support for the maintenance of the present level of n-6 PUFAs intake. At the same time, these results suggest that substitution of plant-derived n-3 PUFAs with marine derived n-3 PUFAs may be considered and further evaluated.

Supplementary Material can be found online: http://aje.oxfordjournals.org/content/early/2015/04/21/aje.kwv021

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3.2 Dietary Fat Composition, Total Body Fat and Regional Body Fat Distribution in Two Caucasian Populations.

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ABSTRACT

Objective: We aimed to study whether dietary fat composition is associated with total body fat (TF) and body fat distribution in middle-aged and elderly women participants in two population-based cohorts in the Netherlands and Australia.

Methods: The study was performed in the Rotterdam Study (RS), a prospective cohort study among subjects aged 55 years and older (N=1,182 women) and the Calcium Intake Fracture Outcome Study (CAIFOS), a 5-year randomized controlled trial among women age 70+ (N=846). At baseline, diet (i.e. polyunsaturated and saturated fatty acids (PUFAs and SFA) was measured by validated food frequency questionnaires. TF was assessed using Dual-energy X-ray absorptiometry in both studies and android abdominal fat (AF), gynoid fat (GF) and the android/gynoid ratio (A/G ratio) in the RS but not the CAIFOS.

Results: No association was found between dietary n-3/n-6 PUFAs ratio or SFA/PUFAs ratio with TF in both cohorts. In the RS, a high n-3/n-6 PUFAs ratio was associated with a higher AF (3rd vs 2ndtertile (reference): β: 0.15; 95%CI: 0.05, 0.24) but not with the A/G ratio. A low SFA/PUFA ratio was associated with a lower AF (1st vs 2ndtertile (reference): β: -0.12; 95%CI: -0.22, -0.06) but not with the A/G ratio.

Conclusion: These findings do not support the hypothesis that dietary fat composition is consistently associated with TF and body fat distribution in a population-based setting.

INTRODUCTION

There is a large variability in body fat distribution in both lean and obese adults. Android obesity is characterized by the accumulation of body fat in the upper truncal region and is most common in men, whereas gynoid obesity is defined as an accumulation of body fat in hips and thighs and is most common in women[1]. Regional fat distribution has been considered to be more important in understanding the association of obesity with chronic disease than overall body fat mass[2-4]. Android obesity has been associated with an increased risk of atherosclerosis, type 2 diabetes mellitus, cardiovascular disease and total mortality[5-9]. Contrary to this, gynoid obesity has been independently associated with a reduced risk of metabolic complications[2,3].

Although epidemiological studies have identified potentially modifiable factors that affect body fat distribution, such as physical activity and cigarette smoking[10-12], the role of dietary intake, particularly dietary fat composition, and its effect on the distribution of body fat remains controversial[13-17]. There is some evidence that different types of fatty acid have different rates of oxidation, depending on their chemical structure[18,19]. Impaired fat oxidation has been found to be associated with the development of obesity[20]. However, whether different dietary fatty acids also affect the distribution of body fat remains unclear. Some but not all studies suggest that high dietary polyunsaturated fatty acids (PUFAs) intake is associated with a greater fat mass loss in the abdominal region[21,22] whereas dietary saturated fatty acids (SFAs) intake is associated with abdominal obesity[23]. Nevertheless, results from long-term studies linking dietary fat intake to regional fat distribution remain scarce. Additionally, previous studies generally used anthropometric measures such as waist circumference or BMI[21,23-27] instead of more accurate measures of body composition by dual-energy X-ray absorptiometry (DXA) [28,29]. Moreover, previous research on the relation of dietary fat with body fat has focused primarily on men [23,27,30,31] whereas there may be gender-specific differences[32]. Furthermore, previous studies have not taken into account the role that chronic disease may play in the association between dietary fat intake and body fat. Chronic disease may alter weight and waist measures and therefore body fat[33,34], as well as dietary intake[35].

We therefore examined the hypothesis that dietary fat composition (i.e. dietary ratios of n-3/n-6 PUFAs and SFAs/PUFAs) is associated with total body fat and regional body fat distribution, as measured by DXA, and the presence of chronic disease modifies this association, in two Caucasian study populations from the Netherlands and Australia.

SUBJECTS AND METHOD

The present study included two geographically distinct study populations, the Rotterdam Study (RS) and the Calcium Intake Fracture Outcome Study (CAIFOS). The RS is a community based prospective cohort study that started in 1990 to examine the determinants of disease and health in the elderly. Inhabitants of a suburb of Rotterdam, the Netherlands (n=7,983), aged 55 years or

older were included. The rationale and design of the RS is described elsewhere[36]. Trained research assistants collected data on current health status, use of medication, medical history, lifestyle and risk indicators for chronic disease during an extensive home interview at baseline examination which took place in 1989-1993. Subsequently, the participants visited the study center for detailed clinical examinations and assessment of diet. Follow up visits were held every 2-3 years[36]. The present study used data from the baseline examination of the original cohort. Out of 6,521 non-institutionalized subjects (85% of the cohort) who visited the study center at baseline were eligible for a dietary interview. Dietary data was not obtained in 271 subjects who participated in the pilot phase of the Rotterdam Study. Also, diet was not assessed in 122 subjects suspected with dementia because of expected difficulties in dietary recall, and in a random group of 481 subjects due to logistic reasons. Additionally, 678 dietary reports were excluded because they were considered to be unreliable by the dietician or because of extreme total energy intake (2 interquartile ranges of the 25th and 75th percentile of the distribution of total energy). Dietary data was thus available for 4,969 subjects. During 14 years of follow up, there were 1,454 participants who died (710 women and 744 men) and 1,488 subjects (454 women and 1,033 men) with no DXA scan examination, leaving 2,028 subjects for analysis. 848 male subjects were further excluded from the main analyses. Therefore, 1,182 women were included in the analysis

The Calcium Intake Fracture Outcome Study (CAIFOS) started in 1998 as a randomized, controlled trial of oral calcium supplements to prevent osteoporotic fractures as described previously[37]. Briefly, 1,460 ambulant women with no medical conditions (e.g. cancer) that may influence 5-year survival were recruited from the Western Australian general population of women older than 70 years. Participants were similar in terms of disease burden but were more often from a higher socioeconomic background relative to the general population[38]. During 5 years of follow-up, participants received 1.2 g of elemental calcium supplementation (calcium carbonate) daily or a placebo. For the present study, 906 participants who completed a food frequency questionnaire (FFQ) at baseline in 1998 and had DXA measured 5 years later in 2003 were included. Forty-six women were further excluded because of missing data on covariates, leaving 860 women for the final analysis.

Assessment of dietary fat composition

In both cohorts, information on dietary intake at baseline was collected by using self-administered FFQ but that were adapted for and validated in the Dutch and Australian setting for each cohort as described in detail previously[30,39]. The FFQ asked respondents to recall how often, on average, they consumed a standard serving size of food in the previous 12 months. Energy and nutrient intakes were estimated based on frequency of consumption, standardized portion sizes and using the Dutch food composition table for RS[40] and the NUTTAB95 food composition database for CAIFOS[41].

Previous studies have demonstrated that the FFQs were a valid instrument for ranking individuals by fat intake in the RS and CAIFOS[30,39]. For example, in the RS, in a comparison of the FFQ with fifteen day-food records collected over a year, partial Pearson correlation coefficients of

this comparison, after adjustment for age, sex, energy, and within-person variation were 0.52 for saturated fat and 0.62 for polyunsaturated fat[30].

Measures of body composition

Total body composition was assessed by DXA during the fourth visit of RS (January 2002-July 2004) and at the 5 year visit (2003) after baseline (1998) in CAIFOS using total body-fat beam densitometer (GE Lunar Corp, Madison, WI, USA for RS and Hologic Corp, Waltham, MA, USA for CAIFOS). Body weight (grams) was divided into bone mineral content, lean (non-fat) and fat mass. In both cohorts, fat distribution was determined by measuring fat mass at the total-body, whereas in the RS was additionally determined by measuring fat mass in android and gynoid regions. Body fat was assessed as percentage of body fat taking into consideration the weight of the individual and total fat as well (when using android and gynoid fat as outcomes). Body fat mass index (BFMI) was calculated as total body fat mass (kg) divided by body surface (the square of the height in m i.e. m^2)[42]. This fat mass index eliminates the differences of the body fat associated with an individual's height. Total body fat mass, android and gynoid fat mass were available in the RS, whereas in CAIFOS, total body fat mass was the only measure of body fat available.

Potential confounding variables

Information on current health status, medical history, medication use, smoking behavior, and socioeconomic status was obtained at baseline for both studies. Participants were asked whether they were currently smoking cigarettes, cigars, or pipes. Education was defined as low (primary education), intermediate (secondary general or vocational education), or high (higher vocational education or university). Alcohol intake was assessed in grams of ethanol per day. Cardiovascular disease was defined as a history of myocardial infarction, coronary artery bypass, or percutaneous transluminal coronary angioplasty. Development of chronic disease was defined as the presence of cardiovascular disease, diabetes mellitus and cancer at the baseline and until the DXA measurements took place. Baseline hypertension was defined as a blood pressure ≥140/90 mmHg or use of blood pressure lowering medication, prescribed for the indication of hypertension. Baseline diabetes mellitus was defined as a serum glucose level ≥11 mmol/L or use of glucose lowering drugs. To take into account overall dietary quality we used The Dutch Healthy Diet (DHD)-index[43] for the Rotterdam Study and the Nutrient-Rich Foods (NRF)-index for CAIFOS, adapted for use with Australian dietary recommendations. In the RS, at the third visit to the research center, the total weekly duration of physical activity was assessed by an adapted version of the Zutphen Physical Activity Questionnaire and the LASA Physical Activity Questionnaire[44,45]. In CAIFOS, women were asked if they participate in any sport, recreational, or regular physical activity. Those who answered 'no' scored zero and those who answered 'yes' were asked to list up to four types of physical activity undertaken in the last 3 months. Activity levels were calculated in kcal/day using a previous method utilizing body weight, number of hours and type of physical activity and energy costs of such activities[46,47]. Physical height (m) and body weight (kg) were measured at baseline with the participants standing without shoes and heavy outer garments. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2).

Statistical analysis

Data are presented as the mean ± SD unless indicated otherwise. Pearson correlations coefficients were computed to identify the major food source of dietary PUFAs and SFAs in both populations. Dietary PUFAs and SFAs intake were adjusted for total energy intake using the residual method to account for systematic measurement error[48]. Dietary fat was analyzed in tertiles and the second tertile was considered the reference group since this included the mean intake of the population.

Multivariable linear regression models were used to examine whether dietary n-3/n-6 PUFAs ratio and the SFAs/PUFAs ratio were independently associated with, total body fat mass (%) in both cohorts. In the RS, additional analyses were performed to investigate the relationship between the these two exposures and android fat mass (%), gynoid fat mass (%) and android/gynoidfat mass ratio. We calculated age-adjusted (model 1) regression coefficients and 95% confidence intervals (CIs) for each type of dietary fatty acid intake and each of the outcomes of interest. To control for potential confounding factors, a second multivariable model was built (model 2), which was adjusted for relevant covariates. The following covariates were tested: baseline total energy intake (continuous), BMI (continuous variable), total alcohol intake (quartiles), total physical activity (quartiles), smoking status (current smokers vs. never/former smokers), education (intermediate, high education vs. primary education), income (intermediate, high income vs. low income), hypertension (yes vs. no), more than 3 kg weight loss in the last 12 months (yes vs. no), the presence of a chronic disease during follow-up (coronary heart disease, diabetes mellitus and cancer: yes vs. no), and overall dietary quality by using the DHD-index (continuous) for the RS and the NRF-index (continuous) for CAIFOS respectively. The components of SFAs and PUFAs were removed from the DHD-index and NRF-index to avoid over- adjustment. Potential confounding factors were considered relevant if their inclusion in the model resulted in a change of at least 10% in the regression coefficient of the exposure[32]. In all analysis for the CAIFOS, we adjusted for the intervention effect by adding treatment (yes vs. no) to the models. We tested for possible nonlinear effects of the exposure of interest using quadratic and cubic effects. The *F* test was used to test whether there were overall significant differences in fat mass (total, android or gynoid fat mass) between levels of dietary fat intake. To test for effect modification by the presence of chronic disease, the product term of tertiles of fatty acids and prevalent chronic disease were added to the model. Effects with a P-value lower than 0.05 were considered statistically significant. In case of significant effect modification, stratified analyses were performed.

The following sensitivity analyses were performed: First, since calcium may affect body weight[49], an interaction term between fatty acids and calcium-treatment was tested in the age-adjusted models for the CAIFOS cohort to evaluate if the intervention had an impact on the relationship between the exposure and outcome. Second, since most of the previous studies were performed in men only[23,27,30,31], to establish whether findings were similar for men and women, we performed the analysis in the male participants of the RS as well.

To reduce the potential bias due to missing covariate data multiple imputation (m=5 imputations) was applied to the RS data. Pooled regression coefficients (β) and 95% CIs were calculated using Rubin's rules[50]. All analyses were performed in IBM SPSS Statistics 21.

Table 1 Selected characteristics of study population in the Rotterdam Study and Calcium Intake Fracture Outcome Study.

Baseline characteristics	**RS (N=1182)**	**CAIFOS (N=860)**
Age	64.12 ± 6.03	74.86 ± 2.57
N-3 PUFAs (g/day)	0.98 ± 0.47	1.29 ± 0.63
N-6 PUFAs (g/day)	11.27 ± 6.43	8.76 ± 4.02
Total PUFAs (g/day)	13.70 ± 6.98	10.79 ± 4.69
Total Saturated Fat (g/day)	28.56 ± 10.12	25.16 ± 10.90
Total energy intake (kcal/day)	1790.54 ± 391.11	1650.10 ± 490.46
BMI (kg/m^2)	26.34 ± 3.8	26.85 ± 4.52
Total alcohol (g/day)*	6.89 (66.82)	2.04 (66.15)
Lower Education, n (%)	398 (33.6)	272 (30.8)
Medium Education (%)	685 (58.0)	368 (41.7)
High Education (%)	99 (8.4)	243 (27.5)
Income (%)		
low	394 (33.3)	301 (34.0)
Medium	442 (37.4)	315 (35.5)
High	346 (29.3)	270 (30.5)
Never/former smokers	959 (81.1)	885 (99.8)
Current smokers	223 (18.9)	2 (0.2)
Physical activity (min/week)	2912.6 ± 1056.4	125.93 (1478.40)*@
Hypertension (%)	(540) 45.7	375 (42.1)
More than 3 kg loss in last 12 months (%)	86 (7.3)	91 (10.3)
Total DHDI/ NRFI	52.75 ± 10.45	37.85 ± 9.55
Characteristics at the end and during the follow-up		
Total whole body fat mass (kg)	23.31 ± 7.56	24.84 ± 7.38
Total body fat mass index (kg/m^2)	10.66 ± 3.35	9.59 ± 2.87
Android Fat (kg)	2.41 ± 0.94	NA
Gynoid Fat (kg)	4.51 ± 1.39	NA
Prevalent chronic disease (%)$	346 (30)	160 (18.0)
Chronic disease development (%)#	225 (19.0)	64 (7.2)

DHDI, Dutch Healthy Diet-index; NRFI, Nutrient-Rich Foods Index, PUFAs: polyunsaturated fatty acids
Plus-minus values are means ±SD
* median (range)
@kcal expended/day
$Percentage of participants who have at least one of the chronic disease (cardiovascular disease, diabetes or cancer) when the body composition was assessed.
#Percentage of participants who developed a chronic disease (cardiovascular disease, diabetes or cancer) from baseline until the measurements of body composition.

Table 2. Correlation coefficients between n-6 and n-3 PUFAs and their dietary food sources*.

n-6 PUFAs (g/day)	**Rotterdam Study Pearson Correlation *(r)***	**CAIFOS Pearson Correlation *(r)***
Vegetable oils (g/day)	0.23	NA
Butter, margarines and hard frying fats (g/day)	0.34	0.60
Fish (g/day)	-	0.16
Poultry(g/day)	0.09	0.16
Processed red meat (g/day)	0.12	0.18
Sweet desserts and confectionary (g/day)	0.13	0.31
Chips (g/day)	-	0.13
Wholegrain (g/day)	0.11	0.31
Total dairy** (g/day)	-	0.12
n-3 PUFAs (g/day)	**Rotterdam Study Pearson Correlation *(r)***	**CAIFOS Pearson Correlation *(r)***
Vegetable oils (g/day)	-	NA
Butter, margarines and hard frying fats (g/day)	0.38	0.47
Fish (g/day)	0.29	0.67
Poultry(g/day)	0.13	0.30
Processed red meat (g/day)	0.27	0.21
Sweet desserts and confectionary (g/day)	0.09	0.31
Chips (g/day)	-	0.10
Wholegrain (g/day)	-	0.31
Total dairy** (g/day)	-	0.18
SFAs (g/day)	**Rotterdam Study Pearson Correlation *(r)***	**CAIFOS Pearson Correlation *(r)***
Vegetable oils (g/day)	-	NA
Butter, margarines and hard frying fats (g/day)	0.58	0.38
Fish (g/day)	-0.09	0.17
Poultry(g/day)	-	0.25
Processed red meat (g/day)	0.37	0.42
Sweet desserts and confectionary (g/day)	0.31	0.59
Chips (g/day)	-	0.33
Wholegrain (g/day)	0.09	0.08
Total dairy** (g/day)	0.12	0.20

PUFA: polyunsaturated fatty acids

*Overall variance explained by these food items: 22% for n-6 PUFAs; 37% for n-3 PUFAs.

**including cheese

-does not significantly contribute to n-3 or n-6 PUFAs (p-value >0.05), otherwise, the p-value<0.05

RESULTS

Characteristics

Table 1 displays the selected characteristics of the 2 populations. In the RS, the mean age of study participants were younger (64.12 ± 6.03 vs. 74.86 ± 2.57 years), used to smoke more (18.9% vs. 0.2%) and consume more alcohol (6.89 vs. 2.04 g/day) than in CAIFOS. Also, the mean intake of total PUFAs was higher in the RS than in CAIFOS (13.70 ± 6.98 g/day vs. 10.79 ± 4.69). Main sources of n-6 PUFAs intake were butter, margarines and frying fats in both the RS and CAIFOS (r=0.34 and 0.60 respectively) (**Table 2**). However, the main sources of n-3 PUFAs in the RS were also butter, margarines and hard frying fats (r=0.38) whereas fish was the main source of n-3 PUFAs in CAIFOS (r=0.67) (Table 2). The main source of SFAs were butter, margarines and hard frying fats in the RS (r=0.58) and sweet desserts and confectionary in CAIFOS (r=0.59).

Dietary fat composition, total body fat and regional body fat distribution

We found no association between dietary intake of n-3/n-6 PUFAs and SFAs/PUFAs ratio and total body fat mass (%) in either study (**Table 3** and **Table 4**). However, in the RS high a dietary n-3/n6 PUFAs ratio was associated with higher android fat (3rd vs. 2ndtertile: β=0.15; 95%CI: 0.05 – 0.24; P for F-test, 0.08, Table 3) but not with higher android/gynoid ratio (Table 3). Low SFAs/PUFAs ratio was associated with lower android fat (1st vs. 2ndtertile: **β**=-0.12; 95%CI: -0.22 – 0.06; P for F-test, 0.30, Table 3) but not with andoid/gynoid ratio (Table 3).

Effect modification by chronic disease

The presence of chronic disease was found to be a significant effect modifier in the analysis of n-3/n-6 PUFAs and total body fat mass in both studies ($P_{interaction}$<0.05, **Table 3** and **Table 4**). In the RS, among women without chronic disease, high n-3/n-6 PUFAs ratio was positively associated with total body fat mass (%) (3rd tertile vs. 2ndtertile: β=0.94; 95%CI: 0.12, 1.76; P for F-test, 0.07) whereas no association was found between n-3/n-6 PUFAs ratio and total body fat mass (%) in participants with the presence of chronic disease (**Figure 1**). Effects in the opposite direction were found in CAIFOS but after stratification by chronic disease, none of the associations were significant (**Figure 1**). No effect modification by presence of chronic disease was observed in the association between n-3/n-6 PUFAs ratio and android and gynoid fat or android/gynoid ratio (Table 3). Also, no effect modification in the association of SFAs/PUFAs ratio and regional body fat distribution was observed (**Table 3**) in the RS.

In the CAIFOS cohort, effect modification by the presence of chronic disease was found in the relationship between SFAs/PUFAs ratio and % body fat (Table 4**;** P-$_{interaction}$=0.02) but this was not confirmed in the RS cohort. After stratification by chronic disease, participants with low SFAs/PUFAs ratio had a lower total body fat % (1st vs. 2ndtertile: β=-2.27; 95%CI: -3.99, -0.55; P for F-test, 0.03; **Figure 2**). Similar effects were found in the RS cohort but none of them was statistically significant (**Figure 2).**

Additional analysis

To evaluate the consistency of our findings, we reanalyzed the data using BFMI as the outcome in both cohorts. The results were similar to the ones observed when using total body fat mass (%) as an outcome (**Supplemental Table S1**, **Supplemental Figure S1**). There was no evidence against a linear relationship between dietary fat composition and body fat composition measures in either cohort (data not shown). Furthermore, no effect modification by treatment was observed in any of the analysis in CAIFOS (all $P>0.05$). Moreover, analyses were not different between strata of gender in the RS (data not shown).

DISCUSSION

By using two large population-based cohort studies, we showed that dietary n-3/n-6 PUFAs and SFAs/PUFAs ratios were not consistently associated with total body fat and its distribution.

Little is known about the effects of dietary fatty acids intake on body fat distribution with few prospective studies evaluating the associations between n-3 PUFAs on body fat composition. Cout at al. reported, in six volunteers, a beneficial effect of a diet rich in n-3 fatty acids on body fat[51] whereas Kratz at al. did not find an association between n-3 fatty acids and body fat among twenty-six overweight or moderately obese individuals[52]. It has been proposed that a diet rich in n-6 PUFAs may promote adiposity[53], a view that is not supported by our study or others. It has been previously reported that a diet rich in n-6 PUFAs may also reduce subcutaneous tissue[21] and trunk adipose tissue[54]. Within contrast to the current study, high consumption of dietary SFAs have been reported to promote weight gain and increases in adiposity, particularly in abdominal adiposity[23], whereas a higher dietary PUFAs has been associated with a greater fat mass loss in the abdominal region [21,22]. A diet higher in PUFAs has been shown to have a greater fat-oxidizing effect [55,56] which may imply a lower fat retention in the body[20]. However, unlike this study, most of the previous studies focused on dietary PUFA intake as an individual component, whereas our study took the dietary fat composition into account by using the n-3/n-6 PUFAs ratio and the SFAs/PUFAs ratio which is suggested to affect regional body fat distribution [22,57].

To our knowledge, this is the first study to account for potential modification by chronic disease on the association between dietary fat intake and body fat distribution. Although we found evidence for effect modification, the results showed differential directions in both populations. Hence, it is unclear whether the effect modification by chronic disease found in our study presents a physiological effect modification, or it's due to confounding factors, which may be differently distributed among subjects with and without chronic disease. It can be suggested that chronic disease may dilute any association between dietary fat intake and regional fat distribution since the presence of chronic disease may be associated with weight change and therefore body fat alterations and/or may also alter dietary intake[58]. Furthermore, factors that affect the metabolism of fatty acids, such as estrogen and blood lipid levels differ between people with and without chronic disease. For example, it has been reported that DHA synthesis is almost threefold grater in women using oral contraceptives than those with it[59]. Additionally, the presence of chronic disease is associated with an inflammatory profile,[60,61] which has been shown to be associated with obesity and in particular abdominal obesity[62] but dietary fat intake may also play a role in the inflammatory

Table 3 Dietary fat composition and body fat distribution, the Rotterdam Study.

Rotterdam Study	**Total body fat (%) β (95% CI)**		**Android fat (%) β (95% CI)**		**Gynoid fat (%) β (95% CI)**		**Android fat/gynoid fat ratio β (95% CI)**	
n-3/n-6 PUFA	MODEL 1	MODEL 2[a]	MODEL 1	MODEL 2[b]	MODEL 1	MODEL 2[c]	MODEL 1	MODEL 2[d]
1st Tertile	0.06 (-0.90, 1.02)	0.42 (-0.29-1.12)	-0.07 (-0.26, 0.13)	-0.03 (-0.13, 0.06)	0.09 (-0.17, 0.35)	0.02 (-0.11, 0.15)	-0.01 (-0.03, 0.01)	-0.01 (-0.02, 0.01)
2nd Tertile	reference	Reference	reference	reference	reference	reference	reference	reference
3rd Tertile	0.50 (-0.46, 1.45)	0.35 (-0.36, 1.05)	0.15 (-0.04, 0.35)	**0.15 (0.05, 0.24)**	0.05 (-0.21, 0.32)	0.05 (-0.08, 0.18)	0.01 (-0.01, 0.03)	0.01 (-0.01, 0.02)
P for F-test (3 df)	0.53	0.41	0.07	0.08	0.79	0.81	0.20	0.35
$P_{Interaction}$		<0.05*		>0.05#		>0.05		>0.05
SFA/PUFA ratio	**MODEL 1**	**MODEL 2[e]**	**MODEL 1**	**MODEL 2[f]**	**MODEL 1**	**MODEL 2[g]**	**MODEL 1**	**MODEL 2[h]**
1st Tertile	-0.28 (-1.24, -0.67)	-0.42 (-1.13, 0.29)	-0.11 (-0.30, 0.09)	**-0.12 (-0.22, -0.06)**	-0.04 (-0.30, 0.23)	-0.03 (-0.28, 0.22)	-0.01 (-0.02, 0.01)	-0.01 (-0.02, 0.01)
2nd Tertile	reference	Reference	reference	reference	reference	reference	reference	reference
3rd Tertile	-0.18 (-1.14, 0.77)	0.11 (-0.60-0.82)	-0.04 (-0.23, 0.16)	0.01 (-0.09, 0.10)	0.05 (-0.21, 0.31)	-0.01 (-0.25, 0.24)	-0.01 (-0.02, 0.01)	-0.002 (-0.02, 0.02)
P for F-test (3 df)	0.84	0.31	0.55	0.30	0.82	0.97	0.79	0.74
$P_{Interaction}$		>0.05		>0.05		>0.05		>0.05

Model 1: Adjusted by age

Model 2: Multivariable adjusted as follows

[a] Model 1 + additional adjusted for baseline energy intake, income status, education status, smoking status, hypertension, DHDI-index excluding PUFA component, BMI and prevalent chronic disease at 4th round. Additional adjustment for other covariates did not change the effect estimate with more than 10%.

[b] Model 1 + additional adjusted for baseline hypertension, BMI and prevalent chronic disease at 4th round. Additional adjustment for other covariates did not change the effect estimate with more than 10%.

[c] Model 1 + additional adjusted for baseline energy intake, hypertension, BMI, and prevalent chronic disease at 4th round. Additional adjustment for other covariates did not change the effect estimate with more than 10%.

[d] Model 1 + additional adjusted for baseline hypertension, income status, smoking status, BMI and prevalent chronic disease at 4th round. Additional adjustment for other covariates did not change the effect estimate with more than 10%.

[e] Model 1 + additional adjusted for baseline energy intake, hypertension, income status, alcohol intake, physical activity, DHDI-index excluding PUFA component and BMI. Additional adjustment for other covariates did not change the effect estimate with more than 10%.

[f] Model 1 + additional adjusted for baseline hypertension, alcohol intake, BMI and prevalent chronic disease at 4th. Additional adjustment for other covariates did not change the effect estimate with more than 10%.

[g] Model 1 + additional adjusted for baseline energy intake, income status, hypertension, smoking status, DHDI-index excluding PUFA component, BMI, and prevalent chronic disease at 4th round. Additional adjustment for other covariates did not change the effect estimate with more than 10%.

[m] Model 1 + additional adjusted for baseline energy intake, income status, education status, hypertension, smoking status, and BMI. Additional adjustment for other covariates did not change the effect estimate with more than 10%.

* $P_{Interaction=}$*0.29* for n-3/n-6 PUFAs ratio Quartile 1 x prevalent chronic disease (yes vs. no); $P_{Interaction=}$*0.02* for n-3/n-6 PUFAs ratio Quartile 1 x prevalent chronic disease (yes vs. no) # $P_{Interaction=}$*0.50* for n-3/n-6 PUFAs ratio Quartile 1 x prevalent chronic disease (yes vs. no); $P_{Interaction=}$*0.09* for n-3/n-6 PUFAs ratio Quartile 1 x prevalent chronic disease (yes vs. no)

Figure 1 The association (β, 95% CI) between n-3/n-6 PUFAs ratio intake and total body fat (%) in participants with and without prevalent chronic disease (fully adjusted model, Model 2).

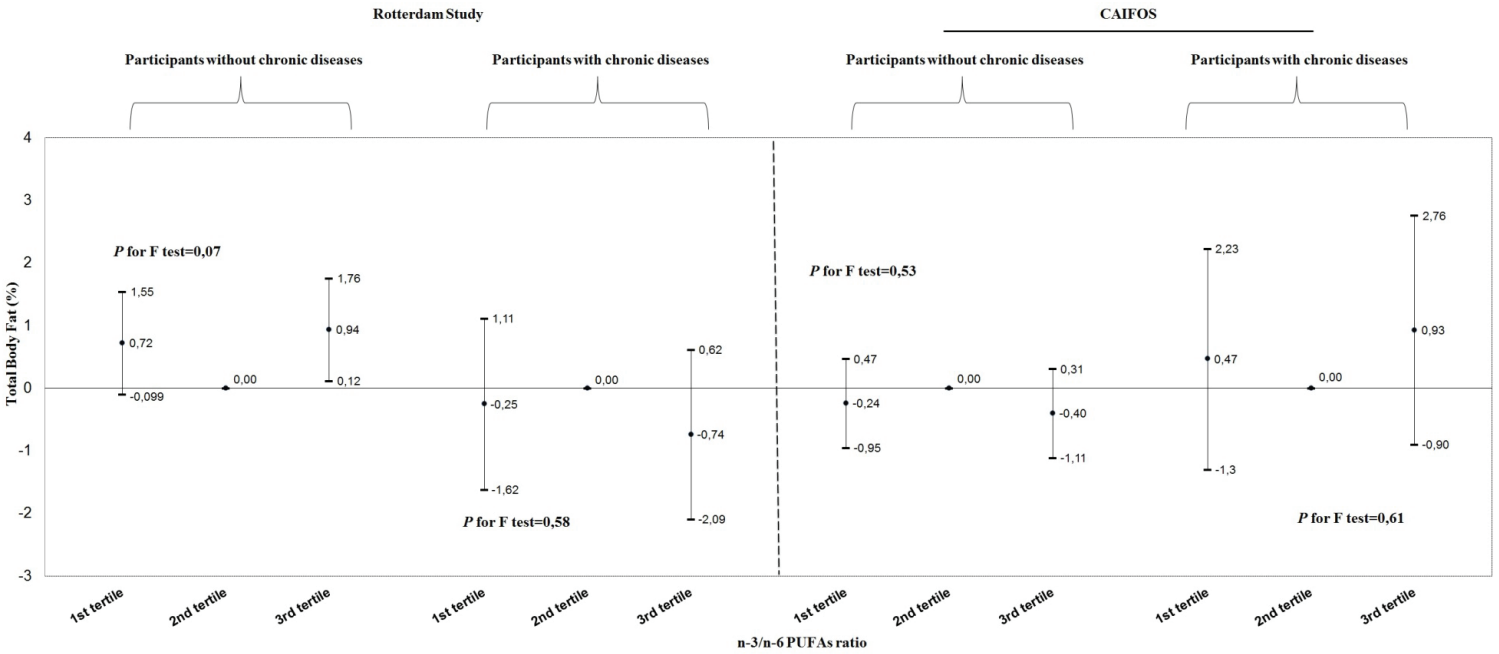

Figure 2 The association (β, 95% CI) between SFAs/PUFAs ratio intake and total body fat (%) in participants with and without prevalent chronic disease (fully adjusted model, Model 2).

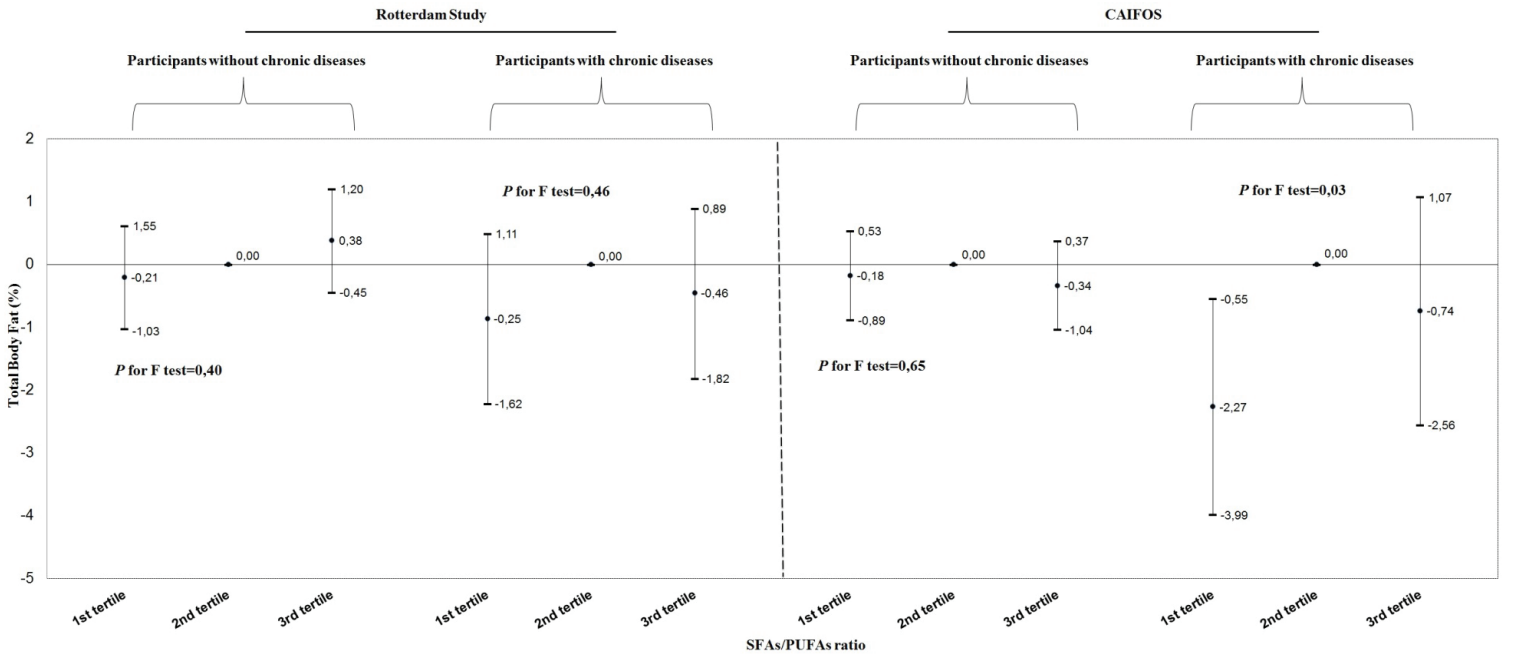

Table 4 Dietary fat composition and total body fat (%), the CAIFOS.

n-3/n-6 PUFAs	Total body fat (%) β (95% CI)			
	MODEL 1	MODEL 2[a]	MODEL 1	MODEL 2[b$]
1st Tertile	-0.43 (-1.35, 0.50)	0.08 (-0.18, 0.35)	-0.48 (-1.41, 0.44)	-0.55 (-1.20, 0.11)
2nd Tertile	reference	reference	reference	reference
3rd Tertile	-0.64 (-1.56, 0.29)	0.11 (-0.16, 0.37)	-0.25 (-1.17, 0.68)	-0.36 (-1.02, 0.29)
P for F-test (3 df)	0.39	0.76	0.59	0.28
$P_{Interaction}$		0.026*		0.022#

Model 1: Adjusted by age

Model 2: Multivariable adjusted as follows

[a] Model 1 + additional adjusted for baseline BMI, energy intake, alcohol intake, education, income status and treatment code. Additional adjustment for other covariates did not change the effect estimate with more than 10%.

[b] Model 1 + additional adjusted baseline BMI, hypertension, more than 3kg loss over 12 months, energy intake, alcohol intake, income status and NRFI. Additional a djustment for other covariates did not change the effect estimate with more than 10%.

*$P_{Interaction=}$ *0.026* for n-3/n-6 PUFAs ratio continuous x prevalent chronic disease (yes vs. no)

*$P_{Interaction=}$ *0.02* for SFA/PUFAs ratio Quartile 1 x prevalent chronic disease (yes vs. no);

$P_{Interaction=}$ *0.76* for SFA/PUFAs ratio Quartile 1 x prevalent chronic disease (yes vs. no)

process [63]. Furthermore, dietary fat composition is linked to other cardiovascular risk factors and health outcomes by the same proposed mechanisms that are linked to obesity[64]. Hence, further large-scale studies should confirm our findings, focusing on the effect of dietary fat intake on body fat distribution and the interaction with the presence of chronic disease.

Although not statistical significant, effects in opposite directions were observed in the two studies for the association between dietary fat composition and total body fat. This may be true to differences in the food sources of n-3 PUFAs in the RS and CAIFOS. In the CAIFOS, the main contribution of n-3 PUFAs was dietary fish intake; whereas the n-3 PUFAs in the RS cohort are mainly plant derived n-3 PUFAs (i.e. 29.3% of women participating in the RS did not consume fish at all). It has been reported previously that the health effects of PUFAs may differ with the food they are consumed in; for example, marine derived n-3 PUFAs have been shown to have differential health effects than n-3 plant derived n-3 PUFAs[65,66]. Therefore, it may be useful to evaluate different sources of fatty acids on body composition in future studies.

Strengths of this study include the large prospective population-based nature of both cohorts, the use of accurate measures of body composition such as DXA, and the extensive adjustment for a broad range of possible confounders Nevertheless, to appreciate the results some limitations need to be discussed. Because dietary intake was self-reported by using a FFQ, measurement error in the exposure can occur due to incomplete nutrient databases, accuracy of memory or willingness to divulge details. Although the FFQ that we used was validated in both studies and showed a relatively high correlation with the intake of fatty acids, errors in the measurement of fat intake have been suggested to lead to an underestimation of the true magnitude of the association[67]. Also, dietary fat intake was assessed at baseline, and there may have been changes in fat consumption over time. Another limiting factor to the current investigation is that body fat in both cohorts was measured only once and that that measurement was taken several years after the exposure was measured. However, in all analyses we adjusted for baseline BMI, which correlates well with total body fat; for example in the RS, partial correlation coefficient between BMI and total body fat percentage at the fourth visit is 0.81. However, we cannot rule out the possibility of selection bias. People who are in poor health are more likely to drop out from the study which may affect the generalizability of our results. Also, it has been shown that using a selected source population for a cohort study may lead to bias towards the null[68]. In addition, in the RS there was no baseline data on physical activity, which is known to be a determinant of body fat composition. Although we used data on physical activity measured that was measured in the third follow-up visit of the RS, we cannot fully exclude residual confounding. Moreover, we did not have data on android fat and gyonid fat from the CAIFOS, and therefore we were not able to investigate the associations of dietary fat composition with this measure of body fat distribution, which may have strengthened our results.

In conclusion, our findings suggest that dietary fat composition is not consistently associated with body fat and its distribution in older individuals. The role of chronic disease warrants further investigation.

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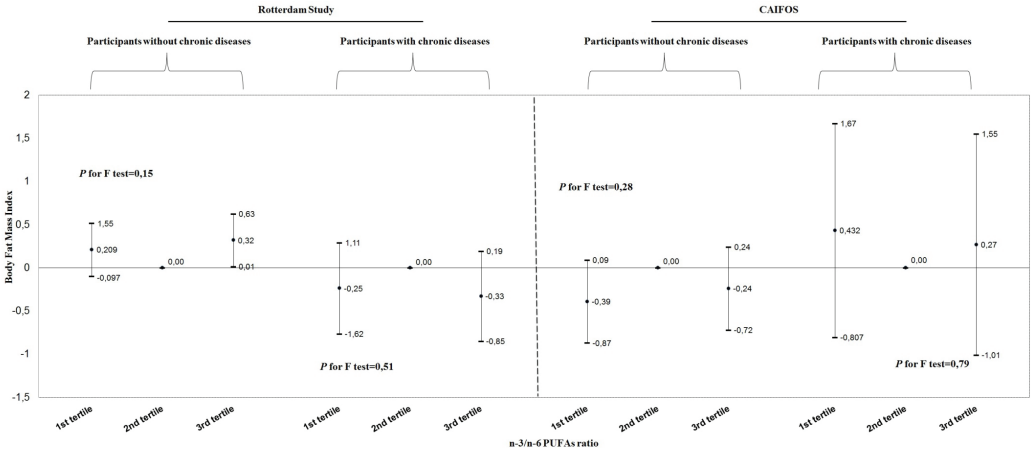
Supplemental material

Supplemental Table S1 Dietary fat composition intake and body fat mass index.

n-3/n-6 PUFA	Body fat mass index (kg/m^2) β (95% CI)				SFA/PUFA	Body fat mass index (kg/m^2) β (95% CI)			
	MODEL 1	MODEL 2[a]	MODEL 1	MODEL 2[b]		MODEL 1	MODEL 2[c]	MODEL 1	MODEL 2[d]
	Rotterdam Study		CAIFOS			Rotterdam Study		CAIFOS	
1st Tertile	-0.12 (-0.59, 0.34)	0.08 (-0.18, 0.35)	-0.20 (-0.66, 0.26)	-0.23 (-0.69, 0.22)	1st Tertile	0.04 (-0.42, 0.51)	-0.06 (-0.33, 0.20)	-0.15 (-0.61, 0.32)	-0.24 (-0.69, 0.22)
2nd Tertile	reference	reference	reference	reference	2nd Tertile	reference	reference	reference	reference
3rd Tertile	0.20 (-0.26, 0.67)	0.11 (-0.16, 0.37)*	-0.18 (-0.64, 0.29)	-0.16 (-0.62, 0.30)	3rd Tertile	-0.09 (-0.55, 0.38)	0.08 (-0.19, 0.35)	0.02 (-0.44, 0.48)	-0.40 (-0.49, 0.41)
P for F-test:	0.38	0.68	0.65	0.59	P for F-test:	0.84	0.31	0.74	0.86

Model 1: Adjusted by age
Model 2: Multivariable adjusted as follows
[a] Model 1 + additional adjusted for baseline energy intake, income status, education status, smoking status, hypertension, DHDI-index excluding PUFA component, BMI and prevalent chronic disease at 4th round. Additional adjustment for other covariates did not change the effect estimate with more than 10%.
[b] Model 1 + additional adjusted for baseline 3kg loss over 12 months, alcohol intake, education status, income status and treatment code. Additional adjustment for other covariates did not change the effect estimate with more than 10%.
[c] Model 1 + additional adjusted for baseline energy intake, hypertension, income status, alcohol intake, physical activity, DHDI-index excluding PUFA component and BMI. Additional adjustment for other covariates did not change the effect estimate with more than 10%.
[d] Model 1 + additional adjusted for baseline hypertension, more than 3kg loss over 12 months, smoking status, alcohol intake, physical activity, education, income status, NRFI and prevalent chronic disease in 2003. Additional adjustment for other covariates did not change the effect estimate with more than 10%.
Significant interaction term, dietary fat composition x prevalent chronic disease

Supplemental Figure S1 The association (β, 95% CI) between n-3/n-6 PUFAs ratio intake and body fat mass index in participants with and without prevalent chronic disease (fully adjusted model, Model 2).



Supplemental Table S2 Selected characteristics of male participants in the Rotterdam Study.

	RS (N=846)
Baseline characteristics	
Age	64.0 ± 5.5
N-3 PUFAs (g/day)	1.23 ± 0.54
N-6 PUFAs (g/day)	15.24 ± 7.62
Total PUFAs (g/day)	18.70 ± 8.26
Total Saturated Fat (g/day)	36.34 ± 12.33
Total energy intake (kcal/day)	2298.4 ± 515.2
BMI (kg/m^2)	27.1 ± 6.4
Total alcohol (g/day)*	15.87 (95.58)
Lower Education, n (%)	152 (17.9)
Medium Education (%)	504 (59.6)
High Education (%)	190 (22.5)
Income (%)	
low	127 (15.0)
medium	330 (39.0)
high	389 (46.0)
Never/former smokers	959 (81.1)
Current smokers	210 (24.8)
Physical activity (min)	2626.47 ± 1130.45
Hypertension (%)	(385) 45.5
More than 3 kg loss in last 12 months (%)	62 (7.3)
Total DHDI-index	48.68 ± 10.58
Characteristics at the end and during the follow-up	
Total whole body fat mass (kg)	23.31 ± 7.56
Total body fat mass index (kg/m^2)	23.31 ± 7.56
Android Fat (kg)	2.58 ± 0.95
Gynoid Fat (kg)	3.24 ± 1.03
Prevalent chronic disease (%)$	366 (43.3)
Chronic disease development (%)#	266 (31.4)

Dutch Healthy Diet-index; PUFAs: polyunsaturated fatty acids
Plus-minus values are means ±SD
* median (range)
$Percentage of participants who have at least one of the chronic disease (cardiovascular disease, diabetes or cancer) when the body composition was assessed.
#Percentage of participants who developed a chronic disease (cardiovascular disease, diabetes or cancer) from baseline until the measurements of body composition.

Supplemental Table S3 Correlations coefficients between n-6 and n-3 PUFAs and their dietary food sources in male participants of the Rotterdam Study*.

Rotterdam Study	n-6 PUFAs (g/day) Pearson Correlation *(r)*	n-3 PUFAs (g/day) Pearson Correlation *(r)*	SFAs (g/day) Pearson Correlation *(r)*
Vegetable oils (g/day)	0.25	-	-
Butter, margarines and hard frying fats (g/day)	0.32	0.47	0.59
Fish (g/day)	-	0.29	-0.04
Poultry(g/day)	-	0.10	-0.07
Processed red meat (g/day)	0.09	0.23	0.36
Sweet desserts and confectionary (g/day)	0.07	0.14	0.36
Chips (g/day)	-	-	0.07
Wholegrain (g/day)	0.16	0.13	0.17
Total dairy** (g/day)	-	0.07	0.22

PUFA: polyunsaturated fatty acids
*Overall variance explained by these food items: 22% for n-6 PUFAs; 37% for n-3 PUFAs.
**including cheese
-does not significantly contribute to n-3 or n-6 PUFAs (p-value >0.05), otherwise, the p-value<0.05

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3.3 Vitamin D and Body Composition in the Elderly: the Rotterdam Study

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ABSTRACT

Objective: To investigate the association between vitamin D status and body composition in the elderly.

Methods: This study was embedded in the Rotterdam Study, a population-based prospective study in Rotterdam, the Netherlands, including subjects aged 55 years and older. Serum 25-hydroxyvitamin D (25(OH)D) was measured between 1997 and 1999. Total body fat, android fat, gynoid fat and lean mass were assessed using dual-energy X-ray absorptiometry (DXA) during a follow-up visit after a median time of 5 years (2002-2004).We calculated body fat percentage, lean mass percentage, and android/gynoid fat ratio. We had 2,158 participants included in our analysis. We used multivariable linear regression models. Serum 25(OH)D was analyzed continuously and after categorization according to cut-offs.

Results: Mean (±SD) serum 25(OH)D concentration of the study population was 52.6 ± 25.4nmol/L. Compared to subjects with an adequate vitamin D status (25(OH)D ≥75nmol/L),vitamin D deficient participants (25(OH)D <50nmol/L) had a higher body fat percentage (β=1.29, 95%CI: 0.55, 2.04) whereas no association was found with lean mass (β=0.01, 95%CI: -0.33, 0.35). Lower 25(OH) D was associated with higher total body fat percentage specifically in participants without cardio-metabolic disease. Each 10 unit increase in serum 25(OH)D was associated with 0.03 unit decrease in android fat (β=-0.03, 95%CI: -0.06, -0.01); after adjustment for BMI the association was no longer significant. Serum 25(OH)D was also associated with the android/gynoid fat ratio but this was also mainly explained by BMI.

Conclusion: Lower serum 25(OH)D concentrations were associated with a higher fat mass percentage. The association between serum 25(OH)D and differential fat distribution in the elderly was mainly explained by BMI and deserves further study.

INTRODUCTION

Low concentrations of Vitamin D (25(OH)D) have been associated with bone health and also with obesity and many obesity-related disorders such as metabolic syndrome, cardiovascular disease, diabetes mellitus, and mortality[1,2].The prevalence of obesity is common worldwide[3] and obesity is a well-known risk factor for diseases like type 2 diabetes mellitus and cardiovascular disease[4,5]. Fat tissue located in the abdominal region, especially around the internal organs, is considered to be hormonally active, causing low grade inflammation, which contributes to the development of insulin resistance and subsequently type 2 diabetes mellitus[6-8].

It is known that circulating concentrations of serum 25(OH)D are lower in obese individuals, most likely due to sequestration of serum 25(OH)D in the fat tissue and due to other lifestyle factors related with sun exposure (e.g. physical activity)[9]. On the other hand, there are numerous mechanisms reported in the literature describing the involvement of vitamin D in adipose tissue metabolism [9]and also mechanisms describing the role of muscle cells in metabolism of vitamin D [10]. For example, it has been suggested there might be increase in catabolism of vitamin D in the adipose tissue[11], while, on the other hand, active 1,25$(OH)_2$D might activate muscle tissue growth and improve muscle function[12-14]. Observational studies found serum 25(OH)D associated with different anthropometric measures and body composition measures such as body mass index, body fat mass, but also visceral fat and lean mass[9,15,16]. Conversely, a study using a bidirectional Mendelian randomization approach found higher body mass index causing lower serum 25(OH)D concentrations but not the other way around[17].

However, it is unclear whether vitamin D status is associated with fat mass stored in specific regions of the body and what is the direction of this association. Furthermore, since most of the research on this topic was performed in younger populations the association between vitamin D status and body composition in the elderly remains unexplored. In addition, the relation between vitamin D status and body composition may be complicated by the presence of cardio-metabolic diseases since these may be associated with both vitamin D and body composition[18,19]. Also, patients who are diagnosed with cardio-metabolic disease might have been advised to lose weight and change their lifestyle in general which might have led to changes in vitamin D status [20].

Knowledge on potential preferential storage of vitamin D in specific fat depots is important to clarify because it might help further elucidate the mechanisms how vitamin D and obesity are associated and may have implications for supplementation of specific populations. Therefore, we investigated whether there is an association between vitamin D status and body composition including measures of fat and lean mass and fat distribution by DXA in the elderly. Additionally, we evaluated whether this association differed by age, gender, presence of cardio-metabolic disease and metabolic syndrome since these are all factors that might relate to vitamin D status and also body composition.

METHODS

The Rotterdam Study

The Rotterdam Study is an ongoing prospective population-based follow-up study comprising of people aged 45 years or older from a suburb of Rotterdam, the Netherlands[21]. Baseline measurements were performed from 1990 to 1993. At this time point we collected the information on current health status, different medication use, medical history, lifestyle factors, and risk indicators for chronic diseases. Subsequently, the participants visited the study center for detailed clinical examinations and assessment of diet. Every 2 to 3 years the participants were invited for the follow-up visits to the research centre[21]. The vital status of the participants was obtained regularly from the municipal population registry. Morbidity and mortality data were collected from the general practitioners records or, in case of hospitalization, from discharge reports by the medical specialists. Data were also collected from pathology registries. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus Medical Center and by the Ministry of Health, Welfare and Sport of the Netherlands , implementing the Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study). All participants provided written informed consent to participate in the study and to obtain information from their treating physicians.

Serum 25-hydroxyvitamin D

Between years 1997 and 1999 (the third examination round of the cohort) serum 25(OH)D concentrations were measured using electrochemiluminescence immunoassay (COBAS< Roche Diagnostics GmbH, Germany). The range of the test for serum 25(OH)D concentrations was 7.5nmol/L to 175nmol/L. The sensitivity of the test was 10nmol/L, within-run precision was <7.8%, and intermediate precision was <13.1%. We categorized serum 25(OH)D concentrations as follows: <50nmol/L as vitamin D deficiency, 50-75nmol/L as vitamin D insufficiency, and ≥75nmol/L as adequate vitamin D status[1].

Anthropometrics

Participants' height, weight, waist circumference, and hip circumference were measured at the third examination round of the cohort (1997-1999) and at the fourth examination round of the cohort (2002-2004). For these measurements the participants were standing without their shoes and heavy clothes, they were asked to empty their pockets and to breathe gently. Waist circumference was measured at the level midway between the lower rib margin and the iliac crest. Hip circumference was measured as the maximum circumference over the buttocks. Based on these measurements we subsequently calculated body mass index (BMI, kg/m^2) and waist-to-hip ratio (WHR).

Measures of body composition

Body composition was assessed using Dual-energy X-ray absorptiometry (DXA) during the fourth examination round of the Rotterdam Study (2002-2004). For the whole body DXA scans we used

ProdigyTM total body-fan beam densitometer (GE Lunar Corp, Madison, WI, USA) [22]. Total body weight (grams) was divided into bone mineral content, lean mass, and fat mass. In addition, we analyzed fat mass of the android body region and gynoid body region. We calculated body fat percentage, android fat percentage, gynoid fat percentage and lean mass percentage as percentages of total body weight. We calculated the ratio of android fat and gynoid fat, and a ratio of fat mass and lean mass as additional measures of body fat distribution.

Potential confounding variables

During the first examination round of the Rotterdam Study cohort (baseline visit) we collected the information on current health status, medical history, smoking behavior, education level attained and socioeconomic status. This was done during the home interviews. Education was defined as low (primary education), intermediate (secondary general education or secondary vocational education), or high (higher vocational education or university). Household income was categorized as low (<1700 euro/month), middle (1700-3000 euro/month) or high (≥3000 euro/month). Cardio-metabolic disease was defined as presence of cardiovascular disease and/or presence of type 2 diabetes mellitus. History of cardiovascular disease was defined as having a history of coronary heart diseases (myocardial infarction, revascularization, coronary artery bypass graft surgery or percutaneous coronary intervention). This information and was verified from the general practitioners' medical records. Baseline diabetes mellitus was defined as having a serum glucose level higher or equal to 11mmol/L or if the use of glucose lowering drugs was reported. We defined chronic kidney disease as an estimated glomerular filtration rate (eGFR) lower than 60mL/min/1.73m^2. The metabolic syndrome was defined according to the interim definition proposed by Alberti and colleagues[23]. Physical activity of the participants was assessed with an adapted version of the Zutphen Physical Activity Questionnaire[24]. We assigned a MET-value according to the 2011 Compendium of Physical Activities[25] to every activity mentioned in the questionnaire. The questionnaire contained questions on walking, cycling, gardening, diverse sports, hobbies and on housekeeping. Total time spent on physical activity was calculated as the sum of minutes per week for each type of activity. At baseline examination round of the cohort the food intake was assessed. For this purpose a semi-quantitative food frequency questionnaire was used. To assess overall quality of the diet, the Dutch Healthy Diet (DHD) index was used[26]. This index is a continuous score representing compliance to the Dutch Guidelines for a Healthy Diet. The following components of the diet were available and included in the DHD index in this study: intake of vegetables, fruits, polyunsaturated fatty acids, saturated fatty acids, trans fatty acids, fish, dietary fiber, alcohol and sodium. Dates when the blood samples were drawn were categorized into summer, autumn, winter and spring according to the Dutch standard seasons. The weight change was calculated as the difference between weight at the third visit and weight at the fourth visit of the cohort.

Study population

Participants from the third visit of the first cohort of the Rotterdam Study (1997-1999) were eligible for inclusion into this analysis (N=4,787). We had data on serum 25(OH)D concentrations available for 3,828 of these participants. During 7 years of follow-up 337 participants from the initial population died and 1,333 participants had no dual-energy X-ray absorptiometry (DXA) scan examinations performed. Thus, we had 2,158 participants eligible for our final analysis.

Statistical analysis

Descriptives are presented as mean ± standard deviation (SD) unless indicated otherwise. Multivariable linear regression was used to examine whether vitamin D status was independently associated with total body fat percentage, lean mass percentage, android fat percentage, gynoid fat percentage, android fat/gynoid fat ratio and total fat mass/lean mass ratio. In the first model, we calculated the age and gender adjusted regression coefficients and their 95% confidence intervals (CIs). Further, for all the outcomes of our analyses we built the second model, which was additionally adjusted for the season when the blood was drawn, total alcohol intake (continuous), total physical activity (continuous), smoking status, education, income, DHD-index (continuous), prevalent cardio-metabolic disease (cardiovascular diseases and diabetes mellitus) and presence of chronic kidney disease. Finally, the third model was built depending on the outcome of interest; for the analysis concerning total body fat percentage, lean mass percentage and total fat mass/lean mass ratio as the outcomes, we additionally adjusted for height (continuous), weight change (continuous) during the follow up and lean mass or total fat mass in kilogram respectively; whereas for the analyses concerning android fat percentage and gynoid fat percentage, and android fat/gynoid fat ratio as outcomes, we additionally adjusted for BMI measured at the third visit (the baseline visit for this analysis). The confounders used in our analyses we chosen based on a 10% change in regression coefficient [27] as well as based on previously published literature on this topic. We tested for possible nonlinear effects by adding a quadratic term of 25(OH)D into the model. To test for effect modification by age, gender, cardio-metabolic diseases or metabolic syndrome, the product term of 25(OH)D with each one of the potential effect modifiers separately was added as independent variable to the models. The analyses were stratified if an interaction term was significant at a P-value lower than 0.05. In order to compare how well the data fit the three models we also reported R^2. To examine the association between serum 25(OH)D concentrations and anthropometric measurements measured repeatedly (waist circumference, waist to hip ratio and BMI) we fitted linear regression models using generalized estimating equations (GEE) with exchangeable correlation structure adjusting for the within-subject correlations due to the repeated measurements of anthropometric measurements in the same individual[28]. To account for the time difference in measurements at the three time points, we entered a time variable in the model and coded as 1,6 and 13. Finally, to account for the potential effect of vitamin D supplementation we performed sensitivity analysis excluding participants with osteoporosis or receiving any vitamin supplementation. To adjust for potential bias associated with missing data, a multiple imputation procedure was performed (N= 5 imputations).

Table 1. Selected characteristics of the study population.

	Deficient (N=1129)	Insufficient (N=621)	Adequate (N=408)	% Missing
Age	71.6 ± 6.2	69.6 ± 5.4	68.8 ± 5.2	0
Female %(n)	66.7 (753)	50.4 (313)	39.5 (161)	0
Smoking status (current smokers) %(n)	16.4 (185)	13.7 (85)	16.7 68	0.5
Physical activity (total MET hours)	85.9 ± 43.7	85.9 ± 44.4	95.9± 48.2	0.6
Alcohol intake (g/day)*	2.1 (10.2)	3.6 (13.6)	4.3 (16.4)	0.5
Education Level				0.9
Low % (n)	46.7 (527)	42.0 (261)	36.5 (149)	
Medium % (n)	43.4 (990)	43.3 (269)	50.7 (207)	
High % (n)	9.9 (112)	14.7 (91)	12.7 (52)	
Income				10.0
Low % (n)	19.3 (218)	13.5 (84)	11.8 (48)	
Medium % (n)	42.3 (478)	40.7 (253)	38.0 (155)	
High % (n)	38.4 (433)	45.7 (284)	50.2 (205)	
BMI 3rd visit (kg/m^2)	27.30 ± 4.1	26.4 ± 3.4	26.2 ± 3.4	0.4
BMI 4th visit (kg/m^2)	27.8 ± 4.4	27.0 ± 3.7	26.2 ± 3.4	1.8
BMI 5th visit	27.9 ± 4.6	27.02 ± 3.7	26.9 ± 3.4	42.8
WC 3rd visit	93.7 ± 12.0	92.7 ± 10.9	92.6 ± 10.29	0.37
WC4th visit (kg/m^2)	93.6 ± 12.1	93.29 ± 11.4	93.8 ± 11.03	0.32
WC 5th visit	92.5 ± 12.9	92.2 ± 12.1	92.2 ± 11.8	43.0
WHR3rd visit (kg/m^2)	0.91 ± 0.10	0.92 ± 0.09	0.93 ± 0.1	0.37
WHR4th visit (kg/m^2)	0.90 ± 0.09	0.91 ± 0.09	0.92 ± 0.09	0.32
WHR 5th visit	0.89 ± 0.09	0.90 ± 0.09	0.91 ± 0.09	43.0
Weight change (%)	-0.83 ± 4.6	-1.2 ± 3.9	-1.5 ± 3.8	2.0
DHDI	49.3 ± 10.2	49.1 ± 10.3	47.3 ± 10.1	11.8
Cardio-metabolic disease %(n)	73.1 (304)	22.2 (138)	22.88 (93	0
Metabolic Syndrome %(n)	46.0 (519)	35.7 (222)	30.1 (123)	1.8
Chronic Kidney Disease %(n)	13.0 (147)	9.8 (61)	11.3 (46)	0.2
Vitamin D (nmol/L)	33.2 ± 10.3	61.2 ± 6.9	93.3 ± 15.5	0

DHDI, Dutch Health Diet Index; BMI, Body mass index; WC, Waist circumference; WHR, Waist hip ratio
Values presented are mean ± SD;
*Median (interquartile range).

For the pooled regression coefficients (β) and 95% CIs we used Rubin's method [29]. All analyses were performed using IBM SPSS Statistics 20. A P-value lower than 0.05 was considered statistically significant.

RESULTS

The mean (± SD) age of the participants was 70.5± 5.9 years; mean time difference between the 25(OH)D measurements and DXA measurements was 4.6 years; 19% of study participants had adequate vitamin D status (25(OH)D≥75 nmol/L), 29% had vitamin D insufficiency (50-75 nmol/L) and 52% had vitamin D deficiency (<50 nmol/L) (Table 1).

Vitamin D, total body fat mass and lean mass

In the age, gender and covariates adjusted model, subjects with vitamin D deficiency had a 1.29 unit higher body fat percentage than participants with adequate vitamin D status (95%CI=0.55, 2.04) (Table 2). When analyzed continuously an inverse association was observed between serum 25(OH) D and body fat percentage: a 10 nmol/L increase in serum 25(OH)D was associated with a 0.24unit lower body fat percentage (95%CI=-0.35, -0.13)in model 3 (the fully adjusted model) (Table 2). A higher serum 25(OH)D was also associated with a lower fat mass/lean mass ratio(β=-0.01, 95%CI= -0.01, -0.00, model 3) (Table 2).In contrast, a 10 nmol/L increase in serum 25(OH)D was associated with a 0.23higherlean mass percentage(95%CI=0.11, 0.35) in model 2 (Table 2), and did not remain significant after further adjustment for height, weight change and total fat mass in kilogram (model 3).The corresponding analyses of 25(OH)D cutoffs yielded very similar results as the analyses of 25(OH)D continuous (see the P for trend, Table 2). Additionally we performed the same analysis using absolute total body fat and absolute lean mass (Supplementary Table S1). The results changed slightly: the association between 25(OH)D and total fat mass weakened but remained significant (β=-0.06, 95%CI= -0.01, -0.00, model 3) while the association between 25(OH)D and lean mass became significant (β=0.06, 95%CI= 0.01, 0.12, model 3). Furthermore, we observed an interaction between 25(OH)D and presence of cardio-metabolic diseases on total body fat percentage (Pinteraction=0.03).The inverse association between 25(OH)D and body fat percentage was present among subjects without cardio-metabolic diseases (β=1.70, 95%CI=0.87, 2.53 for deficiency vs. adequate vitamin D status, P for trend<0.001), but not among participants with cardio-metabolic disease **(Figure 1).** No effect modification by age, gender and metabolic syndrome was present (Pinteraction>0.05).

Vitamin D and regional body fat distribution

There was no consistent association between vitamin D status and android fat percentage, gynoid fat percentage or android/gynoid fat ratio after adjustment for confounders (Table 3). Continuous analyses showed a weak inverse association between 25(OH)D and android fat percentage and android/gynoid ratio, but this was not independent of BMI (P for trend when analyzed by categories of 25(OH)D 0.04 and 0.08 respectively). Also, a significant quadratic term was observed in the association between serum 25(OH)D and android fat and in the association between 25(OH)D and android/gynoid fat ratio suggesting non-linear associations (Table 3).

Table2. Serum 25(OH)D and body composition (2158 subjects).

	Total fat (%) β (95% CI)			**Lean Mass (%) β (95% CI)**			**Total Fat Mass/ Lean Mass Ratio**		
Vitamin D	MODEL 1	MODEL 2	MODEL 3a	MODEL 1	MODEL 2	MODEL 3b	MODEL 1	MODEL 2	MODEL 3
Deficient	**1.56 (0.81, 2.31)**	**1.29 (0.55, 2.04)**	**1.35 (0.63, 2.06)**	**-1.55 (-2.33, -0.78)**	**-1.30 (-2.07, -0.53)**	0.01 (-0.33, 0.35)	**0.04 (0.02, 0.06)**	**0.03 (0.02, 0.05)**	**0.04 (0.02, 0.06)**
Insufficient	0.62 (-0.18, 1.42)	0.35 (-0.44, 1.14)	0.44 (-0.32, 1.20)	-0.42 (-1.25, 0.40)	-0.17 (-0.99, 0.65)	0.03 (-0.33, 0.39)	0.01 (-0.01, 0.03)	0.01 (-0.01, 0.03)	0.01 (-0.01, 0.03)
Adequate	reference	Reference	reference	reference	Reference	reference	reference	Reference	reference
P-trend	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.999	**<0.001**	**<0.001**	**<0.001**
Continuous (per 10 unit increase)	**-0.26 (-0.37, -0.15)**	**-0.22 (-0.33, -0.11)**	**-0.24 (-0.35, -0.13)**	**0.27 (0.15, 0.38)**	**0.23 (0.11, 0.35)**	-0.01 (-0.06, 0.04)	**-0.01 (-0.01, -0.00)**	**-0.01 (-0.01, -0.00)**	**-0.01 (-0.01, -0.00)**
R^2 for vitamin D as continuous	**0.43**	**0.45**	**0.50**	**0.41**	**0.44**	0.89	**0.42**	**0.45**	**0.49**

Model 1: Adjusted for age and gender;
Model 2: Model 1 additionally adjusted for season when the blood was drawn, chronic kidney disease, smoking status, alcohol intake, physical activity, highest education attained, household income, Dutch Healthy Diet-index, prevalent cardio-metabolic diseases;
Model 3a: Model 2 additionally adjusted forheightmeasured at the third visit, weight changeand lean mass in kilogram;
Model 3b: Model 2 additionally adjusted for heightmeasured at the third visit, weight change and total body fat in kilogram.

Table3. Serum 25(OH)D and regional fat distribution (2158 subjects).

	Android fat (%) β (95% CI)			**Gynoid fat (%) β (95% CI)**			**Android/Gynoid fat ratio β (95% CI)**		
				Android fat/Gynoid Fat β (95% CI)					
Vitamin D	MODEL 1	MODEL 2	MODEL 3	MODEL 1	MODEL 2	MODEL 3	MODEL 1	MODEL 2	MODEL 3
Deficient	**0.18 (0.01, 0.35)**	0.17 (-0.00, 0.34)	0.08 (-0.08, 0.24)	-0.08 (-0.29, 0.14)	-0.05 (-0.26, 0.17)	0.09 (-0.11, 0.30)	0.02 (-0.00, 0.03)	0.01 (-0.01, 0.03)	0.00 (-0.02, 0.02)
Insufficient	0.10 (-0.08, 0.28)	0.12 (-0.06, 0.29)	0.11 (-0.06, 0.28)	-0.02 (-0.25, 0.21)	0.00 (-0.23, 0.23)	0.01 (-0.20, 0.22)	0.01 (-0.01, 0.03)	0.01 (-0.01, 0.03)	0.005 (-0.01, 0.02)
Adequate	reference	reference	reference	reference	reference	reference	reference	reference	reference
P-trend	**0.04**	0.06	0.47	0.43	0.54	0.30	0.08	0.15	0.91
Continuous (per 10 unit increase)	**-0.03 (-0.06, -0.01)**	**-0.03 (-0.06, -0.01)**	-0.01 (-0.04, 0.01)*	0.02 (-0.02, 0.05)	0.01 (-0.02, 0.05)	-0.01 (-0.04, 0.02)	**0.00 (-0.01, -0.00)**	**0.00 (-0.01, -0.00)**	-0.00 (-0.00, 0.00)*
R^2 for vitamin D as continuous	**0.46**	**0.48**	0.52	0.23	0.26	0.34	**0.44**	**0.47**	0.52

Model 1: Adjusted for age and gender
Model 2: Model 1 additionally adjusted for season when the blood was drawn, chronic kidney disease, smoking status, alcohol intake, physical activity, highest education attained, household income, Dutch Healthy Diet-index, prevalent cardio-metabolic diseases, weight change;
Model 3: Model 2 additionally adjusted forbody mass indexmeasured at the third visit;
*Quadratic term significant, P<0.05; β (95% Confidence Interval) for the quadratic term: android fat -7.2 10^{-04} (-0.002; -4.1 10^{-05}): android fat/gynoid fat ratio: 0.01 (-3.8 10^{-04}; 0.02)

Additional analysis

We observed an inverse association between vitamin D status and BMI; in the fully adjusted model a10 nmol/L increase in serum 25(OH)D was associated with a 0.16 unit decrease in BMI (95%CI=-022, -0.09) (Supplementary Table S2). When stratified by metabolic syndrome status (Pinteraction=0.001) a decrease in BMI with increasing concentrations of serum 25(OH)D was observed only among subjects with metabolic syndrome (P for trend=0.001), but not in those without (Supplementary Figure S3). The analysis of serum 25(OH)D and waist circumference showed a statistically significant inverse association while in the case of serum 25(OH)D and waist to hip ratio this inverse association was weaker and not independent of BMI (Supplementary Table S2). Sensitivity analysis excluding participants with osteoporosis or receiving any vitamin supplementation did not change the results (data not shown). Even though the gender differences in body composition are to be expected, the association between 25(OH)D and body composition was not significantly different between men and women (Pinteraction=0.051).

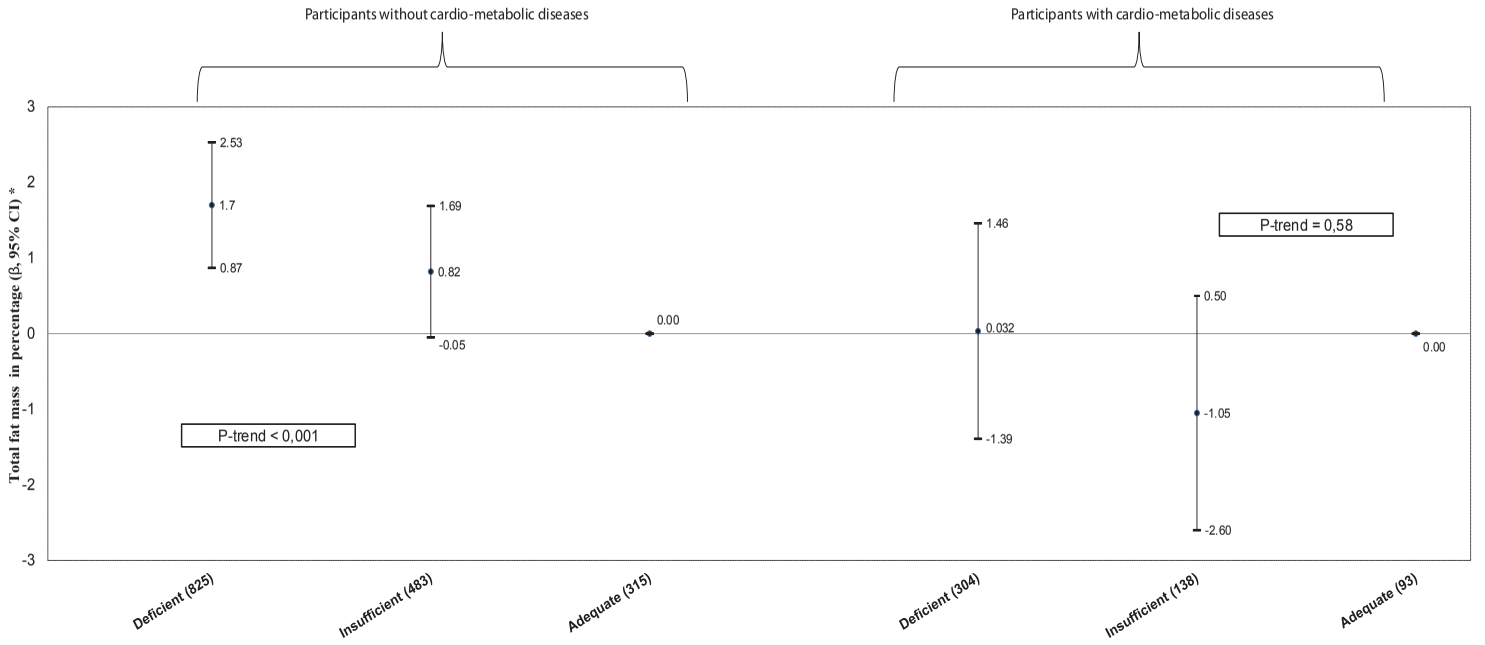
DISCUSSION

We found adequate vitamin D status to be inversely associated with total body fat mass percentage. Our results suggest a BMI dependent role for vitamin D status in the regional distribution of body fat in the elderly.

Comparison with other studies

In our study, it was observed that low concentrations of serum 25(OH)D are associated with a higher body fat percentage. Several previous studies also investigated this association and found similar results however, most of them were of cross-sectional design. For example, a cross-sectional study by Kremer et al. (2008)[30]found that vitamin D insufficiency was associated with increased body fat in young women. Similarly, a study by Snijder et al. (2005)[31]found an inverse association between total body fat and serum 25(OH)D concentrations in older men and women. This latter study was performed in a population similar to ours (mean age ±75 years), but with only 453 participants included in the analysis. However, the conclusions coming from intervention studies are inconsistent. For example, Salehpour et al. (2012)[32]found that supplementation with vitamin D3 for 12 weeks in 77 overweight and obese healthy subjects led to a statistically significant decrease in body fat in the intervention group compared to placebo group. Conversely, Wamberg et al. (2013)[33]found that increasing serum 25(OH)D concentrations with cholecalciferol treatment during 26 weeks in 52 obese subjects had neither an effect on body fat, nor specifically on subcutaneous or visceral adipose tissue. However, not many studies investigated the association between vitamin D status and regional fat distribution specifically in the elderly. We found a cross-sectional study by Moschonis et al. (2009)reporting on vitamin D status as an outcome and body composition, including regional fat mass(measured by calibrated Lange skinfold caliper) as an exposure. This study was performed in 112 non-osteoporotic, overweight, postmenopausal women (mean age 60 years)[15] and showed inverse associations of vitamin D status with total body fat and with all measures of regional body fat mass(assessed as arms fat mass percentage, legs fat

Figure 1. Serum 25(OH)D and total body fat percentage stratified by presence of cardio-metabolic disease*.



*$P_{Interaction}$=0.03 for serum 25(OH)D ^{X} cardio-metabolic disease.

mass percentage and trunk fat mass percentage) and a positive association with fat-free mass. Compared to this study by Moschonis and colleagues[15] we had a bigger sample but we did not find a consistent association between vitamin D status and gynoid fat but we did find an association between vitamin D status and android fat even though it was not independent of BMI. However, our findings of an inverse association between vitamin D status and total body fat are in line with the results reported by Moschonis et al.[15].

Potential mechanisms

Even though many studies report associations between low vitamin D status and obesity, the underlying mechanisms and the direction of this association are not fully understood. There are several pathways by which vitamin D and body composition may be connected in both directions. Firstly, obese people have commonly lower serum 25(OH)D concentrations compared to lean people[9]. This might be a result of decreased bioavailability of vitamin D because of sequestration of vitamin D by the adipose tissue[11]; dilution of vitamin D in the large fat mass of obese people [34]; increased catabolism of vitamin D in the adipose tissue[35]; decreased synthesis of 25(OH)D in the liver[36]; reduced sun-exposure [37]; or decreased hepatic synthesis of 25(OH)D caused by a negative feedback loop of $1,25(OH)_2D$ and parathyroid hormone (PTH)[38].Also, clearance of vitamin D might be increased by inflammation related to obesity[39]. In addition, vitamin D might have anti-inflammatory effects[40] and low vitamin D has been associated with visceral adiposity that is associated with inflammation [41]. Furthermore, a study using bidirectional Mendelian randomization approach found higher BMI leading to lower 25(OH)D but not the other way around[17]. Secondly, it has been suggested that low serum 25(OH)D concentrations lead to increase in PTH levels as a normal physiological response, which in turn may favor the lipid storage metabolism[42] [43]. In vitro studies found that receptors for vitamin D (VDRs) and the enzyme 1-α-hydroxylase needed for production of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), are both present in adipocytes[35] [44] [45] suggesting a role of $1,25(OH)_2D$ in metabolism of the adipose tissue.Also,$1,25(OH)_2D$ was found to regulate expression of some genes involved in genesis of adipose tissue[46]. Thirdly, it was found that skeletal muscles act as a functional store of 25(OH)D - a proportion of circulating 25(OH) D binds to vitamin D binding protein in muscle cells and can be released back to the circulation. This retention by muscle cells protects the 25(OH)D from degradation by the liver[10]. Additionally, active $1,25(OH)_2D$ bound to VDR receptor in muscle tissue activates muscle growth and improves muscle function and may thereby influence overall body composition[12-14]. With this in mind, we speculate that with the increase in body fat percentage the concentration of 25(OH)D in the circulation decreases, subsequently leading to a decrease in the functional store of 25(OH)D in the skeletal muscles. This decrease in the functional store of 25(OH)D might in turn lower serum 25(OH)D concentration that might contribute to more unfavorable body composition.

Interactions

We found an interaction between 25(OH)D, body composition and cardio-metabolic diseases. Several studies have shown vitamin D status to be associated with metabolic syndrome,

cardiovascular disease and type 2 diabetes mellitus[1,18]. Also, body fat and BMI are associated with an increased risk of cardiovascular disease, metabolic syndrome and type 2 diabetes mellitus[7].

We found an association between 25(OH)D and total fat percentage only in subjects free of cardio-metabolic disease. This finding can be explained in several ways. Firstly, it might be possible that people with cardio-metabolic disease at baseline changed their life-style after diagnosis[47]. This change might have resulted in for example weight loss or improvement of vitamin D status and diluting any association between 25(OH)D and body composition. Secondly, it might be that due to inflammatory processes that may come along with cardiovascular disease[48] and type 2 diabetes mellitus[49] the clearance of vitamin D is increased [39]diluting the association with 25(OH)D in individuals with cardiovascular disease and type 2 diabetes mellitus. In contrast, we only found a significant association between 25(OH)D and BMI in those with a metabolic syndrome suggesting that a complex interaction between 25(OH)D, body composition and cardio-metabolic diseases may exist and it needs further elucidation. At last, literature has consistently shown that body composition and vitamin D status are different among men and women [50]. Our study showed that the association between vitamin D and body composition was not significantly different between men and women. Nonetheless, since we found that women had more often vitamin D deficiency, future intervention studies may be targeted to men and women separately.

Strengths and limitations

Main strengths of our study are the prospective, population-based design, large sample size, use of accurate measures of body fat, as well as collection of numerous population characteristics. However, it is also important to mention the limitations of our study. There is a time difference between measurements of 25(OH)D and measurements of body composition, and changes in serum 25(OH)D could have occurred during this time . However, the study on reproducibility of 25(OH)D measurements by Sonderman et al. found the reproducibility of 25(OH)D measurements over time to be relatively high [51]. Another limiting factor to the current investigation is that body fat was measured only once. However, where appropriate we adjusted our analyses for baseline BMI, which correlates well with total fat (partial Pearson correlation coefficient 0.87) and our results were also confirmed by BMI that was repeatedly measured. Another limitation is the fact that we did not have PTH levels measured and therefore could not test if the observed association between 25(OH)D and body fat might be explained by PTH.

Main conclusion and future directions

Lower serum 25(OH)D concentrations were associated with higher total body fat percentage. However, the association between 25(OH)D and fat distribution in this population was mainly explained by BMI. The association between 25(OH)D and body composition might be modified by cardio-metabolic disease. Further studies are needed to confirm our findings and to clarify the underlying mechanisms.

Supplementary Material can be found at Clinical Nutrition

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3.4 Serum Gamma-Glutamyltransferase Within Normal Range, Total Body Fat and Regional Body Fat Distribution: the Rotterdam Study

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ABSTRACT

BACKGROUND Little data is available on gamma-glutamyltransferase (GGT) within normal range and body fat distribution in healthy individuals.

OBJECTIVE We examined whether GGT within normal range is prospectively associated with total body fat (TF) and regional body fat distribution.

METHODS This study was embedded in the Rotterdam Study, a prospective follow-up study among subjects 55 years and older (n= 1715). GGT was measured between 1997-1997 by enzymatic photometry method. TF, android fat (AF), gynoid fat (GF) and android/gynoid ratio (A/G ratio) was assessed using Dual-energy X-ray absorptiometry during follow-up visit (2002-2004). Regression coefficients and 95% Confidence Intervals were calculated using multivariable linear regression models adjusting for confounders.

RESULTS Mean value of GGT of the study population was 21.64 U/L (ranging from 6 to 48 U/L). There was no association between GGT and TF. After adjustments for potential confounders, increased GGT was associated with higher AF (top tertile relative to the lowest: ß=0.35; 95%CI: 0.19, 0.52), lower GF (top tertile relative to the lowest: ß=-0.48; 95%CI: -0.69,.-0.27) and higher AF/GF ratio (top tertile relative to the lowest: ß=0.04; 95%CI: 0.03, 0.06). These associations were independent of C-reactive protein, insulin resistance and non-alcoholic fatty liver disease.

CONCLUSIONS This study suggests that an increase in GGT concentrations within its physiological normal range is a sensitive and early biomarker of unfavorable body fat distribution. As measurement of serum GGT is reliable, easy and inexpensive, its assessment may have clinical utility in identifying individuals at high risk of metabolic disturbances later in life that could benefit from effective preventive interventions.

INTRODUCTION

Gamma-glutamyltransferase (GGT), a marker of alcohol consumption and liver disease, has been strongly associated with obesity related outcomes, including diabetes, hypertension, dyslipidemia, metabolic syndrome, cardiovascular diseases and cancer. [1-6] Also, GGT levels correlate positively with markers of chronic inflammation, such as C-reactive protein (CRP) and fibrinogen[6,7] which are associated with obesity and its phenotypes. [8,9] Furthermore, an increase in serum GGT activity has been suggested to be used as a marker of increased oxidative stress in humans due to the pivotal role of GGT in oxidative stress. [10,11] Recently, it has been reported a causal role of oxidative stress in the development of obesity[12] and a GGT-mediated oxidative stress is capable of inducing lipid oxidation. [11] On the other hand, GGT plays a critical role in cysteine metabolism and is linked to insulin resistance as well, which have been shown to be obesogenic in both animals and human studies. [13-15] Moreover, serum GGT levels seems closely related to liver fat and is included in the diagnostic criteria of fatty liver index. [16]

Despite this evidence, and the global epidemic of obesity, very little attention has been given to GGT within normal range and its role on obesity and/or body fat distribution and healthy individuals. Measurement of serum GGT is accurate, reliable, easy and inexpensive. [17] Therefore, if serum GGT is a marker of total fat and body fat distribution, it might have important implications both clinically and epidemiologically. Thus far, few studies looking at GGT and obesity, have shown a positive correlation between GGT and abdominal obesity in both children and elderly, but the studies mostly used anthropometric measures instead of more accurate measures of body composition, [18] or were often related to a restricted sample [19] or clinical setting. [20,21] Moreover, the studies were limited because of the cross-sectional design and therefore causality could not be addressed.

Hence, in a large cohort study among middle-aged and elderly, we prospectively assessed whether GGT within normal range was associated with total body fat and body fat distribution and whether this association was dependent of CRP, insulin resistance or (non-alcoholic) fatty liver disease.

SUBJECTS AND METHODS

The Rotterdam Study is an ongoing prospective population-based cohort study that focuses on the causes and consequences of chronic and disabling diseases in the elderly. The cohort started enrollment in 1990 and included 7,983 participants aged ≥55 years living in Ommoord, a district of the city of Rotterdam in the Netherlands. Baseline measurements were obtained between 1990 and 1993. Trained research assistants collected data on current health status, use of medication, medical history, lifestyle and risk indicators for chronic diseases during an extensive home interview. Subsequently, the participants visited the study center for detailed clinical examinations and assessment of diet. Follow up visits were held every 3-5 years. [22] This study was approved by the Medical Ethics Committee of the Erasmus Medical Center. All participants gave written informed consent to participate in the study.

Gamma-glutamyltransferase (GGT)

Fasting blood samples were collected by venipuncture during the third visit (RSI-3)(1997-1999), and immediately frozen (−20°C). Serum GGT, ALP, ALT and AST levels were determined within two weeks using a Merck Diagnostica kit (Merck, Whitehouse Station, NJ, USA) on an Elan Autoanalyzer (Merck). All liver biochemistry measurements were obtained in the laboratory of the Department of Epidemiology, Erasmus University Medical Center.

Fatty liver index (FLI)

We used FLI as a surrogate marker for the presence of Non-Alcoholic Fatty Liver Disease (NALFD) in our study, using data from the third visit of the original cohort (RS-I-3; 1997-1999) when ultrasonography was not yet available. The FLI was recently validated in the Rotterdam Study, and was found to correlate closely to the presence of liver steatosis on ultrasonography, performed in a later cohort (AUROC 0.81, sensitivity 64%, specificity 83%). [22] The index uses an algorithm based on BMI, waist circumference, triglycerides (TGs) and GGT with an accuracy of 0.81 (95%CI: 0.80, 0.83) in detecting FL. [16] In accordance with previous studies, presence of FL was defined as a FLI>=60 (probability to have FL is 81%).

Measures of body composition

Body composition was assessed by Dual-energy X-ray absorptiometry (DXA) in the fourth visit of Rotterdam Study (2002-2004). Whole body DXA scans were acquired by using a ProdigyTM total body-fan beam densitometer (GE Lunar Corp, Madison, WI, USA) [23]. Body weight (grams) was divided into bone mineral content, lean (non-fat) and fat mass. Body fat distribution was determined by measuring fat mass at the total-body, and fat mass in android and gynoid regions. Body fat was assessed as percentage of body fat taking into consideration the weight of the individual and total fat as well (when using android and gynoid fat as outcomes).

Potential confounding variables

Information on current health status, medication use, smoking behavior, physical activity and socioeconomic status was obtained between February 1997 - December 1999. Participants were asked whether they were currently smoking cigarettes, cigars, or pipe. Education was defined as low (primary education), intermediate (secondary general or vocational education), or high (higher vocational education or university). Household income was categorized in low (<1700 euro/month), middle (1700-3000 euro/month) or high (≥3000 euro/month). Alcohol intake was assessed in grams of ethanol per day. Cardiovascular disease was defined as a history of myocardial infarction, coronary artery bypass, or percutaneous transluminal coronary angioplasty. Diabetes mellitus was defined as a serum glucose level ≥11 mmol/L or use of glucose lowering drugs. Cardio-metabolic disease was defined as the presence of cardiovascular disease and diabetes mellitus at the third visit and until the DXA measurements took place. Serum cholesterol, CRP, glucose and insulin levels were determined by automated enzymatic procedures in a fasting blood sample. Data on fasting glucose and fasting insulin levels were used to calculate the degree of insulin resistance according to homeostasis model assessment for insulin resistance (HOMA-IR)

which is calculated by dividing the product of fasting levels of glucose and insulin by a constant. [24] Medication use was defined as use of any of the following drugs: antiparkinson, psycholeptic, psychoanaleptics, cytostatics, bile and liver therapy, serum lipid reducing agents, antimycotics for systemic use and use of anti-diabetic therapy at third round and dichotomized: yes or no. The total weekly duration of physical activity was assessed by an adapted version of the Zutphen Physical Activity Questionnaire and the LASA Physical Activity Questionnaire. [25,26] Physical height and body weight 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). To take into account overall dietary quality we used the Dutch Healthy Diet (DHD)-index assessed at the first visit. [27] Briefly, the DHD-index is a continuous score that represents compliance to the Dutch Guidelines for a Healthy Diet as assessed from the FFQ at baseline. [27]

Population of analysis

The present study used data from the third visit of the original cohort (RS-I-3; February 1997 – December 1999). There were 3920 persons eligible for laboratory measurements. Among them, 11 subjects were excluded because of no information available on GGT (because of logistic reasons or no consent). Additionally, 1711 participants died or did not have DXA measures (because of logistic reasons) performed at the fourth round visit (January 2002- July 2004). Moreover, 483 subject were excluded because of GGT values not within the normal range (for men: <12 U/L or >48 U/L and for women: <6 U/L or >28 U/L) and thus leaving 1715 participants for our final analysis.

Statistical analysis

All analyses were performed by IBM SPSS Statistics 21. Data are presented as the mean ± SD unless indicated otherwise. We investigated the association between serum GGT with body fat both as continuous (one-standard deviation increment in GGT) variable and by tertiles. We used natural log-transformed values of serum CRP concentrations and HOMA-index, to better approximate normal distribution. Multivariate linear regression was used to examine whether GGT or FLI were independently associated with, total body fat android fat mass (%), gynoid fat mass (%) and android/gynoid fat mass ratio. We built minimally adjusted models, with age, sex and body mass index (continuous) as covariates (Model 1); and multivariable models with the following additional covariates: education level (low, intermediate, high), income status (low, middle, high), smoking status (ever, never), total physical activity (continuously), alcohol intake (continuously), serum total cholesterol, DHD-index (continuously), presence of cardio-metabolic diseases and medication use (Model 2). In model three to evaluate the effect of intermediate factors we additionally adjusted for CRP (continuous) and HOMA-index (Model 3). For the association between GGT and total body fat and regional body fat distribution, a fourth model was built, which included the covariates within model 2 and the presence of FL (yes vs. no). The *F* test was used to test whether there were overall significant differences in fat mass (total, android or gynoid fat mass) between levels of GGT and FLI. We constructed multivariate linear regression models for pooled sexes because formal tests of interaction (sex×log-GGT) were not statistically

significant for any outcome. Furthermore, to examine if the association between GGT and body fat differs by smoking status, alcohol consumption and BMI, we tested for statistical interaction by adding a product term in model 2. In case of significant effect modification, stratified analysis was performed and the results were presented for model 3 and 4. To check for non-linear relationship, a quadratic term was tested in multivariable model 2. A *P-value* lower than 0.05 was considered as statistically significant. Multiple imputation procedure was used (*N*= 5 imputations) to adjust for potential bias associated with missing data. Rubin's method was used for the pooled regression coefficients (β) and 95% Confidence Intervals. [28]

Sensitivity analyses

To explore the possibility of selection bias, we examined whether there were differences in age, sex, prevalent cardio-metabolic diseases, BMI and GGT levels between participants included in our analysis and those excluded due to no data on DXA measurements. Additionally, using generalized estimated equations with exchangeable correlation structure [29], we evaluated the relation of GGT within normal range to 11-year changes in BMI, waist circumference and waist to hip ratio in participants included in our analysis (n=1715) and further extended to participants of the third visit of the first cohort of the Rotterdam Study and first visit of the second cohort of the Rotterdam Study (n=4595). Our subset included 412 participants who had cardio-metabolic disease at third visit and/or during the follow up and therefore, a sensitivity analysis was performed after removing these subjects. Moreover, additional models were built by adding ALT and AST or wait to hip ratio to covariates in model 2. To see if GGT values not within the normal range could play a role in total body fat and body fat distribution, we rerun all analysis including subjects with values of GGT not within the normal range (n=483). Because the distribution of GGT values was right-skewed when these subjects were included in the analysis, a natural log-transformation was applied and GGT teriles were calculated using the natural log-transformed GGT.

RESULTS

Table 1 displays the selected characteristics of the study population. The mean age was 70.51 ± 5.88 and the mean GGT was 21.64 ± 20.0 U/L (Table 1).

Association between GGT, total body fat and regional body fat distribution

There was no association between GGT analyzed continuously or in tertiles and total body fat (table 2). In multivariable linear models adjusted for age, gender, education level, income status, smoking status, physical activity, body mass index, serum total cholesterol, DHD-index, cardio-metabolic disease, medication use (Model 2), higher GGT was associated with higher android fat mass (per SD increase in GGT: ß=0.17; 95%CI: 0.09, 0.24 and top tertile relative to the lowest: ß=0.35; 95%CI: 0.19, 0.52; *P* for F-test, 3 df: 0.7 10^{-05}, Table 3), lower gynoid fat mass (per SD increase in GGT: ß=-0.25; 95%CI: -0.35, -0.16 and top tertile relative to the lowest: ß=-0.48; 95%CI: -0.69,.-0.27; *P* for F-test, 3 df: 5.9 10^{-07}, Table 3) and higher android/gynoid fat mass ratio (per SD increase in GGT: ß=0.023; 95%CI: 0.015, 0.03 and top tertile relative to the lowest:

Table 1 Selected characteristics of study participants

	(N=1715)
Age	70.71 ± 5.96
Female % (n)	935 (54.5)
Smoking status n (%)	242 (14.1)
Physical activity (min/week)	2783.84 ± 1084.98
Alcohol intake g/day*	2.86 (158732,86)
Education Level n (%)	
Low	753 (43.9)
Medium	762 (44.4)
High	200 (11.7)
Income n (%)	
Low	438 (25.5)
Medium	622 (36.3)
High	655 (38.2)
BMI (kg/m^2)	26.59 ± 3.66
Waist to hip ratio	0.91 ± 0.096
DHD-index	48.85 ± 10.18
Prevalent cardio-metabolic disease n (%)	412 (24%)
Medication use n (%)	266 (15.5)
GGT	21.64 ± 20.0
ALT	21.51 ± 9.12
AST	23.75 ± 5.80
Hepatic Steatosis n (%)**	425 (24.8)
Insulin (pmol/l)*	64 (1124)
Glucose (mmol/l)	5.75 ± 1.19
HOMA-IR (units)*	2.93 (97.18)
C-reactive protein (mg/ml)*	2.05 (144.80)
Serum total cholesterol (mmol/l)	5.80 ± 0.94
Total fat mass (%)	33.60 ± 8.40
Abdominal fat mass (%)	9.43 ± 1.95
Gynoid fat mass (%)	15.28 ± 2.13
Andorid/Gynoid fat mass rato	0.64 ± 0.20

ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; DHD-index: Dutch Healthy Diet Index; HOMA-IR: HOMA-insulin resistance; GGT: gamma glutamyltransferase; kg: kilogram; Plus minus values are mean ± SD

*Median(range)

**Defined by a fatty liver index ≥60

ß=0.04; 95%CI: 0.03, 0.06; *P* for F-test, 3 df: 7.4 10^{-08}, Table 4). The overall results changed only slightly after further adjustments for CRP, HOMA-index or FL (Table 2-4). Significant quadratic terms were observed for the association between GGT and android fat and gynoid fat mass (Table 3). In contrast, no significant quadratic terms were observed for the association of GGT with total body fat mass or android/gynoid fat mass ratio. (Table 2-4).

Effect-modification by alcohol intake, smoking status and body mass index

No significant interaction terms with alcohol intake and smoking status were observed for the association between serum GGT, total body fat and regional body fat distribution (*P*-interactions >0.05). However, for the association of GGT with total body fat mass and gynoid fat mass, significant interaction terms were observed with BMI (*P*-interaction<0.05). After stratification by BMI (*P*-interaction=0.005), higher GGT was associated with higher total fat mass in participants with BMI<median (Model 3-top tertile relative to the lowest: ß=1.45; 95%CI: 0.54, 2.37; *P* for F-test, 3 df: 0.014, **Figure 1**). In contrast, high levels of GGT were associated with lower total body fat in subjects with BMI≥ median (Model 3-top tertile relative to the lowest: ß=-0.93; 95%CI: -1.18, -0.06; *P* for F-test, 3 df: 0.28, **Figure 1**). Similarly, after stratification by BMI (*P*-interaction=0.026), raised GGT was associated with lower gynoid fat mass in participants with BMI<median (Model 3-top tertile relative to the lowest: ß=-0.52; 95%CI: -0.84, -0.19; *P* for F-test, 3 df: 0.0004, **Figure 2)** but not in subjects with BMI≥ median (Model 3-top tertile relative to the lowest: ß=-0.16; 95%CI: -0.41, 0.10; *P* for F-test, 3 df: 0.12, Figure 2). There was no significant interaction term with BMI for the association of GGT with AF or A/G ratio (*P*-interactions>0.05).

Table 2 Gamma-glutamyltransferase (GGT) and total body fat mass

	Total body fat mass (%) β (95% CI)			
GGT	MODEL 1[a]	MODEL 2	MODEL 3	MODEL 4
1st tertile	reference	reference	reference	reference
2nd tertile	0.03 (-0.46, 0.53)	-0.03 (-0.53, 0.47)	-0.10 (-0.59, 0.40)	-0.03 (-0.52, 0.47)
3rd tertile	0.50 (-0.06, 1.06)	0.37 (-0.19, 0.94)	0.19 (-0.38, 0.76)	0.51 (-0.07, 1.09)
P for F-test	0.31	0.43	0.87	0.23
Continuous (per SD increase)*	0.16 (-0.07, 0.39)	0.12 (-0.14, 0.38)	0.003 (-0.26, 0.27)	0.20 (-0.07, 0.47)

Model 1: Adjusted by age, gender and body mass index

Model 2: Model 1 + alcohol intake, Dutch Healthy Diet-index, smoking status, education level, income level, cardio-metabolic diseases, serum total cholesterol, medication use

Model 3: Model 2 + C-reactive protein and insulin resistance

Model 4: Model 2 + presence of fatty liver

*Quadratic term not significant (p>0.05)

Table 3 Gamma-glutamyltransferase (GGT) android fat mass

	Android fat mass (%) β (95% CI)				Gynoid fat mass (%) β (95% CI)			
GGT	MODEL 1	MODEL 2	MODEL 3	MODEL 4	Model 1	MODEL 2	MODEL 3	MODEL 4
1st tertile	reference	reference	reference	reference	reference	reference	reference	reference
2nd tertile	0.22 (0.07, 0.36)	0.20 (0.06, 0.35)	0.15 (0.003, 0.29)	0.20 (0.05, 0.34)	-0.09 (-0.27, -0.10)	-0.07 (-0.26, 0.11)	-0.01 (-0.20, 0.17)	-0.08 (-0.26, 0.11)
3rd tertile	0.39 (0.23, 0.54)	0.35 (0.19, 0.52)	0.23 (0.06, 0.40)	0.29 (0.12, 0.46)	-0.51 (-0.71, -0.30)	-0.48 (-0.69, -0.27)	-0.34 (-0.55, -0.13)	-0.50 (-0.71, -0.28)
P for F-test	$7.3\ 10^{-07}$	$0.7\ 10^{-05}$	0.003	$9.3\ 10^{-05}$	$1.94\ 10^{-07}$	$5.9\ 10^{-07}$	0.0002	$3.07\ 10^{-07}$
Continuous (per SD increase)*	0.18 (0.14, 0.22)	0.17 (0.09, 0.24)	0.10 (0.02, 0.18)	0.13 (0.05, 0.21)	-0.26 (-0.31, -0.21)	-0.25 (-0.35, -0.16)	-0.18 (-0.28, -0.08)	-0.27 (-0.37, -0.17)
Continuous (per SD increase)†	0.52 (0.20, 0.83)	0.48 (0.16, 0.79)			-0.71 (-1.10, -0.31)	-0.69 (-1.08, -0.29)		

Model 1: Adjusted by age, gender and body mass index

Model 2: Model 1 + alcohol intake, Dutch Healthy Diet-index, smoking status, education level, income level, cardio-metabolic diseases, serum total cholesterol, medication use

Model 3: Model 2 + C-reactive protein and insulin resistance

Model 4: Model 2 + presence of fatty liver

* The continous βs are presented for the model without the quadratic term

† Quadratic term significant ($p<0.05$): the continous βs are presented when the quadratic term is in the model

Table 4 Gamma-glutamyltransferase (GGT) and android/gynoid fat mass ratio

	Android/Gynoid fat mass ratio β (95% CI)			
GGT	MODEL 1[a]	MODEL 2	MODEL 3	MODEL 4
1st tertile	reference	reference	reference	reference
2nd tertile	0.02 (0.001, 0.03)	0.01 (-0.001, 0.03)	0.009 (-0.006, 0.02)	0.014 (-0.001, 0.029)
3rd tertile	0.05 (0.03, 0.06)	0.04 (0.03, 0.06)	0.03 (0.01, 0.05)	0.04 (0.02, 0.06)
P for F-test	$6.2\ 10^{-09}$	$7.4\ 10^{-08}$	0.0001	$3.0\ 10^{-06}$
Continuous (per SD increase)*	0.024 (0.02, 0.028)	0.023 (0.015, 0.03)	0.016 (0.01, 0.024)	0.019 (0.01, 0.027)

Model 1: Adjusted by age, gender and body mass index

Model 2: Model 1 + alcohol intake, Dutch Healthy Diet-index, smoking status, education level, income level, cardio-metabolic diseases, serum total cholesterol, medication use

Model 3: Model 2 + C-reactive protein and insulin resistance

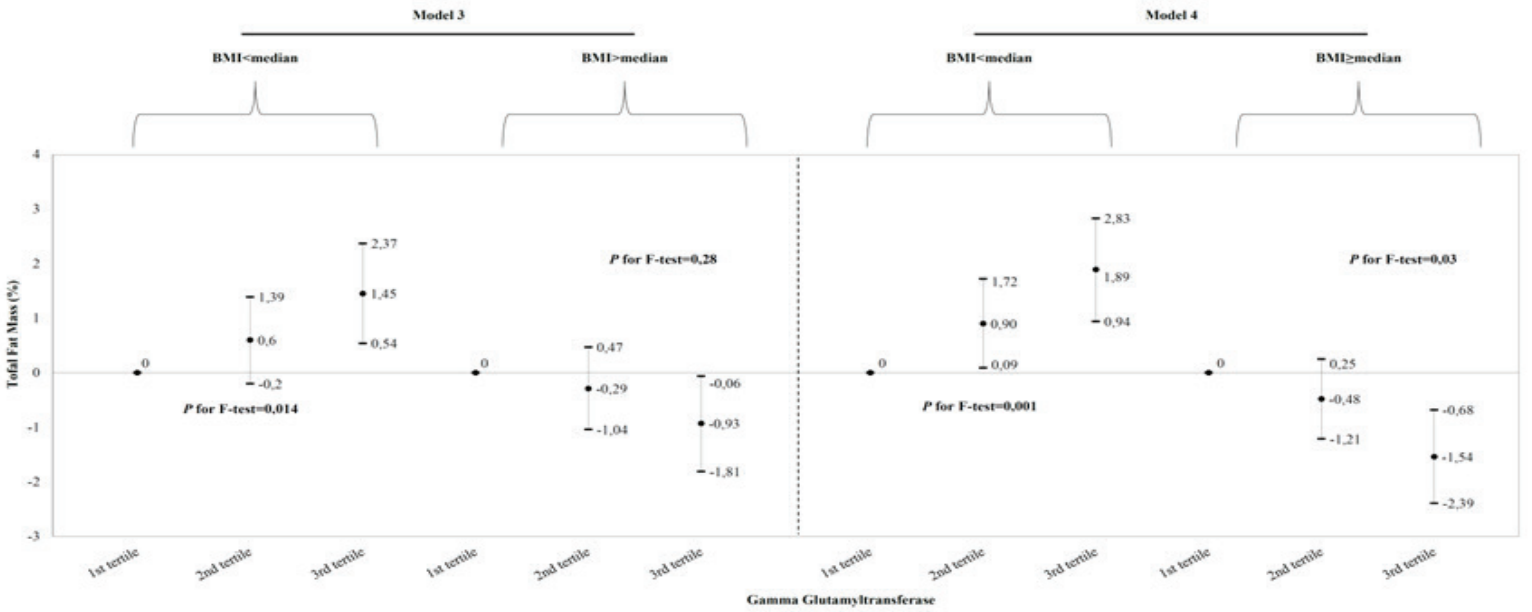
Model 4: Model 2 + presence of fatty liver

* Quadratic term not significant (p>0.05)

Sensitivity analyses

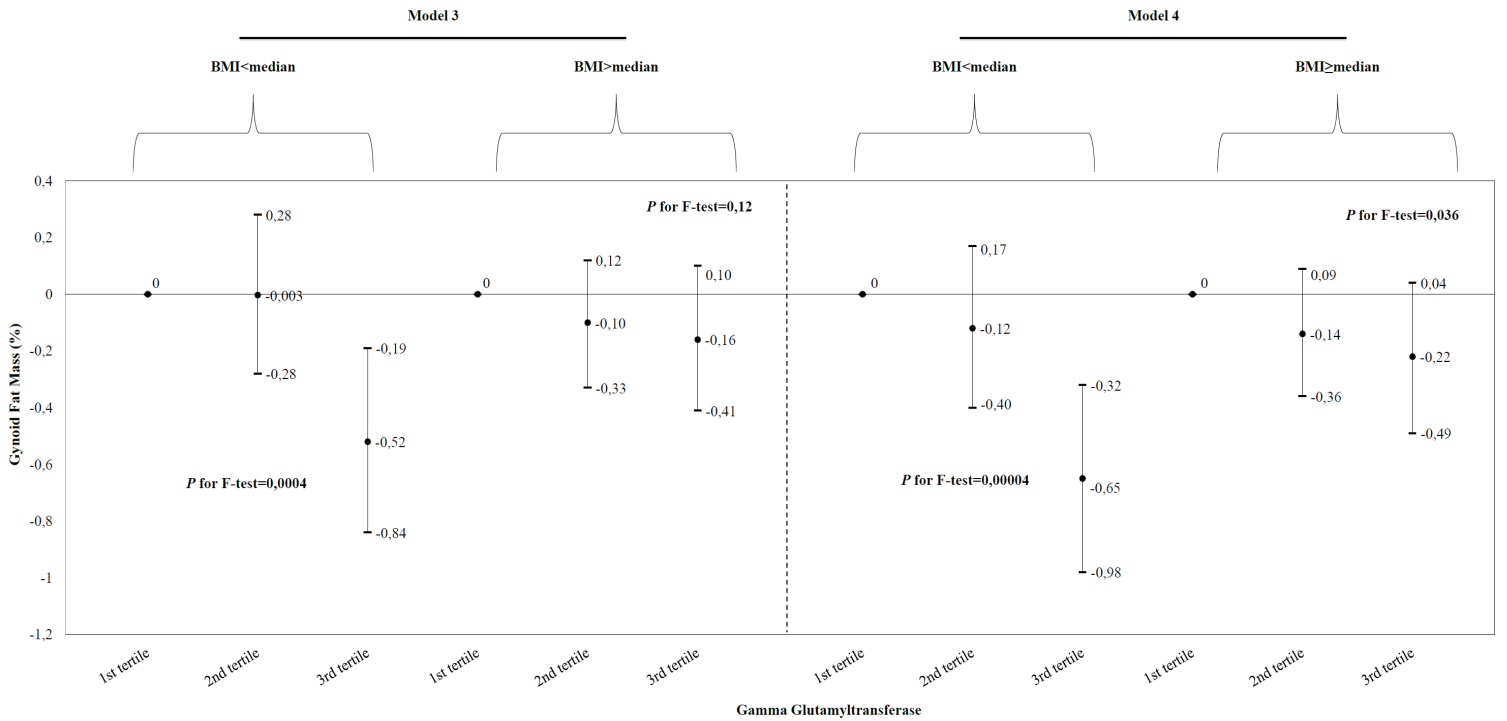
There were significant differences in gender (56.5% female vs. 61.2 %), age (mean age=70.5 vs. 75.3 years old), prevalent cardio-metabolic diseases (26.1% vs. 38.9%) but not with regard to BMI or GGT levels among participants included in our analysis and participants that were excluded because of no data on DXA measurements. All associations that were statistically significant in the main analysis remained unchanged in terms of statistical significance when reanalyzed, excluding subjects with cardiometabolic disease and/or subjects who had (non-alcoholic) fatty liver disease. The association of GGT with regional body fat distribution remained robust in models adjusted additionally for serum AST and ALT or waist to hip ratio measured at third round visit (February 1997- December 1999). Also, the results did not change in terms of statistically significance when we repeated all analysis including subject with abnormal values of GGT (all: data not shown). Similarly to the results with body fat, GGT did not affect long-term changes in BMI, but one unit increase in log GGT was associated with an annual rise of 0.28 cm and 0.004 in waist circumference and waist to hip ratio respectively independent of CRP, HOMA-IR or FL, in the population included in our analysis and as well in the population extended to both cohorts of the Rotterdam Study (Supplementary Table 1 and 2).

Figure 1 The association between gamma-glutamyltransferase and total fat mass by body mass index.



βs (95% CIs) were estimated by using multivariable linear mode ls adjusted for age, gender and body mass index, alcohol intake, Dutch Healthy Diet-index, smoking status, education level, income level, cardio-metabolic diseases, serum total cholesterol, medication use and C-reactive protein, insulin resistance (Model 3) or non-alcoholic fatty liver disease (Model 4).

Figure 2 The association between gamma-glutamyltransferase and gynoid fat mass by body mass index.



βs (95% CIs) were estimated by using multivariable linear models adjusted for age, gender and body mass index, alcohol intake, Dutch Healthy Diet-index, smoking status, education level, income level, cardio-metabolic diseases, serum total cholesterol, medication use and C-reactive protein, insulin resistance (Model 3) or non-alcoholic fatty liver disease (Model 4).

DISCUSSION

In this prospective study, we found that GGT levels within the normal range are associated with unfavorable body fat distribution, independent of age, gender, socioeconomic status, lifestyle factors, and other confounders. These associations were independent of CRP, insulin resistance or non-alcoholic fatty liver disease.

To our knowledge, this is the first prospective study to show an association between levels of GGT and body fat distribution. Previous studies reporting on obesity and GGT have mostly tested the hypothesis that obesity, and in particular central obesity may predict levels of GGT, [21,30-33] and not vice versa. Furthermore, all these studies have been on cross-sectional design and therefore causality could not be addressed. Nevertheless, most of them have reported a strong positive correlation between GGT and abdominal obesity; for example, in a study of 2704 women and men, 35-80 years of age, a positive correlation was observed between GGT, waist circumference and waist to hip ratio. [18] Similar correlations were reported also by Mager and colleagues in a study of 44 children. [19] In addition, Iwasaki et al in a cross-sectional study of 257 Japanese patients, by using more accurate measures of body composition such as by dual-energy X-ray absorptiometry, showed that higher levels of GGT were significantly associated with higher visceral fat whereas no association was observed with subcutaneous fat, proposing that the serum GGT may be useful as a convenient indicator of visceral adiposity.[20] However, in contrast to our study, Iwasaki and his colleagues did not examine the association of GGT with gynoid fat or android/gynoid fat ratio.

Although the current study suggests that GGT may be used as a marker for abdominal fat, we did not measure visceral or subcutaneous fat separately and therefore we were not able to distinguish these fat compartments. It has been shown that visceral adipose tissue is associated with more adverse cardiometabolic risk factor profiles than abdominal subcutaneous adipose tissue. [34] Measurement of serum GGT is reliable, easy and inexpensive and therefore, if serum GGT is a marker of unfavorable body fat distribution, it might have important implications both clinically and epidemiologically. Future research is thus needed to validate our findings and to assess if GGT within normal range can be a specific marker to visceral fat.

It is at present unclear the mechanism underlying the association between GGT and body fat. An increase in concentrations of GGT is conventionally interpreted as a marker of alcohol abuse, insulin resistance and/or liver damage; [35] however, neither of these interpretations explains the observed association in the current study of GGT within what is considered its physiological normal range with body fat distribution. Nor chronic inflammation, as measured by CRP, could explain the association. GGT and body fat distribution has been shown to positively correlate with markers of chronic inflammation, such as CRP and fibrinogen, [6] which on the other hand, closely correlate with obesity. [8,9] However, other mechanisms may explain the association of GGT with body fat distribution. Although GGT has been regarded as marker of liver diseases, GGT is widely distributed in the human body. [36] There is evidence that cellular GGT plays a pivotal role in antioxidant defense system. [11] As a primary function, ectoenzyme GGT maintains

intracellular concentrations of glutathione, the most important non-protein antioxidant of the cell. [11] Increased GGT activity can be a response to oxidative stress, facilitating increased transport of glutathione precursors into cell. [11] Also, ectoplasmatic GGT may be involved in the generation of reactive oxygen species (ROS), particularly in the presence of $Fe3^{+}$ and CU^{2+}[34]. Recently, oxidative stress was shown to induce obesity [37] and a GGT-mediated oxidative stress was reported to be capable of inducing oxidation of lipids. [11] GGT plays an important role in homeostasis of plasma cysteine as well [13] which similar to GGT induces oxidative stress mainly in the presence of cooper ions. [38] Cysteine has been related with body fat in both men and women, independent of GGT. [13] Furthermore, GGT levels have been reported to correlate with adipokines such as adiponectin, [39] which play an important role in obesity by different pathways i.e. increased energy expenditure, insulin sensitivity or fatty acid oxidation. [37] Therefore, it still remains to be established whether the observed association between GGT and body fat distribution reflects other truly detrimental pathways, whether it is intermediate in the pathology of obesity or is a true risk factor initiating a mechanistic pathway on the road to obesity.

The main strength of this study are its prospective design, the relative large sample size, use of accurate measures of body fat and very detailed information elicited on several covariates related to GGT. A further strength is that we enrolled participants randomly selected from the general population. Moreover, there were no differences in GGT levels among participants included in our study and those excluded due to no data on DXA measurements, and therefore minimizing the possibility of selection bias which could have influenced the validity of our results. Furthermore, it has been shown that using a restricted source population for a cohort study does not compromise validity of exposure-outcome association[40]. Several limitations of our approach merit comment. Establishing that GGT is a risk factor for development of abdominal obesity would require additional prospective and experimental studies to understand the potential pathways behind the association. Also, there is a time difference between measurement of GGT and body fat distribution, which is a potential limitation because changes could occur over time. However, we measured serum GGT levels at the baseline visit of The Rotterdam Study (1990-1993) in a subgroup of study participants (n=2753 participants) and at the fifth visit (2009-2011) (n=1395 participants) and observed high correlations with GGT measure at the third visit (1997-1999) (interclass correlation coefficients between the first and the third visit and the third and the fifth visit of the Rotterdam Study were 0.84 and 0.81 respectively), supporting internal consistency and validity. Another limiting factor to the current investigation is that body fat was measured only once and five years later after baseline. However, in all analysis we adjusted for baseline BMI, which correlates well with total fat; for example partial Pearson correlation coefficient between BMI and total fat at the fourth round visit (January 2002-July 2004) was 0.87. Moreover, additional adjustment for waist to hip ratio, a surrogate marker of android/gynoid fat mass ratio, did not affected our results (partial Pearson correlation coefficient between waist to hip ratio and android/gynoid fat mass ratio at the fourth round visit (year 2002-2004) was 0.77. Lastly, the participants enrolled in our study are all white, limiting the generalizability to other ethnicities.

In conclusion, our findings suggest that serum levels of GGT within physiological normal range could be an early biomarker of unfavorable body fat distribution and that the well-known associations of obesity with cardiometabolic and other chronic disease may be modified by serum GGT. However, it is at present unclear whether medications or lifestyle factors that alter GGT metabolism can be usefully used in prevention of obesity and its complications. Also, further investigations are needed to clarify the underlying mechanisms.

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Supplemental material

Supplementary Table 1 The longitudinal association between gamma-glutamyltransferase (GGT) within normal range and 11-year change in anthropometric measures (n=1715)*

GGT Continuous (per unit log increase)	Body Mass Index (kg/m^2)	Waist circumference (cm)	Waist to hip ratio
Model 1 β (95% CI)	-0.01 (-0.04; 0.02)	0.28 (0.18; 0.38)	0.004 (0.002; 0.005)
Model 2 β (95% CI)	-0.01 (-0.03:0.01)	0.28 (0.23; 0.34)	0.004 (0.003; 0.004)
Model 3 β (95% CI)	-0.01 (-0.03:0.01)	0.28 (0.23; 0.33)	0.004 (0.003; 0.004)
Model 4 β (95% CI)	-0.01 (-0.03; 0.01)	0.28 (0.23; 0.34)	0.004 (0.003; 0.004)

*Gamma-glutamyltransferase was measured at baseline (third visit of the first cohort of the Rotterdam Study) whereas body mass index, waist circumference and waist to hip ratio were assessed at baseline, 5 and 10 years later. Linear regression models fitted into generalized estimating equations were used to analyze relations of GGT to average annual individual anthropometric factors changes.
Model 1: Adjusted by age, gender and body mass index
Model 2: Model 1 + alcohol intake, smoking status, physical activity, cardio-metabolic diseases, serum total cholesterol, medication use
Model 3: Model 2 + C-reactive protein and insulin resistance
Model 4: Model 2 + presence of fatty liver

Supplementary Table 2 The longitudinal association between gamma-glutamyltransferase (GGT) within normal range and 11-year change in anthropometric measures (n=4595)*

GGT Continuous (per unit log increase)	Body Mass Index (kg/m^2)	Waist circumference (cm)	Waist to hip ratio
Model 1 β (95% CI)	-0.01 (-0.04; 0.01)	0.24 (0.17; 0.30)	0.003 (0.002; 0.004)
Model 2 β (95% CI)	-0.01 (-0.02:0.00)	0.23 (0.17; 0.30)	0.003 (0.002; 0.004)
Model 3 β (95% CI)	-0.02 (-0.04:0.01)	0.23 (0.17; 0.29)	0.003 (0.002; 0.004)
Model 4 β (95% CI)	-0.02 (-0.04; 0.01)	0.23 (0.16; 0.30)	0.003 (0.003; 0.004)

*Gamma-glutamyltransferase was measured at baseline (third visit of the first cohort of the Rotterdam Study and first visit of the second cohort of the Rotterdam Study) whereas body mass index, waist circumference and waist to hip ratio were assessed at baseline, 5 and 10 years later. Linear regression models fitted into generalized estimating equations were used to analyze relations of GGT to average annual individual anthropometric factors changes.
Model 1: Adjusted by age, gender and body mass index
Model 2: Model 1 + alcohol intake, smoking status, physical activity, cardio-metabolic diseases, serum total cholesterol, medication use
Model 3: Model 2 + C-reactive protein and insulin resistance
Model 4: Model 2 + presence of fatty liver

3.5 Gamma-Glutamyltransferase Levels, Prediabetes and Type 2 Diabetes Risk: a Mendelian Randomization Study

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ABSTRACT

Background: High levels of gamma-glutamyltransferase (GGT) are consistently associated with increased risk of prediabetes and type 2 diabetes in observational studies. Whether levels of GGT are causally associated with prediabetes and type 2 diabetes remains still unknown.

Methods: We used data of the prospective population-based Rotterdam Study, compromising 8,611 individual. Cox models were used for both the observational and genetic analysis. A Mendelian Randomization Study was performed using a genetic risk score consisted of 26 GGT-related loci, based on a genome-wide association study (GWAS) on liver enzymes and using data from the Rotterdam Study and large GWAS on glycemic traits and type 2 diabetes.

Results: During follow-up, 1125 cases of prediabetes (mean follow up 5.7 years) and 811 cases of type 2 diabetes (6.9 years) were ascertained. The corresponding observational predicted hazard ratios in the multivariable model were 1.10 for prediabetes (95% CI: 1.02 to 1.19) and 1.19 for type 2 diabetes risk (95% CI: 1.30 to 1.32). The genetic risk score increa sed GGT levels by 0.09 U/L per SD and explained 3.2% of the GGT variation. However, GGT genetic risk score was not associated with prediabetes (HR= 0.97 [95% CI: 0.91 to 1.04], *P*-value= 0.4) or type 2 diabetes (HR= 0.96 [95% CI: 0.89 to 1.04], *P*-value= 0.3) in the Rotterdam Study. Using data from GWAS on glycemic traits and type 2 diabetes did not change the results.

Conclusion: Our study does not support a causal effect of GGT levels on the risk of prediabetes or diabetes. The association of GGT with diabetes in observational studies is likely to be driven by confounding bias or reverse causation.

INTRODUCTION

Circulating levels of gamma-glutamyltransferase (GGT) are strongly associated with increased risk of prediabetes and type 2 diabetes in observational studies [1]. A meta-analysis of 24 cohorts reported 34% higher diabetes risk in a comparison of extreme thirds of baseline levels of GGT [1]. It is, however, unclear whether the association between GGT and diabetes is free of unobserved confounding. In addition, GGT levels could be changed as a consequence of type2 diabetes pathology (i.e., reverse causality). Therefore, causal role of GGT on type2 diabetes is uncertain.

In recent years, genetic information has commonly been used to infer causality in the pathogenesis of complex diseases. The inference is based on the fact that alleles are allocated randomly during gamete formation; therefore, genetic variants are inherited independent of potential confounding. We are aware of one previous study (4,360 individuals) where a single nucleotide polymorphism (SNP) in the *GGT1* gene was used to support a causal relation between GGT and fasting insulin employing a Mendelian Randomization (MR) approach [2]. This SNP explains only 1.2% of the variation in GGT levels. Its known that a weak instrumental variable makes the association susceptible to false positive findings. So far 26 loci have been identified for GGT. Thus, a genetic risk score (GRS) combining the effect of all these loci could provide a stronger instrument for the Mendelian Randomization analysis.

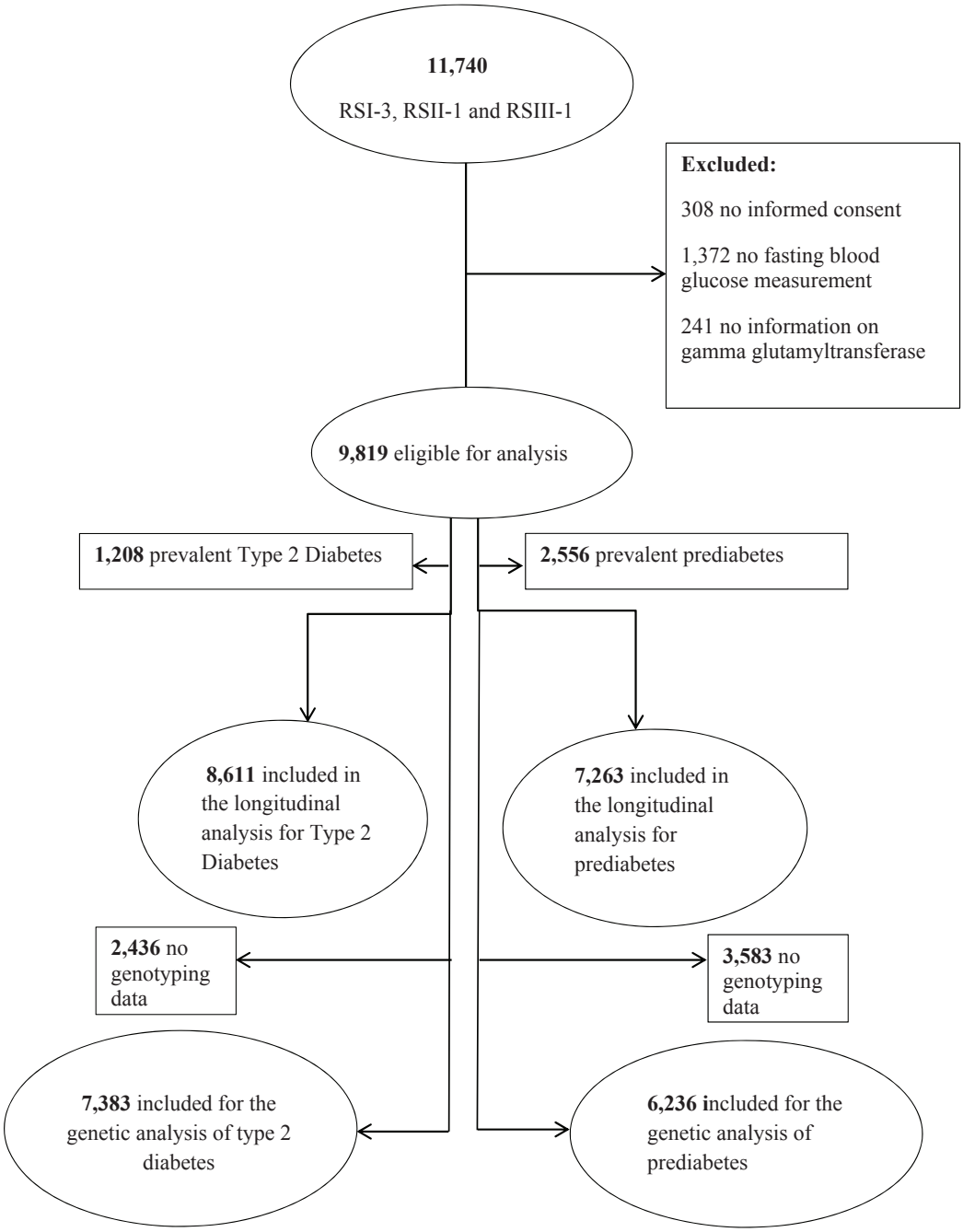
In this study, we investigated the association between serum GGT levels and risk of incidence prediabetes and type 2 diabetes in the Rotterdam Study, a large prospective population-based cohort study of participants ≥ 55 years. Capitalizing on Mendelian Randomization, we created a genetic risk score using 26 variants that are identified for GGT in a recent GWAS and examined its association with prediabetes and type 2 diabetes in the Rotterdam Study. We then boosted the statistical power by using summary-level data from the largest GWAS on glycemic traits and type 2 diabetes [3-5].

METHODS

Study population

This study was performed among participants of the prospective population-based Rotterdam Study. In 1989, all residents aged 55 years or older in Ommoord, a suburb of Rotterdam, were invited to participate in the study (RS-I). Seventy-eight percent of the invitees agreed to participate (n= 7,983). In 1999, the Rotterdam Study was extended by including 3,011 participants from those who either moved to Ommoord or turned 55 (RS-II). The third cohort was formed in 2006 and included 3,932 participants 45 years and older (RS-III). There were no eligibility criteria to enter the Rotterdam Study cohorts except the minimum age and residential area based on ZIP codes. Participants have been re-examined every 3-4 years and have been followed up for a variety of diseases. A more detailed description of the Rotterdam Study can be found elsewhere [6]. The Rotterdam Study has been approved by the medical ethics committee according to the Population Screening Act: Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of

Figure 1. Flow Chart of Study Population



the Netherlands. All participants in the present analysis provided written informed consent to participate and to obtain information from their treating physicians.

Population for analysis

We used the third visit of the first cohort (RSI-3, 1997-1999, n= 3,877) and the first center visit for both the second cohort (RSII-1, 2000-2001, n= 2,507) and the third cohort (RSIII-12006-2008, n= 3,437) as baseline. We excluded 1,372 participants with no fasting glucose measurement and 241 other participants with no information on GGT. Next, we excluded individuals with prevalent type 2 diabetes or prediabetes depending on the studied outcome (**Figure 1**). To perform the genetic analysis for prediabetes, we further excluded 3,583 participants due to lack of genotyping data; for type 2 diabetes, we excluded 2,436 participants (**Figure 1**).

Measurement of gamma-glutamyltransferase.

Fasting blood samples were collected by venipuncture, and immediately frozen −20ºC. Serum GGT was determined within two weeks using a Merck Diagnostica kit on an Elan Autoanalyzer Merck. All liver biochemistry measurements were obtained in the laboratory of the Department of Epidemiology, Erasmus University Medical Center.

Ascertainment of prediabetes and type 2 diabetes.

The participants were followed from the date of baseline center visit onwards. At baseline and during follow-up, cases of prediabetes and type 2 diabetes were ascertained through active follow-up using general practitioners' records, glucose hospital discharge letters and glucose measurements from the Rotterdam Study visits which takes place approximately every 4 years. According to the WHO guidelines, prediabetes was defined as a fasting blood glucose between 6.0 mmol/L and 7.0 mmol/L and type 2 diabetes was defined as a fasting blood glucose > 7.0 mmol/L, or the use of blood glucose lowering medication[7]. Information regarding the use of blood glucose lowering medication was derived from both structured home interviews and linkage to pharmacy records. At baseline, more than 95% of the Rotterdam Study population were covered by the pharmacies in the study area. All potential events of prediabetes and type 2 diabetes were independently adjudicated by two study physicians. In case of disagreement, consensus was sought with a specialist. Follow-up data was complete until January 1st 2012 [8].

Covariates

Body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared. Information on current smoking was acquired from questionnaires. Alcohol intake was assessed in grams of ethanol per day from the questionnaires. Participants were asked for the average daily consumption. Lipid-lowering and antihypertensive medication use was assessed during a structured home interview. Blood pressure was measured twice by an oscillometric device after five minutes of rest and the mean was taken as the subject's reading. Data on fasting glucose and insulin levels were used to calculate the degree of insulin resistance according to homeostasis model assessment for insulin resistance (HOMA-IR) which is calculated by dividing the product

of fasting levels of glucose and insulin by a constant [9]. Serum total cholesterol, triglycerides and high-density lipoprotein (HDL) cholesterol levels were determined using an automated enzymatic method. Blood glucose and insulin levels were quantified using standard laboratory technics. At RSI-1, C-reactive protein (CRP) was measured using a rate near-infrared particle immunoassay (IMMAGE Immunochemistry System, Beckham Coulter, Fullerton, CA).In RSII-1 and RSIII-1, CRP was measured using a particle enhanced immunoturbidimetric assay (Roche Diagnostics, Mannheim, Germany). Prevalent cardiovascular disease (CVD) was defined as having a history of coronary heart disease (CHD) including myocardial infarction or coronary revascularization procedures or heart failure, as previously described [8].

Genotyping

Genotyping was conducted, in self-reported white participants in all three cohorts using the Illumina Infinium HumanHap550K Beadchip in RS-I-1and RS-II-3 and the Illumina Infinitum HumanHap 610 Quad chip in RS-III-1at 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 phenotyping sex, or recognized as being outlier with identical-by-state clustering analysis. Before imputation, SNPs with minor allele frequency MAF < 0.01, call rate $< 95\%$ and departure from Hardy-Weinberg equilibrium cut off P-value 1×10^{-6} were excluded. SNPs were imputed based on the 1000 Genomes cosmopolitan phase 1 version 3 reference.

Construction of the genetic risk score

In this study, we selected 26 single-nucleotide polymorphisms (SNPs) that passed the genome-wide significance threshold (P value $< 5 \times 10^{-8}$) in a genome-wide association study (GWAS) of GGT levels on more than 61,089 individuals of European descent [10]. SNPs were not in linkage disequilibrium with each other ($R^2 > 0.8$). **Supplementary Table 1** provides an overview of the SNPs included in the genetic score and weights assigned to each SNP. The effect allele (coded 0-2) was the GGT raising allele. We then calculated the GRS by multiplying the number of risk alleles at each locus by corresponding reported beta coefficient and sum the products. The total score was then divided by twice the average effect size multiplied by 100 to rescale it.

Statistical Analysis

For the observational association of GGT with prediabetes and type 2 diabetes, we estimated hazard ratios (HR) in four adjustment Cox proportional hazards models. Model 1 was adjusted only for age, sex and cohort effect. Model 2 was further adjusted for BMI and ALT. In model 3, potential risk factors and confounders of type 2 diabetes were added to model 2 including total and HDL cholesterol, triglycerides, CRP, waist circumference, current smoking status, systolic blood pressure, antihypertensive medication use, lipid lowering medication use and prevalent CVD. Model 4 was further adjusted for fasting insulin and fasting glucose levels. Participants with prevalent prediabetes and type 2 diabetes at baseline were excluded accordingly. CRP, ALT, GGT, fasting insulin were naturally log transformed. Hazard ratios were also calculated for sex-specific

quartiles of GGT levels for prediabetes and type 2 diabetes. Results were reported per SD of log transformed GGT levels.

Associations of individual SNPs and GGT genetic score with GGT levels were assessed with linear regression analysis among participants in the Rotterdam Study. SNPs were modeled per GGT-increasing allele (additive model). Participants with prevalent prediabetes and type 2 diabetes at baseline were excluded, and hazard ratios were computed using Cox proportional hazard models adjusted for sex, age and cohort effect. We further investigated the association of GGT genetic score with glycemic traits (fasting glucose, fasting insulin, HOMA-IR).In a sensitivity analysis, participants with prevalent CVD were excluded. To increase our sample size, we examined the association of genetic risk score with a combination of both prevalent and incident prediabetes and type 2 diabetes cases. Taking to consideration any pleiotropic effect of the score genes, we reanalyzed the genetic estimate excluding SNPs that have been reported to be associated with any other cardiometabolic trait (rs1260326, rs10513686, rs4074793, rs17145750, rs7310409, rs516246) [10].

Mendelian Randomization using data from MAGIC and DIAGRAM Consortia

To maximize the statistical power, we examined the association of the genetic risk score and glycemic traits and diabetes using data from the largest GWAS meta-analyses. For diabetes, we used data from the DIAGRAM consortium, which meta-analyzed genetic variants in 34, 840 case subjects with diabetes and 114,981 control subjects from 37 studies [4]. For fasting glucose (n= 58, 074), fasting insulin (n= 133,000) and HOMA- insulin resistance (n=37,073), we used data form the Meta-Analyses of Glucose and Insulin-related Traits Consortium (MAGIC), which is a collaborative effort that combined data from 55 studies to identify genetic determinants that affect glycemic traits. Participants were of European ancestry and genotyped with the Metabochip [11]. We selected the same 26 SNPs as described previously [3] and extracted the odds ratios (ORs) and accompanying SEs. Then we estimated the association between GGT- genetic score using data from MAGIC for glycemic traits and DIAGRAM for type 2 diabetes.

We estimated the power for the Mendelian randomization analysis for a binary outcome at a two sided α of 0.05 based on the proportion of cases in the study, strength of the genetic instrument, and the true odds ratio of the outcome variable per standard deviation of the exposure variable using the online tool mRnd (http://cnsgenomics.com/shiny/mRnd/).

Tests were considered statistically significant at *P*- values lower than 0.05. Proportional hazard assumptions were inspected visually using log-minus-log plots, with no deviations detected for both the observational and genetic analysis. Statistical analysis were done using SPSS version 20 (IBM, Armonk, NY, USA) and R version 2.15.1 (R Foundation for Statistical Computing, Vienna, Austria). Missing values for all covariates were imputed using expectation maximization in SPSS.

Table 1. Baseline characteristics of participants

Characteristics	Total
Women (n, %)	3753 (58)
Age (years)	65.6 (9.8)
Waist circumference (cm)	93 (11.8)
BMI (kg/m^2)	26.9 (3.9)
Total Cholesterol (mmol/L)	5.7 (0.9)
Triglycerides (mmol/L)	1.4 (0.7)
HDL cholesterol (mmol/L)	1.4 (0.4)
Lipid lowering medication (n, %)	907 (13.9)
Systolic blood pressure (mmHg)	139.4 (20.8)
Antihypertensive medication use (n, %)	1885 (29)
Alcohol (g/day)	10 (13.3)
Prevalent CVD (n, %)	478 (7)
Current smoking (n, %)	1042 (16)
ALT (U/L)	21 (16 - 27)
GGT (U/L)	24 (17 - 36)
CRP (mg/L)	1.6 (0.6 - 3.5)
Fasting insulin (pmol/L)	73 (51 - 106)
Fasting glucose (mmol/L)	5.4 (0.7)

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; CRP, C-reactive protein; CVD, cardiovascular disease; HDL, high density lipoprotein; GGT, gamma-glutaryltransferase;
Values are mean ± standard deviation or median (interquartile range) for characteristics with skewed distributions.

Table 2. Association of log transformed GGT levels (per SD) with incident prediabetes and incidence type 2 diabetes.

	Incident prediabetes (n= 1125/7263)		**Incident type 2 diabetes** (n= 811/8628)	
	HR (95% CI)	***P*-value**	**HR (95% CI)**	***P*-value**
Model 1	1.26 (1.19 - 1.34)	1.0×10^{-14}	1.39 (1.3 - 1.4)	2.0×10^{-16}
Model 2	1.16 (1.09 - 1.25)	1.3×10^{-5}	1.23 (1.14 - 1.33)	1.7×10^{-7}
Model 3	1.10 (1.02 - 1.19)	0.008	1.19 (1.1 - 1.3)	2.6×10^{-5}
Model 4	1.08 (1.01 - 1.17)	0.02	1.11 (1.02 - 1.21)	0.01

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; CI confidence interval; CRP, C-reactive protein; CVD, cardiovascular disease; HDL, high density lipoprotein; HR, Hazard Ratio; SD, standard deviation.

Model 1: age, sex and cohort effect

Model 2: Model 1 and BMI, ALT

Model 3: Model 2 and additionally adjusted for triglycerides, CRP, BMI, waist circumference, cholesterol, HDL, smoking, alcohol, systolic blood pressure, indication for hypertension, lipid lowering medication and prevalence CVD

Model 4: Model 3 and additionally adjusted for fasting insulin, fasting glucose

Results

Baseline characteristics of the population used for analysis from the Rotterdam Study are shown in **Table 1**. Mean (SD) age of participants was 65.6 (9.8), 58% of the population were women and mean (SD) serum GGT level was 24 (SD) U/L. During a mean follow up time of 5.7 years, 1,125 individuals progressed to prediabetes (incidence rate: 27.1 per 1000 person-years). For type 2 diabetes, the mean follow up was 6.9 years and 811 individuals developed diabetes (incidence rate: 13.6 per 1000 person-years).

The age and sex adjusted hazard ratio (HR) for prediabetes per 1 standard (SD) change in natural logarithm of GGT was 1.26 (95% CI: 1.19 to 1.34; *P*-value < 1.0×10^{-14}) , as shown in **Table 2**. Further adjustments for BMI and ALT yielded a HR of 1.16 (95% CI: 1.09 to 1.25; *P*-value < 1.3×10^{-5}) in model 2. Controlling for conventional risk factors in model 3 diminished the magnitude of the association (HR=1.10; 95% CI: 1.02 to 1.19; *P*-value < 0.008) , however, adjustment for fasting glucose and fasting insulin did not materially change the stimate in model 4 (HR =1.08, 95% CI: 1.0 to 1.16; *P*-value < 0.02). For type 2 diabetes, the age- and sex- adjusted HR was 1.39 (95% CI: 1.3 to 1.4; *P*-value < 2×10^{-16}). The results were largely attenuated after adjustment for BMI and ALT in model 2 (HR = 1.27; 95% CI: 1.07 to 1.2; *P*-value 3.2×10^{-7}). The association further attenuated after adjustment for confounders in model 3 (HR=1.19; 95% CI: 1.1 to 1.3; *P*-value 2.6×10^{-5}) and for fasting glucose and fasting insulin in model 4 (HR=1.11; 95% CI: 1.02 to 1.21; *P*-value < 0.01). However, the association remained significant in all these models. Prediabetes and Type 2 diabetes risk were significantly increased across sex-specific quartiles when compared with the first quartile (**Figure 2**).

Figure 2.

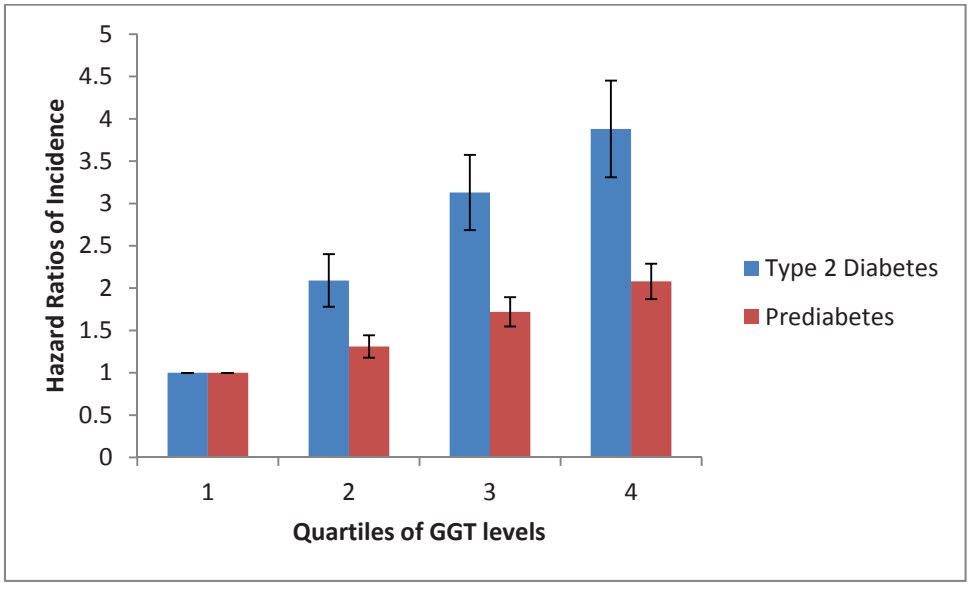


Table 3. Association of GGT related genetic risk score and rs2017869 genotype with glycemic traits, incidence prediabetes and type 2 diabetes in the Rotterdam Study and in the GWAS (MAGIC for glycemic traits and DIAGRAM for type 2 diabetes).

	The Rotterdam Study			***GWAS***		
	N	**HR/ β* (95% CI)**	***P*-value**	**N**	**OR/ β (SE)**	***P*-value**
			Genetic risk score			
Glucose (mmol/L)	7383	-0.01 (-0.02 – 0.002)	0.1	58,074	-0.002 (0.008)	0.1
Insulin (mmol/L)	7383	-0.002 (-0.01 – 0.01)	0.6	133,000	-0.001 (0.008)	0.3
HOMA-IR (log units)	7383	-0.005 (-0.01 – 0.008)	0.4	36, 466	-0.001 (0.0009)	0.2
Prediabetes	976/ 6236	0.97 (0.91 - 1.04)	0.4	-	-	
T2D	679/ 7383	0.96 (0.89 - 1.04)	0.3	34,840 cases	0.99 (0.001)	0.2
			rs2017869			
Glucose (mmol/L)	7383	-0.002 (-0.02 – 0.02)	0.8	58,074	4.5×10^{-3} (4.2×10^{-3})	0.2
Insulin (mmol/L)	7383	-0.0003 (-0.02 – 0.02)	0.9	133,000	-1.7×10^{-3} (4.5×10^{-3})	0.7
HOMA-IR (log units)	7383	-0.0009 (-0.02 – 0.02)	0.9	36, 466	-3.8×10^{-3} (4.7×10^{-3})	0.4
Prediabetes	976/ 6236	1.03 (0.93 – 1.15)	0.5	-	-	
T2D	679/ 7383	1.09 (0.96 – 1.23)	0.18	34,840 cases	1.01 (0.23)	0.5

Abbreviations: β, beta estimate for continues traits; CI, confidence interval; GGT, gamma-glutamyltransferase; GWAS, genome-wide association studies; HOMA-IR, homeostatic model assessment- insulin resistance; HR, hazard ratio; OR, odds ratio; SD, standard deviation; SE, standard error

Model: age, sex and cohort effect

β* are per SD increase in GGT related genetic risk score in the Rotterdam Study

GGT genetic risk score and risk of prediabetes and type 2 diabetes.

Nearly all GGT SNPs were associated with GGT levels in the Rotterdam Study (**Supplementary Table 2**), however, none of them were associated with prediabetes and type 2 diabetes. The genetic risk score composed of 26 SNPs was normally distributed among the study participants. The genetic risk score was associated with GGT levels (Beta per SD= 0.09, 95% CI: 0.07 to 0.1, *P* value= 2×10^{-16}) and explained 3.2 % of the variation in serum GGT levels *(F* statistic = 93). **Supplementary Figure 1** shows increasing mean levels of GGT in quartiles of the genetic risk score. The genetic risk score did not associate with the risk of prediabetes (HR = 0.98; 95% CI: 0.91 to 1.04) and type 2 diabetes (HR = 0.96; 95% CI: 0.88 to 1.03) in the sex- and age-adjusted model (**Table 3**). Furthermore, there was no evidence for an effect of GGT genetic score on glycemic traits in the Rotterdam Study (**Table 3**). Excluding prevalent CVD cases did not affect the estimates (**Supplementary Table 3**). Combining prevalent and incident cases of prediabetes and type 2 diabetes did not change the results (**Supplemental Table 3**). Excluding SNPs which had a pleiotropic effect did not affect association between GGT genetic risk score, prediabetes and type 2 diabetes (**Supplementary Table 4**). Nevertheless, adjusting for these variables in the genetic analysis model did not change the estimates (HR for prediabetes 0.95 [95% CI: 0.89 to1.01]; HR for type 2 diabetes 0.93 [95% CI: 0.83 to1.01]). Rs2017869 of *GGT1* gene, previously reported to be associated with fasting insulin, was negatively associated with GGT levels (Beta= - 0.1; 95% CI: -0.1 to -0.08; *P*-value < 2×10^{-16}) and it was explaining 2.3% of the variance in the GGT levels. In a separate genetic analysis of rs2017869, we could not evidence any significant relation between our outcomes of interest (**Table 3**).

The GGT genetic score was not associated with the risk of type 2 diabetes using data from the DIAGRAM Consortium (OR = 0.99; *P*-value= 0.2). As shown in **Table 3**, we observed no association with fasting glucose (Beta = -0.002, *P*- value = 0.1), fasting insulin (Beta = -0.001, *P*- value = 0.3), and HOMA-IR (Beta = -0.001, *P*- value = 0.2). Excluding pleiotropic SNPs from this analysis did not alter the results (**Supplementary Table 5**). Relation between individual SNPs and type 2 diabetes, fasting glucose, fasting insulin, insulin resistance are shown graphically in **Supplementary Figure 2** and annotation of SNPs with their effect estimates on **Supplementary Table 6.**

Discussion

In this cohort study, we found a 10% higher risk of prediabetes and19% higher risk of diabetes per SD increase in GGT levels. Instrumental variable analysis through GGT genetic risk score did not support this association to be causal. Altogether, our results suggest that the association could be mainly due to confounding or reverse causation.

Although a large and broadly consistent body of evidence has established GGT as strongly linked to the development of type 2 diabetes, its causal role is uncertain. It is suggested that GGT links to type 2 diabetes through hepatic lipid accumulation and non-alcoholic fatty liver disease (NAFLD), both implicated in the impaired hepatic insulin resistance, major features of pathophysiology of type 2 diabetes [12]. In addition, GGT is involved in the catabolism of glutathione and is associated

with increased oxidative stress, which is involved in the development of insulin resistance and diabetes [13,14]. This evidence is confirmed from animal models that relate dysregulated glutathione metabolism with impaired insulin action in adipocytes [15]. However, GGT could also be associated with type 2 diabetes and glycemic traits based on reverse causality. We observed in our study that further adjustment for potential confounder including fasting glucose and insulin as well, considerably diluted the association. In agreement with this observation, a recent study from Scott et al. found a significant association between a genetic risk score for insulin resistance and GGT levels [16] suggesting a causal role of insulin resistance in increasing GGT levels.

The direction of estimates in the observational analysis is in line with a previous meta-analysis of 24 cohort studies [1] that reported a pooled relative risk of top vs bottom tertiles of GGT levels with type 2 diabetes incidence of 1.34 (1.27 – 1.42). However, the substantial heterogeneity (I^2 >70%) that was observed between studies, together with the younger mean age of participants (50 years old vs 65.6 years old in our study) might be the reasons to explain the difference in magnitude with our study. The effect estimates in our analysis were substantially and steeply attenuated by adjustment for conventional risk factors and confounders across models. This suggests that potential residual and unmeasured confounders remain a concern and might explain the association. On the other hand, we do not rule out the possibility of reverse causation, despite adjusting for fasting glucose and insulin at the fourth model.

Our genetic analysis provides evidence that GGT is not causally affecting the glycemic traits, prediabetes or risk of type 2 diabetes. There is evidence to suggest that the *GGT1* locus, which is the main protein-coding gene for GGT, may account for GGT levels variation [17,18] and therefore variants within this locus have been used as instrumental variables for Mendelian Randomization (MR) studies. Conen and colleagues [2] used a MR approach and found evidence for a causal effect of *GGT1* variant on fasting insulin levels in a sample size of ~ 4000 participants. The instrumental variable (rs2017869) was explaining only ~1.6% of the variance of GGT levels. These results were not replicated in the Rotterdam Study. Similarly, we could not replicate the causal analysis when using the multi-locus genetic instrument (explaining 3.2% the variance of GGT levels). Although the Rotterdam study sample size couldn't help yield high precision estimates from the genetic analysis, we were unable to confirm an association in the GWAS data where we had 83% power to detect an HR of 1.34 of GGT levels on type 2 diabetes risk [19] meaning that the estimates derived from the replication analysis were well powered. Mendelian randomization is a valid way to explore evidence for causality when certain assumptions are met. First, there should be a strong association between the genetic risk score and the risk factor of interest. All SNPs that we used in our study showed to be strongly associated with GGT levels in a large meta-analyses of GWAS [10]. Second, the instrumental variable must be independent of confounders that are associated with both GGT and type 2 diabetes which is met since the alleles are inherited in random. Third, genetic risk score affects the outcome only though the risk factor of interest. This assumption cannot be tested and should be considered using information on the underlying biology. However, *GCKR, SLC2A2,*and *HNF1A* have been reported to be associated with glucose [20] and type 2 diabetes [21]. Other reason that might have influenced our results is the pleiotropic effect of the genetic variants with serum CRP [22], low

density lipoprotein cholesterol, and coronary artery disease [23] . We excluded the pleiotropic variants from the genetic risk score in a sensitivity analysis and we controlled for potential confounders in these pathways. Thus, it is unlikely that our results are affected by pleiotropic effects of the genes.

The major strength of this study is the large sample size for measurements of both GGT and glycemic indices, prediabetes and diabetes in the Rotterdam Study and the boosted power through utilization of the GWAS data. By also examining associations with incident prediabetes, we provided insight into the early development of subclinical disease. Additionally, we used data from a well-characterized prospective population-based cohort study, which allowed us to have a comprehensive assessment of this association using both observational and genetic data. Nevertheless, several issues may compromise our approach in assessing causality. We only used GGT measured in serum but there have been reported differences in plasma levels and different types of GGT fractions [24,25]. Furthermore, the results may not be valid for all ethnic groups since our population consisted of Caucasian individuals.

In conclusion, our results provide no evidence that genetically elevated GGT levels affect the risk of prediabetes and type 2 diabetes, and thus do not support a causal role for GGT levels. The observed association between GGT levels and the risk of prediabetes and type 2 diabetes is possibly due to residual confounding or reverse causation. These findings may further highlight the importance of adequate sample size in Mendelian Randomization studies.

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Supplemental material

Supplementary Table 1. Alleles used in the genetic risk score.

SNP	Coded Allele	Beta	Weight used for deriving externally weighted genetic score	Gene
rs1497406	G	3.8	4.9	RSG1, EPHA2
rs12145922	A	2.8	4.8	CCBL2, PKN2
rs1335645	A	4.3	3.7	CEPT1, DENND2D
rs10908458	T	3.7	2.8	DPM3, EFNA1, PKLR
rs1260326	T	3.2	3.2	C2orf16, GCKR
rs13030978	T	3.7	4.8	MYO1B, STAT4
rs2140773	A	2.9	3.7	EFHD1, LOC100129166
rs10513686	A	4.9	4.3	SLC2A2
rs4547811	C	6.4	3.8	ZNF827
rs6888304	A	2.7	4.5	CDH6
rs4074793	G	5.5	12.3	ITGA1
rs9296736	T	3	2.9	MLIP
rs17145750	C	4.5	3.7	MLXIPL
rs754466	T	3.5	4.5	DLG5
rs7310409	G	6.8	5.5	HNF1A, C12orf27
rs944002	G	6.3	3.6	C14orf73
rs339969	A	4.5	6.4	RORA
rs8038465	T	2.4	3.2	CD276
rs4581712	A	3.2	2.3	DYNLRB2
rs9913711	C	2.4	2.7	FLJ37644, SOX9
rs12968116	C	4.8	6.8	ATP8B1
rs4503880	T	3.6	3.5	NEDD4L
rs516246	T	2.3	2.4	FUT2
rs1076540	C	4.8	3	MICAL3
rs2739330	T	3.7	6.3	DDT, DDTL, GSTT1, GSTT2B, MIF
rs2073398	G	12.3	2.4	GGT1, GGTLC2

Supplementary Table 2. Associations of individual GGT SNPs with circulating GGT levels, glycemic traits, incident prediabetes and type 2 diabetes in the Rotterdam Study.

SNP	Chr	R/E	Gene	β (SE) for GGT levels	P-value	OR (SE) for incidence prediabetes	P-value	OR (SE) for incidence diabetes	P-value
rs1497406	1	A/G	RSG1, EPHA2	0.08 (0.016)	< 0.001	0.90 (0.05)	0.06	0.99 (0.06)	0.87
rs12145922	1	C/A	CCBL2, PKN2	0.03 (0.016)	6.6×10^{-2}	0.97 (0.05)	0.64	1.00 (0.05)	0.96
rs1335645	1	G/A	CEPT1, DENND2D	0.05 (0.027)	4.9×10^{-2}	1.01 (0.08)	0.86	1.02 (0.09)	0.82
rs10908458	1	C/T	DPM3, EFNA1, PKLR	0.05 (0.017)	7.0×10^{-3}	1.01 (0.05)	0.84	0.96 (0.06)	0.56
rs1260326	2	C/T	C2orf16, GCKR	-0.02 (0.017)	3.6×10^{-1}	0.98 (0.05)	0.8X	1.1 (0.06)	0.1X
rs13030978	2	C/T	MYO1B, STAT4	-0.03 (0.018)	1.0×10^{-1}	1.03 (0.05)	0.52	1.0 (0.06)	0.99
rs2140773	2	C/A	EFHD1, LOC100129166	0.03 (0.017)	1.2×10^{-1}	0.98 (0.05)	0.72	0.96 (0.06)	0.51
rs10513686	3	G/A	SLC2A2	-0.02 (0.024)	3.4×10^{-1}	0.95 (0.07)	0.58	1.07 (0.08)	0.44
rs4547811	4	T/C	ZNF827	-0.08 (0.021)	< 0.001	0.95 (0.06)	0.52	1.03 (0.07)	0.68
rs6888304	5	G/A	CDH6	0.05 (0.018)	7.0×10^{-3}	0.90 (0.05)	0.08	0.99 (0.06)	0.98
rs4074793	5	A/G	ITGA1	-0.1 (0.032)	2.0×10^{-3}	0.85 (0.09)	0.09	0.90 (0.11)	0.37
rs9296736	6	C/T	MLIP	-0.02 (0.018)	3.1×10^{-1}	0.94 (0.05)	0.34	0.88 (0.06)	0.05
rs17145750	7	T/C	MLXIPL	0.07 (0.023)	4.0×10^{-3}	0.97 (0.07)	0.73	0.87 (0.08)	0.09
rs754466	10	A/T	DLG5	-0.11 (0.018)	< 0.001	0.96 (0.05)	0.5X	1.18 (0.07)	0.01
rs7310409	12	A/G	HNF1A, C12orf27	-0.08 (0.016)	< 0.001	1.11 (0.05)	0.03	1.01 (0.06)	0.81
rs944002	14	A/G	C14orf73	-0.15 (0.022)	< 0.001	0.96 (0.07)	0.58	0.89 (0.07)	0.16
rs339969	15	C/A	RORA	0.06 (0.017)	< 0.001	1.05 (0.05)	0.3X	1.08 (0.06)	0.17
rs8038465	15	C/T	CD276	0.07 (0.017)	< 0.001	1.06 (0.05)	0.24	1.09 (0.06)	0.13
rs4581712	16	C/A	DYNLRB2	0.02 (0.018)	1.9×10^{-1}	0.95 (0.05)	0.45	1.04 (0.06)	0.51
rs9913711	17	G/C	FLJ37644, SOX9	-0.02 (0.017)	1.8×10^{-1}	1.03 (0.05)	0.51	1.02 (0.06)	0.67
rs12968116	18	T/C	ATP8B1	0.08 (0.025)	1.0×10^{-3}	1.07 (0.08)	0.37	1.03 (0.09)	0.74
rs4503880	18	C/T	NEDD4L	-0.05 (0.02)	2.5×10^{-2}	0.97 (0.06)	0.64	1.06 (0.07)	0.37
rs516246	19	C/T	FUT2	0.04 (0.016)	2.0×10^{-2}	1.08 (0.05)	0.1X	1.01 (0.05)	0.76

rs1076540	22	T/C	MICAL3	0.08 (0.019)	< 0.001	0.99 (0.06)	0.91	1.05 (0.07)	0.42
rs2739330	22	C/T	DDT, DDTL, GSTT1	0.03 (0.017)	7.4×10^{-2}	0.97 (0.05)	0.57	0.89 (0.06)	0.07
rs2073398	22	C/G	GGT1, GGTLC2	0.18 (0.018)	< 0.001	0.95 (0.05)	0.39	0.93 (0.06)	0.29
rs2017869*	22	C/G	GGT1	-0.18 (0.019)	< 0.001	0.98 (0.02)	0.55	1.08 (0.07)	0.26

* rs2017869 of GGT1 gene, previously reported

Supplementary Table 2. Associations of individual GGT SNPs with circulating GGT levels, incident prediabetes and type 2 diabetes in the Rotterdam Study. (Continued)

SNP	Chr	Reference/Effect Allele	Gene	β (SE) for glucose levels	*P*-value	β (SE) for insulin levels	*P*-value	β (SE) for HOMA-IR	*P*-value
rs1497406	1	A/G	*RSG1, EPHA2*	0.006 (0.01)	0.58	-0.008 (0.009)	0.35	-0.008 (0.009)	0.42
rs12145922	1	C/A	*CCBL2, PKN2*	-0.005 (0.01)	0.63	-0.017 (0.009)	0.06	-0.018 (0.009)	0.06
rs1335645	1	G/A	*CEPT1, DENND2D*	0.026 (0.01)	0.17	0.03 (0.014)	0.02	0.039 (0.016)	0.01
rs10908458	1	C/T	*DPM3, EFNA1, PKLR*	0.004 (0.01)	0.73	-0.008 (0.009)	0.39	-0.007 (0.01)	0.45
rs1260326	2	C/T	*C2orf16, GCKR*	0.036 (0.01)	0.00	0.02 (0.009)	0.03	0.026 (0.01)	0.01
rs13030978	2	C/T	*MYO1B, STAT4*	0.013 (0.01)	0.31	0.006 (0.01)	0.56	0.0082(0.01)	0.45
rs2140773	2	C/A	*EFHD1, LOC100129166*	-0.001 (0.01)	0.92	0.002 (0.009)	0.76	0.0021 (0.009)	0.83
rs10513686	3	G/A	*SLC2A2*	0.019 (0.01)	0.26	-0.006 (0.01)	0.63	-0.002 (0.01)	0.86
rs4547811	4	T/C	*ZNF827*	0.002 (0.01)	0.85	-0.0006 (0.01)	0.96	-0.0009 (0.01)	0.94
rs6888304	5	G/A	*CDH6*	-0.011 (0.01)	0.39	0.002 (0.01)	0.83	0.0001 (0.01)	0.99
rs4074793	5	A/G	*ITGA1*	0.008 (0.02)	0.71	-0.003 (0.01)	0.82	-0.002 (0.01)	0.90
rs9296736	6	C/T	*MLIP*	-0.014 (0.01)	0.27	-0.01 (0.01)	0.29	-0.01 (0.01)	0.22
rs17145750	7	T/C	*MLXIPL*	0.0007 (0.01)	0.96	-0.01 (0.01)	0.34	-0.01 (0.01)	0.35
rs754466	10	A/T	*DLG5*	0.035 (0.01)	0.01	0.003 (0.01)	0.77	0.008 (0.01)	0.45

rs7310409	12	A/G	*HNF1A, C12orf27*	0.01 (0.01)	0.39	-0.015 (0.009)	0.10	-0.01 (0.009)	0.17
rs944002	14	A/G	*C14orf73*	0.002 (0.01)	0.89	0.014 (0.01)	0.22	0.01 (0.01)	0.23
rs339969	15	C/A	*RORA*	0.018 (0.01)	0.12	0.015 (0.009)	0.10	0.01 (0.01)	0.07
rs8038465	15	C/T	*CD276*	0.019 (0.01)	0.11	0.009 (0.009)	0.31	0.01 (0.01)	0.21
rs4581712	16	C/A	*DYNLRB2*	0.008 (0.01)	0.52	0.002 (0.01)	0.79	0.004 (0.01)	0.71
rs9913711	17	G/C	*FLJ37644, SOX9*	-0.009 (0.01)	0.45	0.01 (0.009)	0.30	0.008 (0.01)	0.40
rs12968116	18	T/C	*ATP8B1*	0.01 (0.01)	0.47	-0.01 (0.01)	0.47	-0.008 (0.014)	0.57
rs4503880	18	C/T	*NEDD4L*	-0.007 (0.01)	0.62	0.002 (0.01)	0.80	0.001 (0.01)	0.90
rs516246	19	C/T	*FUT2*	0.012 (0.01)	0.28	0.02 (0.009)	0.01	0.02 (0.009)	0.01
rs1076540	22	T/C	*MICAL3*	-0.006 (0.01)	0.65	-0.01 (0.01)	0.09	-0.01 (0.01)	0.09
rs2739330	22	C/T	*DDT, DDTL, GSTT1*	-0.006 (0.01)	0.62	-0.02 (0.01)	0.06	-0.021 (0.01)	0.06
rs2073398	22	C/G	*GGT1, GGTLC2*	-0.027 (0.01)	0.04	0.001 (0.01)	0.85	-0.002 (0.01)	0.82
rs2017869*	22	C/G	*GGT1*	0.001 (0.01)	0.94	-0.0009(0.01)	0.93	-0.001 (0.01)	0.92

* rs2017869 of GGT1 gene, previously reported

Supplementary Table 3. Sensitivity analysis of GGT-related genetic score association with diabetes and prediabetes in the Rotterdam Study

Sensitivity Analysis	N	Risk Estimate (95% CI)	*P*-value
All T2D (and prediabetes)	3,126/ 8,386	*0.97 (0.92- 1.01)	0.18
Excluding cases with CHD[T2D as outcome]	611/ 6,815	**0.95 (0.85- 1.06)	0.37
Excluding cases with CHD [Prediabetes as outcome]	903/ 5,784	**0.96 (0.89- 1.03)	0.26

*Odds Ratio ** Hazard Ratio

Supplementary Table 4. Sensitivity analysis of GGT-related genetic score association with prediabetes and diabetes, after exclusion of pleiotropic SNPs

Sensitivity Analysis	**HR (95% CI) of prediabetes**	**HR (95% CI) of T2D**
Excluding pleiotropic SNPs (rs1260326, rs10513686, rs4074793, rs17145750, rs7310409, rs516246)	1.0 (0.93- 1.07)	0.95 (0.88- 1.03)

*HR, Hazard Ratio

Supplementary Table 5. Sensitivity analysis of GGT-related genetic score with diabetes and glycemic traits in GWAS data excluding the pleiotropic genes from the score.

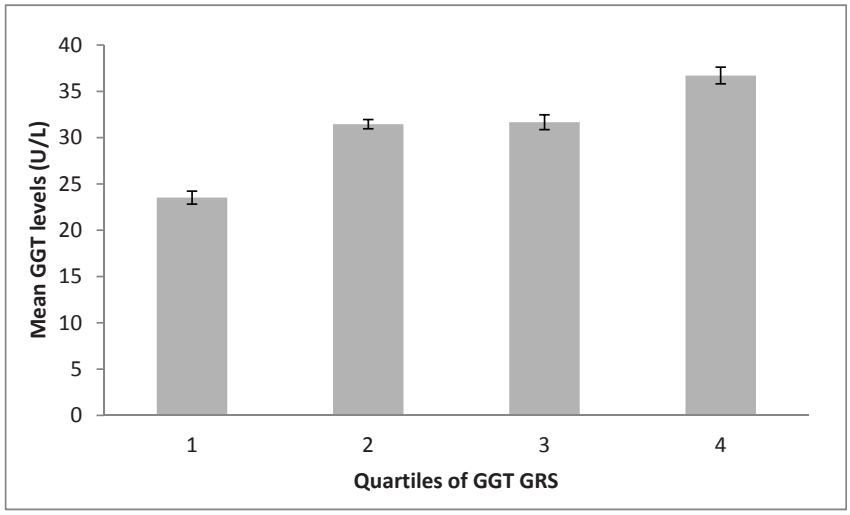
	OR (SE)	***P*-value**
T2D	0.99 (0.23)	0.5
	β (SE)	***P*-value**
Glucose (mmol/L)	4.5×10^{-3} (4.2×10^{-3})	0.2
Insulin (mmol/L)	-1.7×10^{-3} (4.5×10^{-3})	0.7
HOMA-IR (log units)	-3.8×10^{-3} (4.7×10^{-3})	0.4

Supplementary Table 6. SNPs and their estimates from the GWAS data.

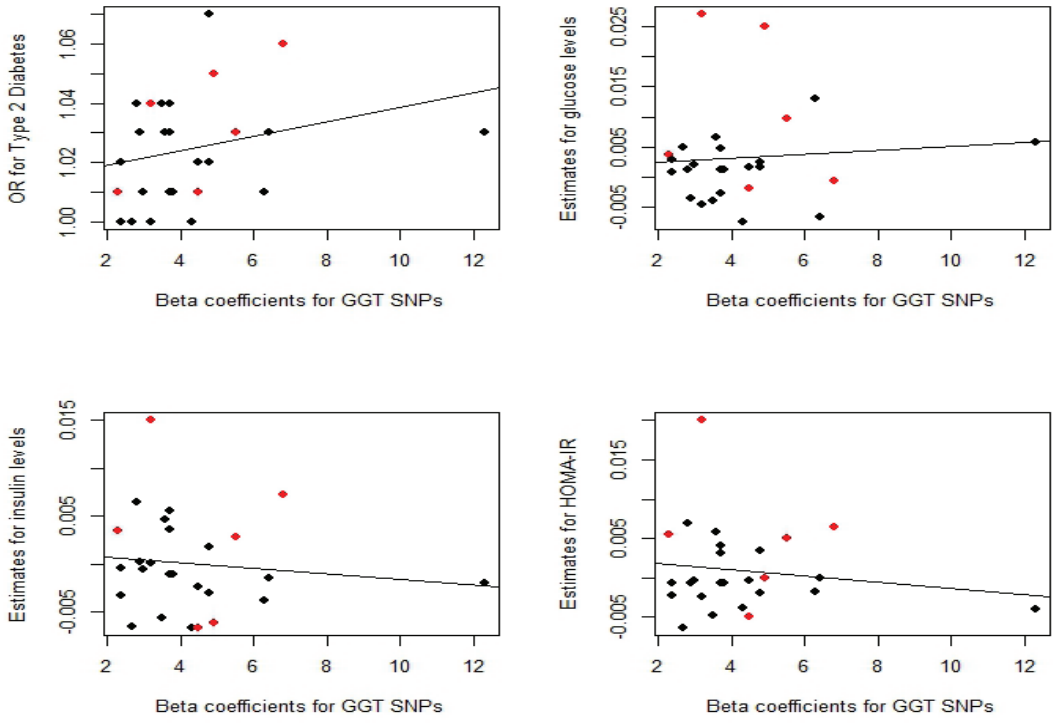
SNP	OR type 2 diabetes	β Fasting Insulin	β HOMA-IR	β Fasting glucose	β GGT
rs10513686*	1.05	0.0061	0.0	-0.025	4.9
rs1076540	1.02	0.003	0.002	-0.0016	4.8
rs10908458	1.01	0.001	6.00×10^{-4}	-0.0047	3.7
rs12145922	1.04	-0.0065	-0.007	-0.0012	2.8
rs1260326*	1.04	-0.015	-0.02	-0.027	3.2
rs12968116	1.07	-0.0018	-0.0035	-0.0024	4.8
rs13030978	1.04	-0.0056	-0.0041	-0.0012	3.7
rs1335645	1.00	0.0066	0.0039	0.0074	4.3
rs1497406	1.01	0.001	7.00×10^{-4}	-0.0012	3.8
rs17145750*	1.01	0.0066	0.0049	0.0019	4.5
rs2073398	1.03	0.002	0.004	-0.0057	12.3
rs2140773	1.03	-3.00×10^{-4}	6.00×10^{-4}	0.0036	2.9
rs2739330	1.03	-0.0036	-0.0031	0.0027	3.7
rs339969	1.02	0.0023	4.00×10^{-4}	-0.0016	4.5
rs4074793*	1.03	-0.0029	-0.005	-0.0096	5.5
rs4503880	1.03	-0.0046	-0.0059	-0.0067	3.6
rs4547811	1.03	0.0014	0.0	0.0067	6.4
rs4581712	1.00	-1.00×10^{-4}	0.0024	0.0046	3.2
rs516246*	1.01	-0.0035	-0.0055	-0.0037	2.3
rs6888304	1.00	0.0065	0.0063	-0.0049	2.7
rs7310409*	1.06	-0.0073	-0.0065	7.00×10^{-4}	6.8
rs754466	1.04	0.0056	0.0048	0.0039	3.5
rs8038465	1.02	4.00×10^{-4}	7.00×10^{-4}	-7.00×10^{-4}	2.4
rs9296736	1.01	5.00×10^{-4}	4.00×10^{-4}	-0.002	3.0
rs944002	1.01	0.0038	0.0017	-0.013	6.3
rs9913711	1.00	0.0033	0.0022	-0.0028	2.4

*pleiotropic genes

Supplementary Figure 1. GGT levels in relation to quartiles of genetic risk score. (P for trend = 4.9 x 10-4. Error bars represent 95% confidence intervals)



Supplementary Figure 2. Relationship between GGT SNPs effect estimates and type 2 diabetes Odds Ratio(OR),fasting insulin, fasting glucose, HOMA-IR: insulin resistance across all 26 GGT SNPs in the GWAS data. Regression line are fitted. Annotation of the SNPs are found in Supplementary Table 7. Red points annotate pleiotropic SNPs.



Chapter 4

Cardiometabolic Health in Women

4.1 Associations of Steroid Sex Hormones and Sex Hormone-Binding Globulin with the Risk of Type 2 Diabetes in Women: a Population-Based Cohort Study and Meta-Analysis

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ABSTRACT

Background: The relation between endogenous sex hormones and type 2 diabetes (T2D) in women is unclear. We examined whether sex hormones were associated with the risk of T2D in women.

Methods and Findings: Data of 3117 postmenopausal women participants of the Rotterdam Study (RS), a prospective population based cohort, were available. At baseline of the RS, sex hormone-binding globulin (SHBG), total testosterone (TT) and total estradiol (TE) were measured. Free androgen index, calculated as (T/SHBG)*100 was used as a surrogate measure of bioavailable testosterone (BT). T2D events were diagnosed on the basis of medical records. Relative risks (RRs) and 95% Confidence Intervals (CIs) were calculated using Cox regression models adjusted for confounders. For the systematic review and meta-analysis, six electronic databases (Medline, Embase.com, Web of Science, the Cochrane Library, PubMed and Google Scholar) were searched for studies assessing the association of endogenous sex hormones with T2D in women. During a median follow-up of 11.1 years, we identified 384 incident cases of T2D in the RS. No association was observed between TT or BT with T2D. SHBG was inversely associated with the risk of T2D (3rd tertile vs. 1st tertile: RR: 0.56; 95%CIs=0.40-0.79) whereas TE was associated with increased risk of T2D (3rd tertile vs. 1st tertile: RR: 1.42; 95%CIs=1.01 -2.00). In a meta-analysis of 12 population-based prospective studies involving 1912 incident T2D cases, the pooled multivariable-adjusted RRs comparing 3rd tertile vs. 1st tertile were 0.44 (0.30-0.66), 1.32 (0.79-2.21), 1.75 (0.92-3.33), 1.99 (1.21-3.27) and 3.58 (0.86-14.84) for SHBG, TT, BT, TE and bioavailable estradiol respectively.

Conclusions: Our findings suggest that endogenous sex hormones are independent risk factors for the development of T2D in women; specifically lower levels of SHBG and higher levels of TE are associated with increased risk of T2D.

INTRODUCTION

The menopause transition is marked by changes in hormonal patterns, including a marked decline in endogenous estradiol levels, leading to a period of relative androgen excess[1]. This shift in hormonal balance contributes to an increase in visceral adiposity that is associated with glycemic traits, and therefore may be associated with the risk of type 2 diabetes (T2D)[2].

Although an inverse association between sex hormone-binding globulin (SHBG) and T2D has been previously reported[3,4], studies of the association between endogenous estradiol and testosterone and T2D are limited. Ding and his colleagues, in a nested case-control study of 719 postmenopausal women, showed a positive association between plasma estradiol and testosterone and the risk of T2D [5]. In contrast, no association was reported between these sex hormones and the risk of T2D in two other case cohort studies of postmenopausal women, including up to 1728 participants[6,7]. SHBG, T and E have been associated with glucose tolerance and development of insulin resistance[8-11]. Furthermore, pregnancy, a state of high endogenous estrogen and polycystic ovary syndrome, a condition of anovulation and hyperandrogenism, have been both associated with insulin resistance and increased risk of T2D [12,13]. Besides the contradictory results, to date, no large studies have examined simultaneously the association of T2D with SHBG, T and E in healthy postmenopausal women.

We aimed to investigate the association between sex hormones and T2D in postmenopausal women. Furthermore, to contextualize these findings and clarify the contradictory results, we systematically review and meta-analyse studies evaluating the association between sex hormones and T2D in women.

SUBJECTS AND METHOD

The Rotterdam Study

The Rotterdam Study is a prospective cohort study which started since 1990 in the Ommoord district, in the city of Rotterdam, The Netherlands. Details regarding the design, objectives, and methods of the Rotterdam Study have been described in detail elsewhere[14]. In brief, in 1990 all inhabitants of a well-defined district of Rotterdam were invited, of whom 7,983 agreed (78.1%). In 2000, an additional 3011 participants were enrolled (RS-II), consisting of all persons living in the study district who had become 55 years of age. Follow up examinations were performed periodically, approximately every 3-5 years[14]. There were no eligibility criteria to enter the Rotterdam Study cohorts except the minimum age and residential area based on ZIP codes. The Rotterdam Study has been approved by the medical ethics committee according to the Population Screening Act: Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. All participants in the present analysis provided written informed consent to participate and to obtain information from their treating physicians.

Ascertainment of type 2 diabetes

The participants were followed from the date of baseline center visit onwards. At baseline and during follow-up, cases of T2D were ascertained through active follow-up using general practitioners' records, glucose hospital discharge letters and glucose measurements from Rotterdam Study visits which take place approximately every 4 years [15]. T2D was defined according to recent WHO guidelines, as a fasting blood glucose ³ 7.0 mmol/L, a non-fasting blood glucose ³ 11.1 mmol/L (when fasting samples were absent), or the use of blood glucose lowering medication [16]. Information regarding the use of blood glucose lowering medication was derived from both structured home interviews and linkage to pharmacy records [15]. At baseline, more than 95% of the Rotterdam Study population was covered by the pharmacies in the study area. All potential events of T2D were independently adjudicated by two study physicians. In case of disagreement, consensus was sought with an endocrinologist. Follow-up data was complete until January 1st 2012.

Sex steroid measurements

All blood samples were drawn in the morning (≤ 11:00 am) and were fasting. Total estradiol (TE) levels were measured with a radioimmunoassay and SHBG with the Immulite platform *(Diagnostics Products Corporation Breda, the Netherlands)*. Serum levels of total testosterone (TT) were measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Free androgen index (FAI), calculated as (T/SHBG)*100 is used as a surrogate measure of bioavailable testosterone (BT)[17].

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 (RSII-1) cohort. Overall, there were 3683 women eligible for blood measurements. Among them, 122 women did not have information on sex hormones and 32 did not have T2D follow-up, and were therefore excluded from the analysis. Furthermore, 412 women with prevalent T2D were excluded, leaving 3117 for our final analysis.

Potential confounding variables

Potential confounding variables are described in detail in **S1 Appendix**.

Statistical analysis

Person years of follow-up were calculated from study entrance (March 1997- December 1999 for RSI-3, February 2000-December 2001 for RSII-1) to the date of diagnosis of T2D, death or the censor date (date of last contact of the living), whichever occurred first. Follow-up was until January 1st 2012. Cox proportional hazard modelling was used to evaluate whether SHBG, TT, TE and BT were associated with T2D. Relative Risks (RR) and 95% confidence intervals (95% CIs) were reported. All sex hormones variables were assessed in separate models, continuously and in tertiles. To study the relations across increasing tertiles, trend tests were computed by entering the categorical variables as continuous variables in multivariable Cox's proportional hazard models. To achieve approximately normal distribution, skewed variables (SHBG, TT, BT, plasma triglyceride, low density lipoprotein cholesterol (LDL-C), C-reactive protein (CRP), thyroid-stimulating hormone

(TSH) and insulin) were natural log transformed. In the base model (Model 1), we adjusted for age, cohort (1 and 2), fasting status (fasting sample vs. non-fasting sample). Model 2 included terms for model 1, body mass index (BMI) (continuous), glucose (continuous) and insulin (continuous). BMI and waist circumference were highly correlated (Pearson's correlation coefficient = 0.81, P<0.001), so only BMI was used as a measure of adiposity, consistent with previous studies [5,18]. Model 3 included all covariates in model 2 and further potential intermediate factors including: metabolic risk factors (total cholesterol, systolic blood pressure (continuous), indication for hypertension (yes vs. no) and use of lipid-lowering medications (yes vs. no)), lifestyle factors (alcohol intake (continuous) and smoking status (current vs. former/never)), prevalent coronary heart disease (yes vs. no), age of menopause, hormone replacement therapy (yes vs. no), CRP (continues) and sex hormones for each other. Effect modifications of sex hormones by BMI and years since menopause were tested in the final multivariable model in addition to performing stratified analysis. We also performed sensitivity analysis (i) substituting BMI for waist circumference, (ii) substituting total cholesterol with HDL-C, triglycerides and LDL-C, (iii) further adjusting for THS, physical activity or type of menopause (non-natural vs. natural) and (iv) excluding the first three years of follow up. Multiple imputation procedure was used (*N*= 5 imputations) to adjust for potential bias associated with missing data. Rubin's method was used for the pooled regression coefficients (β) and 95% Confidence Intervals [19]. 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).

Systematic Review and Meta-Analysis

Data sources and search strategy

The review was conducted using a predefined protocol and in accordance with the PRISMA[20] and MOOSE[21] guidelines (**S2 and S3 Appendix**). Medline, Embase.com, Web of Science, the Cochrane Llibrary, PubMed and Google Scholar were searched from inception until November 2nd 2015 (date last searched) with assistance of an experience biomedical information specialist. The computer-based searches combined terms related to the exposure (eg, sex hormone binding globulin, testosterone, estradiol) with outcomes (eg, type 2 diabetes), without any language restriction. Details on the search strategy are provided in **S4 Appendix**. Furthermore, in order to identify further studies, we checked reference lists and contacted authors and experts in the field.

Study selection and eligibility criteria

Studies were included if they (i) were observational cohort, case-cohort studies, or prospective nested case control studies; (ii) had reported on at least one of the sex hormones as exposures: SHBG, TT, BT, TE and bioavailable estradiol (BE); and (iii) had assessed associations with risk of T2D in women (pre and postmenopausal). Two independent reviewers screened the titles and abstracts of all initially identified studies according to the selection criteria. Full texts were retrieved from studies that satisfied all selection criteria.

Data extraction and quality assessment

Data were extracted by two independent reviewers and a consensus was reached with involvement of a third. A predesigned data abstraction form was used to extract relevant information. This included questions on study size; study design; baseline population; location; age at baseline; duration of follow-up (for cohort studies) and menopausal status. Additionally, in the case of multiple publications, the most up-to-date or comprehensive information was included. Study quality was assessed based on the nine-star Newcastle–Ottawa Scale (NOS)[22] using three pre-defined domains namely: selection of participants (population representativeness), comparability (adjustment for confounders), and ascertainment of outcomes of interest. The NOS assigns a maximum of four points for selection, two points for comparability, and three points for outcome. Nine points on the NOS reflects the highest study quality.

Data synthesis and analysis

For the meta-analysis, we used the risk estimates of the most adjust models reported by each study. To enable a consistent approach for the meta-analysis, we used previously described methods[23] to transform RR estimates for associations between sex hormones with T2D risk which were often differentially reported by each study (for example, per unit change, per one standard deviation change, or comparing quarters or thirds, and other groupings), and therefore, to consistently correspond to comparison of the top third versus the bottom of the baseline distribution by sex hormone levels in each study. Briefly, we transformed the log RR by assuming a normal distribution, with the comparison between extreme thirds being equivalent to 2.18 times the log risk ratio for one standard deviation increases (or equivalently as 2.18/2.54 time the log RR for a comparison of extreme quarters). We calculated standard error of the log RR by using published CIs and standardised them in the same way. Hazard ratios, RRs, and odds ratios were assumed to approximate the same measure of RR.

The inverse variance weighted method was used to combine relative risks to produce a pooled relative risk using random-effects models to allow for between study heterogeneity. Also, additionally we reported the estimates using fixed effect models. Heterogeneity was assessed using the Cochrane χ^2statistic and the I^2 statistic. Publication bias was evaluated through a funnel plot and Egger's test. All tests were two-tailed and p-values of 0.05 or less were considered significant. STATA release 12 (Stata Corp, College Station, Texas) was used for all statistical analyses.

RESULTS

Table 1 summarizes the baseline characteristics of the participants included in the analysis. Of the 3117 women without diabetes at baseline, 384 women developed diabetes over a median follow-up of 11.1 years.

Table 1 Selected Characteristic of Study Participants, the Rotterdam Study.

Age (years)	69.7 ± 8.7
Years since menopause (years)	20.9 ± 10.0
Age of menopause (years)	48.9 ± 5.2
Natural menopause, n (%)	2433 (78.1)
Current smokers, n (%)	218 (9.2)
Alcohol intake g/day	1.3 (10)[a]
BMI (kg/m^2)	27.0 ± 4.3
Waist circumference (cm)	89.4 ± 11.6
Prevalent coronary heart disease, n (%)	86 (2.8)
Estradiol (pmol/l)	34.2 (41.62)[a]
Total testosteron (nmol/l)	0.8 (0.56)[a]
Sex-hormon binding globuline (nmol/l)	69.6 ± 33.0
Free androgen index	1.3 (1.1)[b]
Thyroid-stimulating hormone (mU/l)	1.95 (1.7)[a]
Hormone replacement therapy, n (%)	159 (5.3)
Insulin (pmol/l)	67 (47)[a]
Glucose (mmol/l)	5.5 ± 0.6
C-reactive protein (mg/ml)	1.7 (2.93)[a]
Total cholesterol (mmol/l)	6.0 ± 1.0
Low density lipoprotein cholesterol (mmol/l)	4.2 (1.22)[a]
High density lipoprotein cholesterol (mmol/l)	1.5 ± 0.4
Statin use, n (%)	681 (14)
Triglycerides (moml/l)	1.27 (0.74)[a]
Systolic Blood pressure (mm/Hg)	142.0 ± 21.1
Indication for hypertension, n (%)	794 (25.5)

BMI, body mass index; HRT, hormone replacement therapy; NA, non-applicable. Plus minus values are mean ± SD

[a] Median (interquartile range)

Sex-hormones and the risk of developing T2D

In models adjusted for age, cohort effect and fasting status, lower SHBG levels (3rd tertile vs.1st tertile: RR=0.33, 95% CI=0.25 -0.43, *p*-trend<0.001) and higher levels of BT (3rd tertile vs.1st tertile: RR=2.01, 95% CI=1.55-2.60, *p*-trend<0.001) and TE (3rd tertile vs.1st tertile: RR=2.02, 95% CI=1.50 -2.70, *p*-trend<0.001) were associated with an increased risk of T2D (**Table 2**).

Table 2. Associations of sex hormone-binding globulin, testosterone, free androgen index and estradiol with the risk of type 2 diabetes in peri- and postmenopausal women, the Rotterdam Study.

		Sex hormone-binding globulin		***Continuous***	***Ptrend***
	Tertile 1	Tertile 2	Tertile 3		
Cases	191	119	74		
Model 1, HR, 95% CI	1.00	**0.56 (0.45-0.71)**	**0.33 (0.25-0.43)**	**0.37 (0.30-0.46)**	**<0.001**
Model 2, HR , 95% CI	1.00	0.82 (0.64-1.04)	**0.56 (0.41-.0.77)**	**0.63 (0.49-0.81)**	**<0.001**
Model 3, HR , 95% CI	1.00	0.82 (0.64-1.05)	**0.56 (0.40-.0.79)**	**0.66 (0.51-0.86)**	**0.001**
		Total Testosterone		*Continuous*	*Ptrend*
	Tertile 1	Tertile 2	Tertile 3		
Cases	126	139	119		
Model 1, HR , 95% CI	1.00	1.04 (0.82-1.32)	0.90 (0.69-1.16)	0.91 (0.75-1.10)	0.40
Model 2, HR , 95% CI	1.00	0.94 (0.74-1.20)	0.82 (0.63-1.07)	0.87 (0.71-1.07)	0.15
Model 3, HR , 95% CI	1.00	0.96 (0.75-1.24)	0.88 (0.67-1.16)	0.93 (0.76-1.14)	0.36
		Free androgen index		*Continuous*	*Ptrend*
	Tertile 1	Tertile 2	Tertile 3		
Cases	87	124	173		
Model 1, HR , 95% CI	1.00	**1.39 (1.05-1.82)**	**2.01 (1.55-2.60)**	**1.54 (1.32-1.79)**	**<0.001**
Model 2, HR , 95% CI	1.00	1.06 (0.79-1.42)	1.17 (0.87-1.57)	1.13 (0.94-1.36)	0.28
Model 3, HR , 95% CI	1.00	1.05 (0.78-1.42)	1.15 (0.85-1.54)	1.10 (0.92-1.32)	0.34
		Total estradiol		*Continuous*	*Ptrend*
	Tertile 1	Tertile 2	Tertile 3		
Cases	109	132	143		
Model 1, HR , 95% CI	1.00	**1.28 (0.99-1.65)**	**2.02 (1.50 -2.70)**	**1.003 (1.001-1.004)**	**<0.001**
Model 2, HR , 95% CI	1.00	1.00 (0.74-1.34)	**1.39 (1.004-1.93)**	**1.003 (1.001-1.004)**	0.07
Model 3, HR , 95% CI	1.00	1.05 (0.78-1.41)	**1.42 (1.01-2.00)**	**1.002 (1.001-1.004)**	0.055

Model 1: Adjusted for age, cohort, fasting status

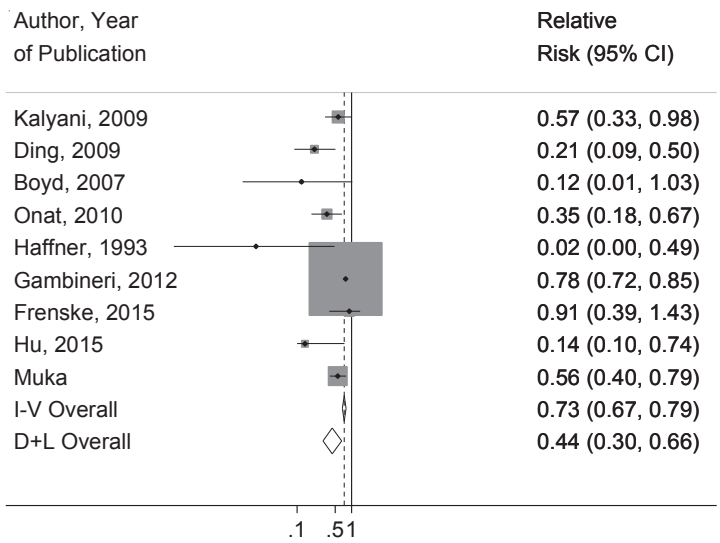
Model 2: Model 1 + insulin, glucose and body mass index

Model 3: Model 2 + alcohol intake, smoking status, coronary heart disease, serum total cholesterol, statin use, systolic blood pressure, treatment for hypertension, hormone replacement therapy, age of menopause, C-reactive protein and sex hormones for each other.

Further adjustments for BMI, insulin and glucose attenuated but did not abolish the association between SHBG (3rd tertile vs.1st tertile: RR=0.56, 95% CI=0.41 -0.77, *p*-trend<0.001) or TE and incident T2D (3rd tertile vs.1st tertile: RR=1.39, 95% CI=1.004 -1.93, *p*-trend=0.07). On the other hand, adjustment for obesity and glycemic traits weakened the associations of BT with T2D such that they were not longer statistically significant (**Table 2**). Controlling for metabolic risk factors, lifestyle factors, inflammatory markers and prevalent coronary heart disease did not materially affect these associations (**Table 2**). No association was found between TT and incident T2D in any of the models (**Table 2**).

Because associations of continues hormone variables with T2D in the Model 1 appeared linear, RRs stratified and sensitivity analyses were expressed per unit log or unit increase in hormone biomarkers. In the sensitivity analysis, substituting BMI with waist circumference as a measure of adiposity, substituting total cholesterol for other blood lipids, adjusting further for serum TSH, physical activity or menopause type, and excluding the first three years of follow up did not affect any of the associations (**S1 Table**). Also, in the stratification analysis, no significant interactions were found for SHBG and TE with BMI or years since menopause (**S1 Table**). Significant interaction terms were found for TT (p-interaction = 0.019) and FAI (p-interaction = 0.03) with years since menopause. However, no association was found between these hormones and T2D after stratification for time since menopause (**S1 Table**). Also, no effect modification by BMI was found for TT and BT (**S1 Table)**.

Figure 1 Relative risks of type 2 diabetes comparing top vs. bottom thirds of baseline plasma sex hormone-binding globulin.



X^2= 36.2, I^2=77.9%; P < 0.001

Systematic Review and Meta-Analysis

Literature Search, Characteristics and Quality of Eligible Studies

Initial search identified 3209 potentially relevant citations. After screening and detailed assessment, 14 articles based on 11 unique studies were included (**S1 Figure and S5 Appendix**). Therefore, we meta-analysed estimates from 12 studies (including the current study) involving a total of 12,972 unique participants with 1912 incident T2D cases reporting on the association between sex hormones and T2D risk. Detailed characteristics of these studies and quality assessment have been summarized in **S2 Table**.

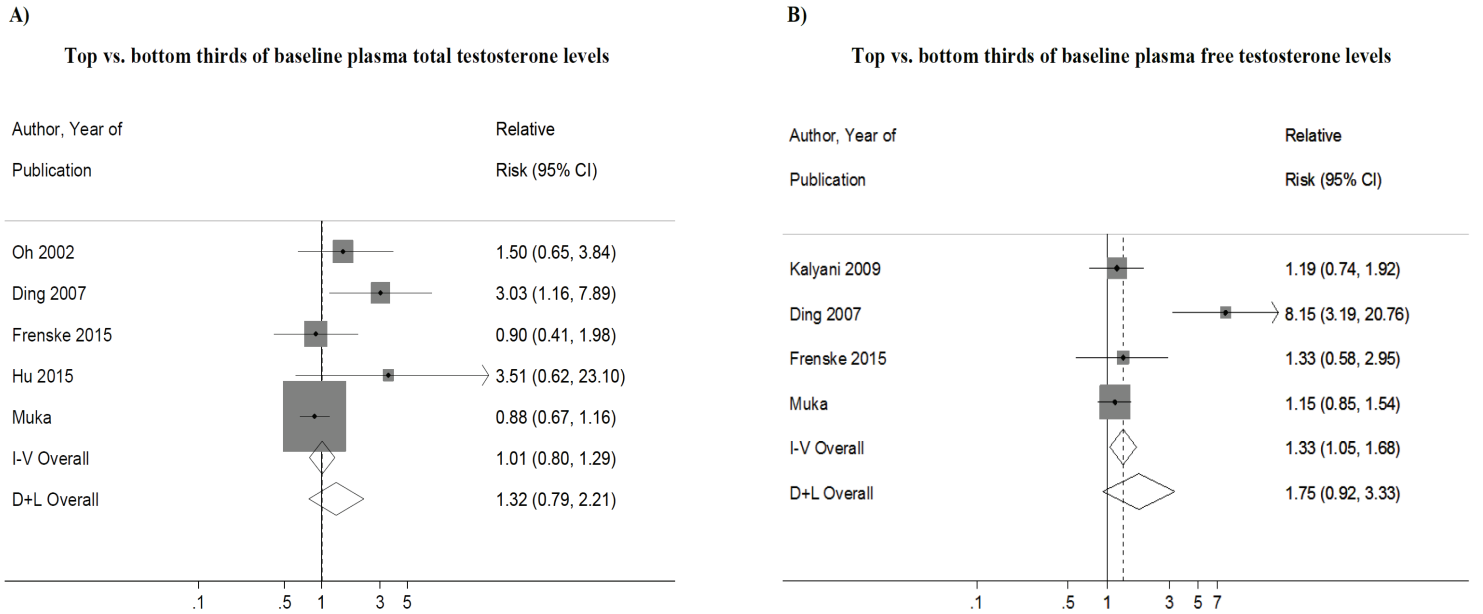
Sex hormones and T2D in Pooled Analysis

The meta-analysis for BT, TE and BE is based only on studies examining postmenopausal women; the meta-analysis for TT is based on 4 studies including postmenopausal women and 1 study including pre and postmenopausal women, whereas the findings for SHBG derive from studies including premenopausal women (n=2), postmenopausal women (n=4) and combined (3). The pooled RR for T2D adjusted for several metabolic risk factors comparing 3rd tertile vs. 1st tertile of SHBG, TT, BT, TE and BE were 0.44 (95%CI: 0.30-0.66, I^2=77,9%, p<0.001), 1.32 (95%CI: 0.79-2.21, I^2=53.8%, p=0.07), 1.75 (95%CI: 0.92-3.33, I^2=80.7%, p=0.001), 1.99 (95%CI: 1.21-3.27, I^2=55.1%, p=0.06) and 3.58 (95%CI: 0.86-14.84, I^2=81.0%, p=0.02) (**Figure 1-3**). There was evidence of between-study heterogeneity for all these analyses with possible exception of the meta-analysis on the association between TE and the risk of T2D (**Figure 1-3**). Four studies were not possible to include in the meta-analyses. Soriguer found that, in pre- and postmenopausal women, per one unit log increase in SHBG, TT and BT, the corresponding RRs were 0.23 (0.1 to 0.53), 1.04 (0.59 to 1.83) and 1.12 (0.59 to 2.13) respectively. Boyd-Waschinko at al. reported a 5-fold increase in T2D incidence in the lowest quintile of SHBG. Similarly, Lindstedt et al. found that among patients in the low SHBG terile, 18% converted to T2D as compared with 5% in mid SHBG tertile and 2.5% in high SHBG tertile. Okubo et al reported lower levels of SHBG in T2D converters (59.7 ± 8.4 nmol/l) than non-converters (69.5 ± 2.5 nmol/l) during 3 years of follow-up but that was not significant different after adjusting for age, body mass index and waist to hip ratio[24].

Publication bias

The appearance of funnel plots was asymmetrical for the analysis on SHBG and T2D, and Egger's test results were significant (P = 0.014) (**S2 Figure**). This suggested that publication bias may be present. After exclusion of the four studies that included 50 or fewer T2D cases findings were not statistically significant (Egger's test, P=0.93, data not shown). No evidence of publication bias was observed for the analysis of TT or TE and T2D (**S2 Figure**).

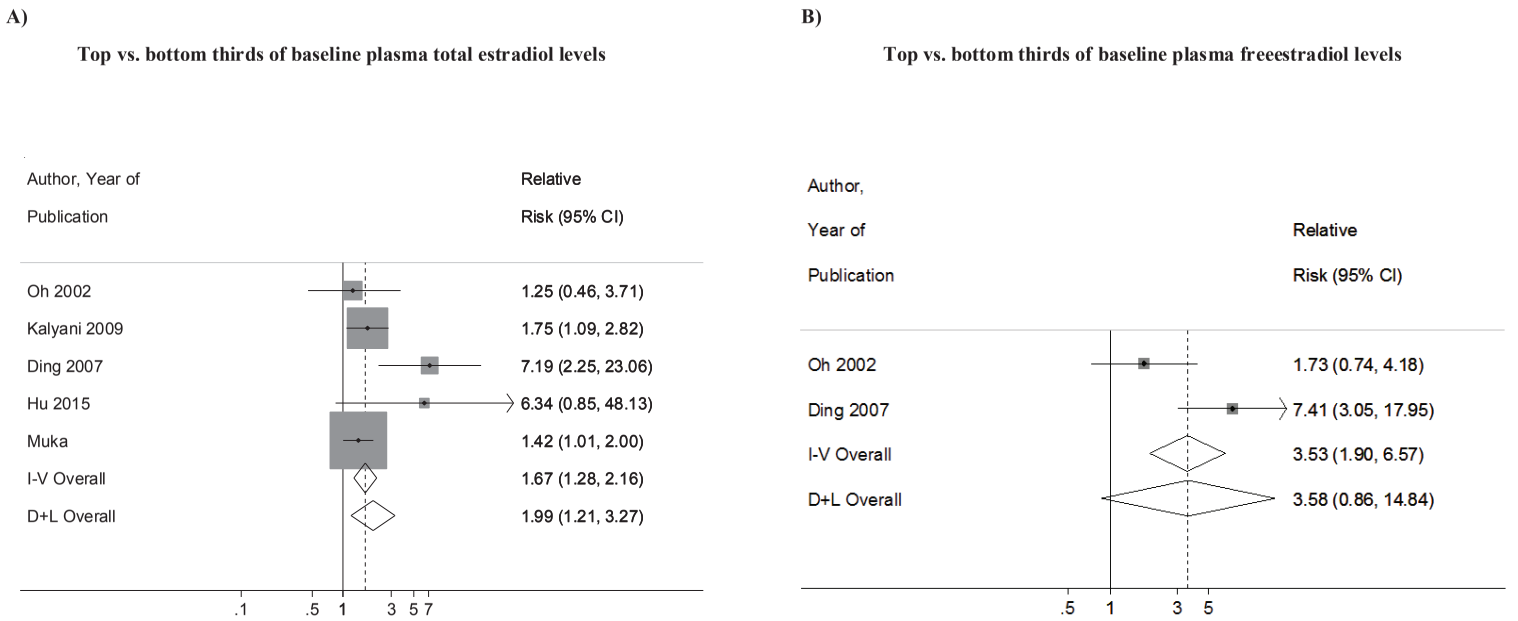
Figure 2 Relative risks of type 2 diabetes comparing top vs. bottom thirds of baseline plasma total and free testosterone levels.



A) X^2= 8.6, I^2=53.8%; P =0.07;

B) X^2= 15.5, I^2=80.7%; P =0.001

Figure 3 Relative risks of type 2 diabetes comparing top vs. bottom thirds of baseline plasma total and free estradiol levels.



A) X^2= 8.91, I^2=55.1%; P =0.06;

B) X^2= 5.26, I^2=81.0%; P =0.02

DISCUSSION

Our findings in the Rotterdam Study generally concur and further extend previous studies, providing relevant findings that have not been previously addressed. In this large population based study of postmenopausal women free of T2D at baseline, we showed that lower levels of SHBG and higher levels of TE are associated with the risk of T2D. These associations were independent of established risk factors, including body mass index, glucose and insulin. In our study, however, we did not find any association between testosterone and the risk of T2D.

Pooled results from the systematic meta-analysis of 12 studies reinforce the validity and generalizability of our findings, suggesting that SHBG and TE are robust risk markers of T2D in women. Unlike the previous meta-analysis by Ding EL et al, which was based mainly on studies with cross-sectional design and examined only mean differences between T2D cases and non T2D controls, our current pooled analysis is based on findings from 11 prospective studies (only two studies were included in the previous review were eligible), including 10873 participants with 1623 T2D cases. Therefore, our meta-analysis provides a more detailed assessment of the nature and magnitude of the association between sex hormones and T2D in women.

SHBG levels have been associated with metabolic syndrome, glucose and insulin levels, established risk factors for T2D[9,10,25]. Also, women with PCOS, a condition of anovulation and hyperandrogenism, are at increased risk of T2D, and levels of SHBG are decreased in these women [13]. The complex biological mechanisms that explain the association between circulating SHBG levels and the risk for T2D are not fully understood. Classically, the primary function of SHBG was thought to be the binding of circulating hormones in order to regulate free sex hormone bioavailability to target tissues. Therefore, it has been hypothesized that the relation between SHBG and T2D may results from indirect influence of alterations in SHBG on sex hormone bioavailability. However, in our study, the association between SHBG and T2D risk remains significant after adjustment for TT, BE and TE, implicating SHBG levels as a risk factor for T2D independent of serum androgen levels. Additional evidence in support of an independent effect of SHBG on T2D comes from recent studies that have found several polymorphisms in the SHBG to associate with insulin resistance and T2D, suggesting that altered SHBG physiology may be a primarily defect in the pathogenesis of disease[26-29]. Furthermore, a growing body of evidence show that SHBG may directly mediate cell-surface signalling, cellular delivery and biologic action of sex hormones via activation of a specific plasma receptor[30-32]. At the target tissue level, the fraction of SHBG that is not bound to sex steroid has the ability to bind plasma membrane high-affinity receptors ($R_{SHBG)}$ [30]. Sex steroids of variable biologic potency can activate the anchored SHBG- R_{SHBG} complex and the activated complex can have either an agonist or antagonist effect. For example, SHBG- R_{SHBG} complex can have direct cellular antagonistic properties against estrogen; SHBG may interact with cellular estrogen receptors which can trigger a biologic antiestrogenic response[30]. Specific downstream effects of the SHBG-receptor complex merit further investigation since may help to clarify the underlying mechanisms linking SHBG to T2D.

Our result for a positive relation between estradiol and T2D are in contrast with the results from previous trials of oral estrogen therapy, which showed a lower risk of T2D among postmenopausal women who used estrogen treatment[33-35]. In contrast, pregnancy, a state of high endogenous estrogen, is associated with insulin resistance[12]. Exogenous estrogen may have different physiological effects depending on type, route, duration and dose of estrogen therapy[36-39]. For example, opposing effects of oral estrogen on fasting glucose vs glucose tolerance have been reported[36,37]. Also, transdermal estradiol elevated CRP levels up to 12 months of treatment but not oral estrogen in a randomized trial of postmenopausal women[39]. Moreover, a bimodal relationship of estrogen dose may exist. In a clinical trial of postmenopausal women, lower dose of estrogen therapy increased insulin sensitivity whereas higher dose had the opposite effect[40].

Endogenous estradiol may be associated with diabetes risk through its relation to glucose, insulin, adiposity and inflammation. Indeed, previous cross-sectional studies have linked both BE and TE with higher glucose and insulin resistance levels in postmenopausal women, independently of obesity[11,41,42]. Also, animal studies show that estradiol may have a direct effect on adipocyte enlargement and weight gain[43], although other studies[44] do not support this hypothesis. Furthermore, elevated inflammatory factors such as CRP have been reported in women on oral estrogen treatment[39]. However, in our study, the association between TE and T2D, although attenuated, remained significant after adjustment for plasma levels of glucose and insulin, BMI and CRP, suggesting that estradiol may play a direct role in the pathophysiology of T2D. Furthermore, additional adjustment for TT did not affect this association, suggesting that estradiol may be more than just a marker of increased aromatase conversion. Explicit mechanisms of estrogen in relation to T2D require further study.

Our study showed no association between TT and the risk of T2D whereas a suggestive positive association was observed between BT and T2D. The lack of association between FT and the risk of T2D in our study might be due to lack of a direct measure of BT in the blood which could have biased our results toward the null. These findings are in line with previous studies reporting higher levels of insulin resistance with increasing levels of BT in postmenopausal women, while no association has been observed between TT and insulin resistance[15,42]. Similarly, BT has been related to increased odds of having impaired fasting glucose[15].

Strengths of our study include its prospective design; the long follow-up and adequate adjustments for a broad range of possible confounders. We also performed several sensitivity analyses such as excluding the first three years of follow up to avoid potential bias of undiagnosed disease at baseline. Furthermore, our study included in addition to analysis of primary data also a systematic review of all available published prospective cohorts, which is the first-ever quantitative synthesis of these associations thus far in women. Also, most of the studied included in our meta-analysis adjusted for potential confounding. However, there are several limitations that need to be taken into account. First, we did not have measures of bioavailable estradiol in the Rotterdam Study, which could have strengthened our results. Second, 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[45]. Third, we observed a moderate to high level of heterogeneity across the included studies. Since the number of available studies included in each meta-analysis was generally small, it precluded our ability to investigate the sources of the observed heterogeneity by subgroup analyses involving various study-level characteristics (such as age). Fourth, there was evidence of publication bias for the association between SHBG and the risk of T2D, so it is possible that our results constitute an overestimation of the performance of the test. However, when we excluded small studies differences were not statistically significant and therefore, the effect of publication bias may be only minor. Fifth, the meta-analyses on BE, BT and T2D included estimates from four or less studies. The use of random-effects models is limited in the presence of few studies for analysis and the use of fixed-effects models may be preferred[46]. Contrary to the results of random-effects models, the fixed-effects models showed a significant association of both BE and BT with the risk of T2D. Last, except for SHBG, the other findings come from studies conducted mainly in postmenopausal women, and therefore these results cannot be extended to pre or perimenopause women.

In conclusion, findings of this review indicate that endogenous sex hormones are associated with the risk of T2D in postmenopausal women. Further studies are needed to establish hormones thresholds at which diabetes risk is increased, because this may aid in identifying high-risk postmenopausal women in the clinical setting. Also, future studies are needed to investigate the effect of medication or lifestyle factors the affect sex hormone levels on glucose metabolism and T2D, which may help in development of novel glucose-lowering therapies and diabetes prevention.

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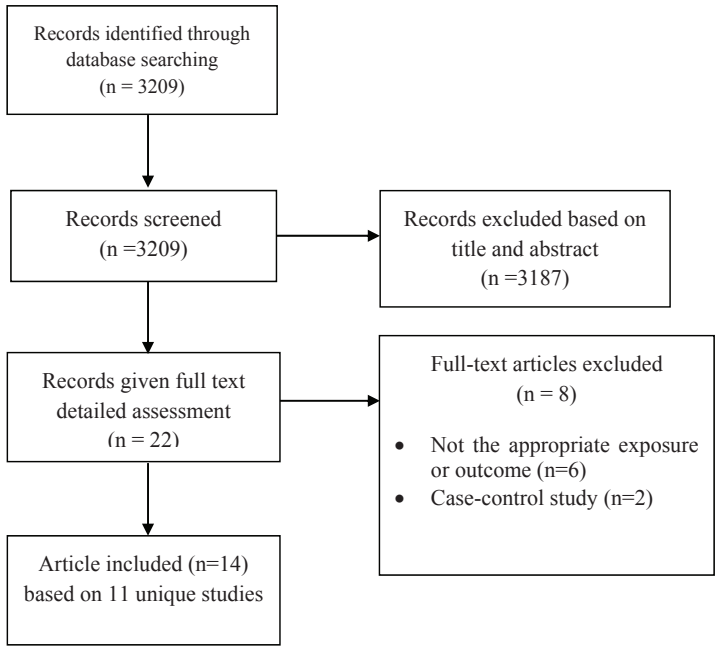
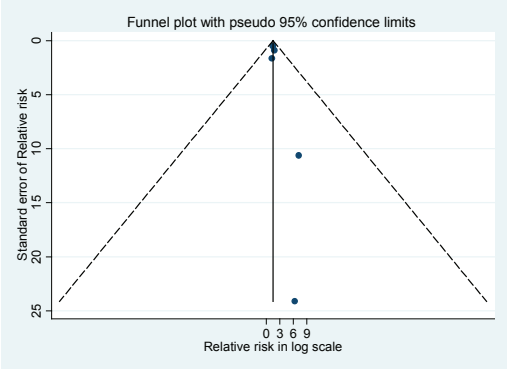
Supplemental material

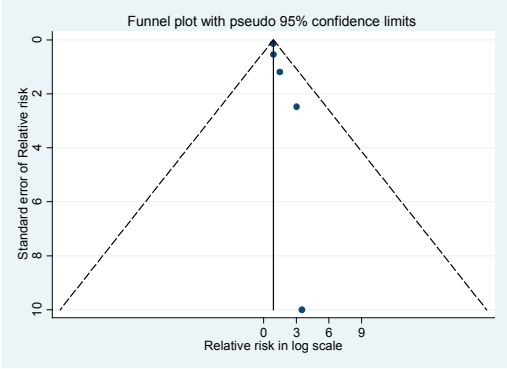
S1 Figure Flowchart of studies investigating the association between endogenous sex hormones and the risk of type 2 diabetes.



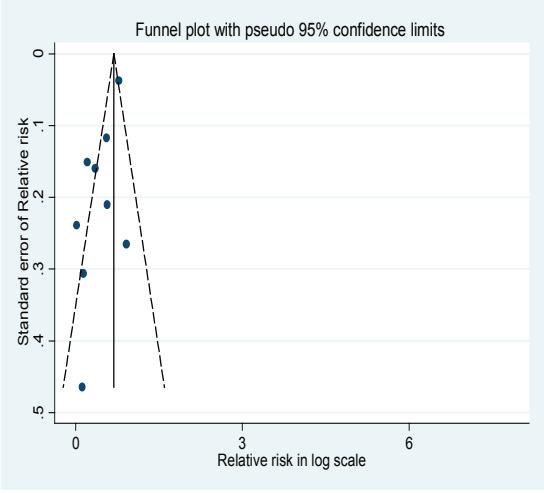
S2 Figure Assessment of small study effects by funnel plots and Egger's test in prospective studies of age at menopause and cardiovascular disease outcomes



Total testosterone



Total estradiol



Sex hormone-binding globulin

The dotted lines show 95% confidence intervals around the overall summary estimate calculated using a fixed effect model; *p*-values for bias calculated using Egger's test was 0.014, 0.08 and 0.18 for sex hormone-binding globulin, total testosterone and total estradiol respectively.

S1 Table Sensitivity analysis of sex and the risk of type 2 diabetes postmenopausal women, the Rotterdam Study.

	Sex hormone-binding globulina[a]	Total Testosterone[a]	Estradiol[b]	Free androgen index[a]
Multivariable model	0.66 (0.51-0.86)	0.93 (0.76-1.14)	1.002 (1.001-1.004)	1.15 (0.85-1.54)
Multivariable model + waist circumference	0.69 (0.53-0.90)	0.96 (0.78-1.19)	1.003 (1.001-1.005)	1.11 (0.92-1.33)
Multivariable model + HDL + TG + LDL	0.72 (0.55-0.95)	0.94 (0.76-1.16)	1.003 (1.001-1.004)	1.07 (0.89-1.29)
Multivariable model + serum thyroid stimulating hormone	0.66 (0.51-0.87)	0.89 (0.73-1.09)	1.002 (1.001-1.004)	1.11 (0.93-1.33)
Multivariable model + physical activity	0.66 (0.50-0.86)	0.89 (0.73-1.09)	1.003 (1.001-1.004)	1.12 (0.94-1.34)
Multivariable model + menopause type (surgical vs. natural)	0.65 (0.50-0.85)	0.89 (0.72-1.08)	1.003 (1.001-1.004)	1.12 (0.94-1.34)
Multivariable model excluding the first 3years of follow-up	0.68 (0.51-0.91)	0.98 (0.79-1.23)	1.003 (1.001 -1.005)	1.13 (0.93-1.37)
BMI (kg/m^2)[c]				
<25	0.50 (0.28-0.91)[d]	0.90 (0.56-1.45)[d]	1.003 (1.001-1.006)[d]	1.18 (0.78-1.78)[d]
25-29.9	0.80 (0.54-1.18)	1.20 (0.88-1.62)	1.003 (1.00-1.006)	1.22 (0.94-1.58)
≥30	0.76 (0.48-1.22)	0.86 (0.59-1.24)	1.001 (1.00-1.005)	0.99 (0.72-1.36)
Time since menopause[c]				
<15	0.84 (0.47-1.50)[d]	1.26 (0.84-1.89)[e]	1.004 (1.001-1.007)[d]	1.22 (0.86-1.73)[k]
15-25	0.78 (0.51-1.19)	1.01 (0.73-1.40)	1.002 (0.998 -1.006)	1.09 (0.83-1.44)
>25	0.46 (0.28-0.75)	0.78 (0.55-1.10)	1.003 (0.996 -1.01)	1.08 (0.79-1.48)

Multivariable model adjusted for variables in model 3 of Table 2.
[a] Values are + 1 log increase
[b] Values are per 1 unit increase
[c] Results are adjusted for variables in model 3 of Table 2
[d] P-interaction >0.05
[e] P-interaction=0.019
[k] P-interaction=0.03

S2 Table Characteristics of the included studies that assessed the association of sex hormones with the risk of type 2 diabetes

Lead Author, Publication Date	Source of participants	Location	Year of baseline survey	Baseline age range/ average age (years)	Study design	Follow up years	Total participants	No. of study events	Menopausal Status	Covariates adjusted for	Study quality*
Oh 2002	RBS	USA	1984-1987	72.4 ± 6.14	Case-cohort	8	233	17	Postmenopausal	Age, BMI, systolic blood pressure,	7
Chen BH et al.2012	WHI-OS	USA	1994-1998	61.1 ± 5.2	Case-cohort	5.9 (median)	1928	642	Postmenopausal	Age, race/ethnicity, clinical center, time of blood flow, duration of follow-up, postmenopausal hormone therapy, physical activity, smoking status, alcohol intake, history of hypertension, family history of diabetes, body mass index, C-reactive protein, HOMA-IR, testosterone, LDL, HDL-cholesterol, glycated haemoglobin.	8
Ding 2009	MESA	USA	2000-20002	65.2 ± 9.0	Prospective	6	1612	116	Postmenopausal	Age, race/ethnicity, education, income, family history of diabetes, examination site, BMI, HOMA-IR, LDL, HDL, triglycerides, use of lipid-lowering medications, systolic blood pressure, antihypertensive medication, total daily caloric intake, physical activity, smoking, IL-6, CRP, age at menopause, years since menopause, type of menopause, age at first live birth, five or more live births, and past use of hormone replacement therapy r oral contraceptive pills.	9
Onat 2010	TARFS	Turkey		48.4 ± 11.7	Case-cohort	9	1067	202	Pre- and post-menopausal	Age, BMI, C-reactive protein, HDL-cholesterol, lipid lowering drugs and Apolipoprotein A-1	7
Haffner 1993	SAHS	USA	1979-1982	25-64	Case-cohort	8	109	38	Pre- and post-menopausal	Fasting glucose and insulin	7
Soriguer 2011	PCS	Italy	1995-1997	36 ± 23	Case-cohort	11	691	106*	Pre- and post-menopausal	Age, body mass index and waist circumference	8

S2 Table Characteristics of the included studies that assessed the association of sex hormones with the risk of type 2 diabetes

Lead Author, Publication Date	Source of participants	Location	Year of baseline survey	Baseline age range/ average age (years)	Study design	Follow up years	Total participants	No. of study events	Menopausal Status	Covariates adjusted for	Study quality*
Gambineri 2012	NA	Italy	1978-1999	23.4 ± 6.3	Prospective	16.9	255	42	Pre-menopausal	Age, BMI, fasting glucose, waist to hip ratio, systolic blood pressure, diastolic blood pressure, fasting insulin, HbA1C, total cholesterol, HDL-cholesterol, triglycerides, luteinizing hormone, follicle-stimulating hormone, estradiol, testosterone, SHBG	8
Lindstedt G et al. 1991	NA	Sweden	1968-1969	38-60	Prospective	12	1462	43	Pre and postmenopausal		7
Boyd-Woschinko	NA	USA		36-43	Case-cohort	8	119	10	Pre-menopausal		5
Okubo M et al. 1999	Medical survey	Hawaii and USA	1992-1993	65.4 ± 0.5	Prospective	3	280	23	Postmenopausal	Age, body mass index and waist to hip ratio	6
Fenske B et al. 2015	Study of Health in Pomerania	Germany	1997-2001	48.8 ± 16.2	Prospective	5	1925	202*	Pre and postmenopausal	Age, waist circumference, physical inactivity and alcohol consumption.	8
Hu J et al. 2015	EIMDS	China	2008	60 ± 11.1	Prospective, nested case-control	5	174	87	Postmenopausal	BMI, systolic blood pressure, diastolic blood pressure, current smoking, alcohol use, hypertension, exercise frequency, family history of T2D, fasting plasma glucose, total cholesterol, high-density lipoprotein cholesterol and low density lipoprotein cholesterol.	8
Total							12,972	1912			

BMI, Body mass index, EIMDS, The Environment, Inflammation and Metabolic Diseases Study; MESA, Multi-Ethnic Study of Atherosclerosis; PCS, Pizarra Cohort Study; RBS, Rancho Bernardo Study; SAHS, San Antonio Heart Study; TARFS, Turkish Adult Risk Factor Study; WHS, Women Health's Study; *approximation

S1 Appendix Potential confounding variables

Information on current health status, medical history, medication use, smoking behaviour, and socioeconomic status was obtained at baseline for both studies. During the home interview, women were asked a special section of questions pertaining to menopausal status. One set of questions dealt with timing of the last menstrual period, gathering information on whether the respondent had a natural menstrual period within the 12 months, the past 3 months, and the age at last period for women who had no period for at least 3 months. One question addressed period regularity and the number of menstrual cycles. Postmenopausal women were defined women who reported absence of menstrual periods for 12 months. 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 was defined as a history of coronary heart diseases (myocardial infarction, revascularization, coronary artery bypass graft surgery or percutaneous coronary intervention) and was verified from the medical records of the general practitioner. Blood pressure was measured in the sitting position at the right upper arm with a random-zero-sphygmomanometer. Physical height (m) and body weight (kg) were measured at baseline with the participants standing without shoes and heavy outer garments. Body mass index (BMI) was calculated as weight divided by height squared (kg/m2). 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), triglycerides (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%. Physical activity was assessed with an adapted version of the Zutphen Physical Activity Questionnaire[1]. Every activity mentioned in the questionnaire was attributed a MET-value according to the 2011[2].The questionnaire contained questions on walking, cycling, gardening, diverse sports, hobbies and on housekeeping. Total time spend on physical activity was calculated as the sum of minutes per week for each type of activity.

S4 Appendix Search strategy

Embase.com

('sex hormone'/de OR androgen/de OR 'androgen blood level'/de OR 'androgen deficiency'/de OR testosterone/de OR 'testosterone blood level'/de OR 'sex hormone binding globulin'/de OR estradiol/de OR 'estradiol blood level'/de OR hypogonadism/exp OR 'gonad dysfunction'/de OR 'ovary insufficiency'/exp OR 'testis function'/de OR 'hyperandrogenism'/exp OR (((sex OR sexual OR gonad* OR testicular* OR ovar*) NEXT/3 hormone*) OR androgen* OR hyperandrogen* OR hypoandrogen* OR testosterone* OR estradiol* OR oestradiol* OR hypogonad* OR hypergonad* OR ((gonad OR testis OR testes OR testicular OR ovar*) NEAR/3 (dysfunction* OR insufficien* OR failure* OR hypofunct* OR function*))):ab,ti) AND ('non insulin dependent diabetes mellitus'/exp OR (((diabet* OR dm) NEAR/3 ('type 2' OR type2 OR 'type ii' OR 'non insulin' OR noninsulin OR 'adult onset' OR 'slow onset' OR 'maturity onset')) OR T2DM OR dmt2 OR dm2 OR T2-DM OR dm-t2 OR dm-2 OR niddm OR nid-dm):ab,ti) NOT ([animals]/lim NOT [humans]/lim) NOT ([Conference Abstract]/lim OR [Letter]/lim OR [Note]/lim OR [Editorial]/lim)

S5 Appendix References of the articles included in the Systematic Review

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Association of vasomotor and other menopausal symptoms with risk of cardiovascular disease: a systematic review and meta-analysis

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4.2 Estrogen Receptor β Actions in the Female Cardiovascular System: a Systematic Review of Animal and Human Studies

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**Contributed equally*

ABSTRACT

Importance: Estrogen receptor beta (ERβ) has been described to play a role in the female cardiovascular system; however, little work has been done to appraise the existing evidence on its function.

Objective: To systematically review studies evaluating the role of ERβ in the female cardiovascular system and the influence of age and menopause on ERβ functioning.

Methods: Five medical databases (Embase, Medline, Web of Science, PubMed, Cochrane and Google Scholar) were used to identify studies. We included female animal and human (women) model experimental studies, randomized trials, cohort, case-control and cross-sectional studies carried out in female adults that assessed the role of ERβ in the cardiovascular system.

Results: Of 9472 references, 88 studies met our inclusion criteria: 71 animal model experimental studies, 15 human model experimental studies and 2 population-based studies. ERβ-signaling was shown to possess vasodilator and antiangiogenic properties by regulating the activity of nitric oxide, altering membrane ionic permeability in vascular smooth muscle cells (VSMC), inhibiting VSMC migration and by regulating adrenergic control of the arteries. A possible protective effect of ERβ-signaling against left ventricular hypertrophy and ischemia/reperfusion injury via genomic and non-genomic pathways was suggested in 27 studies. Five studies reported that the vascular effects of ERβ may be vessel specific and may differ by age and menopause status.

Conclusions: ERβ seems to possess multiple functions in the female cardiovascular system and further studies are needed to evaluate whether isoform-selective ERβ-ligands may contribute to cardiovascular disease prevention.

INTRODUCTION

Despite improvements in prevention and treatment, cardiovascular disease (CVD) remains the leading cause of death for women worldwide[1]. Epidemiological studies have revealed that, compared with age-matched men, premenopausal women have a lower risk of coronary heart disease (CHD) which gradually increases after menopause so that by the sixth decade women have the same incidence of CHD as men[2].

The disparity between the incidence of CVD among women in pre- and post-menopause has been largely ascribed to the actions of estrogens on the cardiovascular system and, particularly, on the vascular endothelium. Extensive evidence from observational studies suggest cardio-protective effects of endogenous and exogenous estrogens in premenopausal women[3]. Similarly, results from clinical trials suggest that estrogen therapy started within the first years after menopause reduce cardiovascular risk',[4,5] whereas initiation of estrogen therapy many years after menopause may have no or even deleterious cardiovascular effects[5,6]. Therefore, the vascular response to estrogens appears to change with increasing age and to depend on years after menopause. In line with this hypothesis, it has recently been suggested that favorable vascular effects of estrogen depend on the endothelium and are mediated mainly via estrogen receptors alpha (ERα) and beta (ERβ) [7]. Therefore, age- and menopause-related endothelial injury, changes in vascular estrogen receptors expression, intracellular signaling or genomics may alter the cardiovascular effects of this sex hormone[8]. To date, however, whereas the role of ERα has been extensively studied, the actions of ERβ on the cardiovascular system and the age- and menopause-related changes of vascular ERβ actions remain unclear and may explain in part the sex differences observed in the influence of estrogen on the cardiovascular system[9-11].

Therefore, we aimed to systematically review the literature evaluating the role of ERβ in the cardiovascular system in females and the role of age and menopause on ERβ actions.

METHODS

Literature search and selection criteria

We conducted a systematic search of electronic databases (Embase, Medline, Web of Science, PubMed, Cochrane and Google Scholar) until February 5th 2015 (date last searched) to retrieve scientific articles assessing the actions of ERβ on the cardiovascular system in females and the age- and/or menopause-related changes in vascular ERβ functions, intracellular signaling or its genomic actions (**Appendix 1, 2 and 3** in the **Online Supplemental Material**). Reference lists of identified articles were also searched and experts in the field were contacted to identify further studies. In total, we found 9472 potentially unique relevant citations.

Studies were included if the following criteria were fulfilled: (1) the study assessed the effect of ERβ on vascular reactivity (vasoconstriction, vasodilatation, actions on sympathetic nervous system, pulse wave velocity, blood pressure, vasopressin release, carbon dioxide, adenosine), endothelial

function and healing (angiogenesis, vascular smooth muscle cell proliferation and migration or collagen synthesis), endothelium- derived relaxing factor expression (nitric oxide or expression of other endothelium-derived compounds), inflammation, regulation of protein biosynthesis or expression and gene expression in the cardiovascular system, atherosclerosis (including proxies such as carotid intima-media thickness, coronary artery calcium, ankle brachial index, pulse wave velocity) or CVD (CHD, cardiomyopathy, heart failure, cerebrovascular disease, peripheral artery disease and inflammatory heart disease), (2) the study was conducted in females (animal or human) or (if both females and males were included in the studies) the studies that reported the results by gender (or no gender differences were shown), (3) the role of ERβ was assessed by its gene deletion, use of estrogen receptor agonist, antagonist, antibodies/vectors and its expression levels (4) there was a restriction to the original study results (review and duplicate publications were excluded). The step-wise inclusion and exclusion procedure outline is shown in **Figure 1.**

Study selection and data extraction

Two independent reviewers screened the retrieved titles and abstracts and selected eligible studies. Any disagreement between the two reviewers was resolved through consensus or consultation with a third independent reviewer. A predesigned data collection form was prepared to extract the relevant information from the selected studies, including study design (for human studies), sample type (animal species, number of animal/human participants, age and weight of participants, tissue used, method to assess ERβ function, outcome measures and results of the study (author's conclusion).

Outcome assessment and statistical methods

For each animal model study, we defined whether a positive or negative effect (no effect) result was reported. For population-based studies, we reported the effect magnitude, direction and significance. Heterogeneity permitting, we sought to pool the results using a random effects meta-analysis model. If pooled, the results were expressed as the pooled estimate and the corresponding 95% confidence intervals.

RESULTS

In total we identified 15283 potentially relevant citations via Embase (n=7161), Medline (n=3240), Web of Science (n=4590), PubMed (n=64), Cochrane (n=28), Google Scholar (n=200) **(Figure 1).** After exclusion of duplicates, 9472 references were identified and screened.Based on title and abstracts, full texts of 389 articles were selected for detailed evaluation. Of those, 88 articles met our eligibility criteria and were therefore included in the analysis (**Table 1A-B**).

General characteristics of included studies

Detailed characteristics of the 88 included studies (86 experimental studies based on animal models or human models and 2 population-based studies) are listed in **Table 1A-B**. There were 71 experimental studies that used animal models (44 studies used tissue from mice, 21 from rats, 1 study used combined tissue from mice and rats and 5 studies used other animal tissue-ewe, rabbit, ovine,

monkey and swine), 15 experimental studies used human tissue and 2 studies were population-based studies. Of the 88 studies included in this review, 17 focused on vascular reactivity, 21 on endothelial function and healing, 1 on endothelial-derived relaxing factors, 5 on inflammation, 12 on regulation of protein/gene expression in the cardiovascular system, 1 on atherosclerosis and 31 of the included studies assessed two or more outcome measures. Among the 88 studies included in this review, 4 studies assessed the role of menopause or age on ERβ function whereas no study assessed the role of ERβ on cardiovascular disease outcomes in women as endpoint.

Figure 1. Flowchart of studies investigating the role of estrogen receptor beta in the female cardiovascular system.

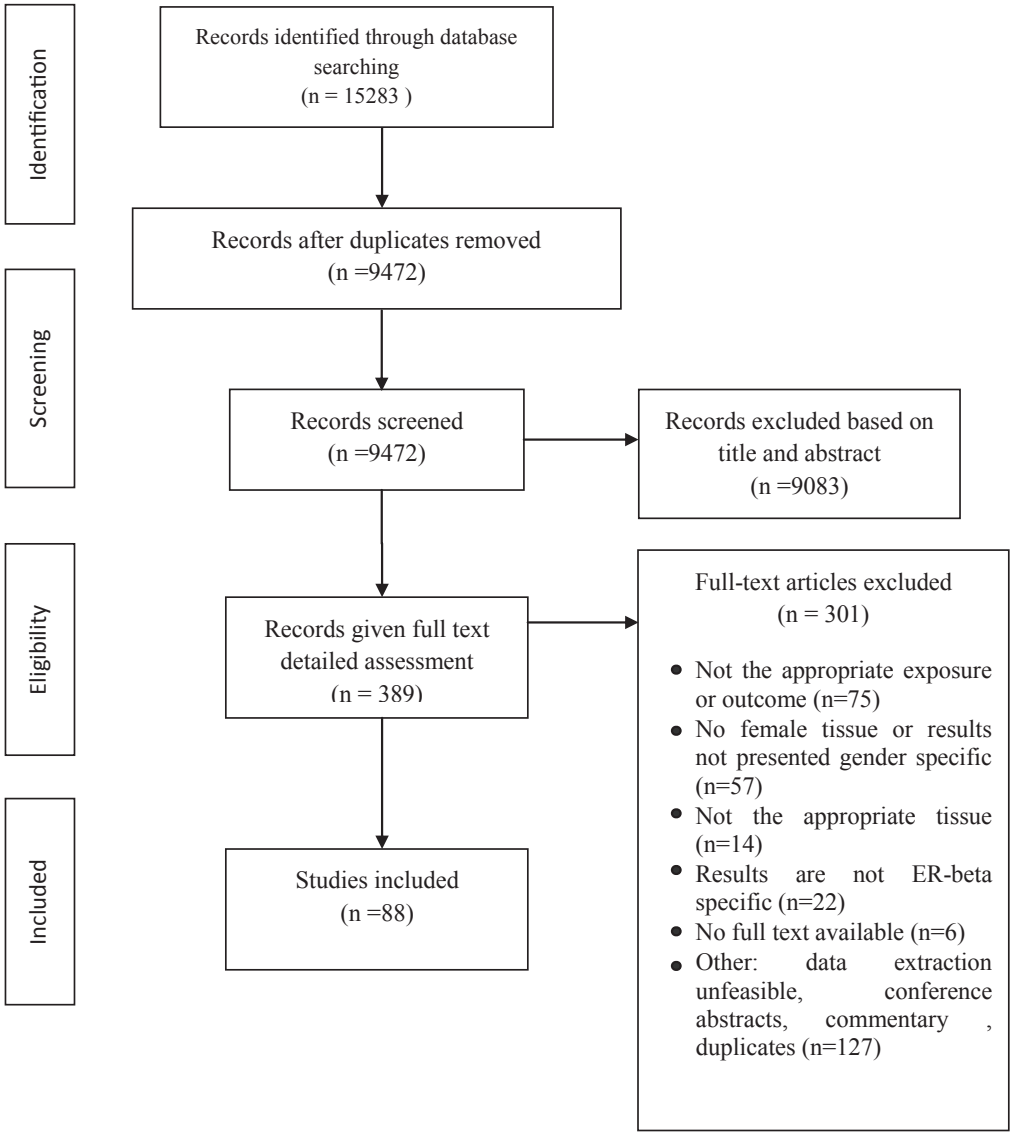


Table 1A. General Characteristics of the Animal Studies Included in this Review

No	Author	Animal Type	N	Age	Intact/ Ovariectomized	Tissue	Outcome
1	Akhayeva, 2012	Wistar Rat	ND	8 weeks	Intact & ovariectomized	Thoracic Aorta	Vascular reactivity: Vasodilatation
2	Zubair, 2005	Wistar Rat and Mouse	29	ND	Intact	Thoracic aorta and mesenteric artery	Vascular reactivity: Vasodilatation
3	Arias-Loza, 2007	Wistar Rat (Aldosterone-Salt treated)	60	12 weeks	Ovariectomized	Thoracic aorta	Vascular reactivity: Blood Pressure
4	Babiker, 2006	Mouse	34	10 weeks	Ovariectomized	Heart (Transverse aortic constriction)	Protein/Gene expression in cardiovascular system
5	Bansal, 2014	Sprague Dawley Rat	54-72	ND	Ovariectomized	Thoracic aorta	• Vascular reactivity: Vasodilatation • Inflammation/Oxidative stress
6	Bolego, 2005	Sprague Dawley Rat	15	ND	Intact & ovariectomized	Aorta and mesenteric artery	Vascular reactivity: Vasodilatation
7	Booth, 2005	New Zealand White Rabbits (with ischemia reperfusion injury)	15	ND	Intact & ovariectomized	Coronary artery (ligation)	• Ischemia reperfusion injury • Vascular reactivity: Blood Pressure
8	Bowling, 2014	Mouse	ND		10 weeks and 52 weeks	NA	Vascular smooth muscle cell (aorta) • Inflammation • Role of age
9	Broberg, 2011	Mouse	63		ND	Intact	Heart • Endothelial function and healing: angiogenesis • Inflammation
10	Brouchet, 2001	Mouse	16		4-6 weeks	Ovariectomized	Common Carotid Artery Endothelial function and healing: angiogenesis

Table 1A. General Characteristics of the Animal Studies Included in this Review (continued)

No	Author	Animal Type	N	Age	Intact/ Ovariectomized	Tissue	Outcome
11	Burek, 2010[55]	Mouse	30	12	Ovariectomized	Cerebral, cerebellar and microvascular myocardial endothelial cells	Endothelial function and healing: protein/ gene expression
12	Burek, 2014[56]	Mouse	12	12	Ovariectomized	Cerebral endothelium cells	Endothelial function and healing: protein/ gene expression
13	Chen, 2013[122]	Mouse	5	Fetus (21 days to 3 months)	Intact	Heart	Protein/Gene expression in cardiovascular system
14	Cheng, 2009[60]	Sprague Dawley Rat	11	ND	Intact	Vascular smooth muscle cell from aorta	Endothelial function and healing: • Smooth muscle cell proliferation/ apoptosis • protein/gene expression
15	Cong, 2014[90]	Mouse	40	6-8 weeks	Intact & ovariectomized	Cardiomyocyte	Protein/Gene expression in cardiovascular system
16	Cruz, 2005[27]	Mouse	25	14-22 week	Intact	Femoral artery	Vascular reactivity: Vasodilatation
17	Darblade, 2002[23]	Mouse	15	4 weeks	Ovariectomized	Thoracic aorta	• Vascular reactivity: Vasodilatation • Endothelial-derived relaxing factor
18	Douglas, 2008[123]	Mouse	12	14-22 weeks	Intact	Mesenteric artery	Vascular reactivity-Vasodilatation
19	Dworatzek, 2014[9]	Mouse	45	12 weeks	Intact	Heart and human cardiomyocyte	Protein/Gene expression in cardiovascular system
20	Flienger, 2010[10]	Mouse	60	9 weeks	Intact	Heat/ cardiomyocyte	Protein/Gene expression in cardiovascular system
21	Gabel, 2005[30]	Mouse	4	Adult	Ovariectomized	Heart	Protein/Gene expression in cardiovascular system

Table 1A. General Characteristics of the Animal Studies Included in this Review (continued)

No	Author	Animal Type	N	Age	Intact/ Ovariectomized	Tissue	Outcome
22	Gao, 2013[72]	Sprague Dawley Rat	6	Adult	Intact	Ventricular myocytes from heart	Protein/Gene expression in cardiovascular system
23	Guo, 2005[26]	Mouse	52	80.6 ± 9.7 days	Ovariectoized	Femoral and carotid artery	Vascular reactivity: Vasodilatation
24	Gurgen, 2011[11]	Mouse	5	10 week	Intact	Aorta/Heart	Vascular reactivity: Blood Pressure
25	Hamada, 2006[51]	Mouse	17		Ovariectomized	Coronary artery ligation and bone marrow transplantation	Endothelial function and healing: angiogenesis
26	Hogg, 2012[63]	Sprague Dawley Rat	ND	8 week	Intact	Smooth muscle cells from aorta	• Endothelial function and healing: angiogenesis • Endothelial-derived relaxing factor
27	Huang, 2011[89]	Sprague Dawley Rat	34	9-11 weeks	Intact & ovariectomized	Heart	Protein/Gene expression in cardiovascular system
28	Jayachandran, 2010[91]	Mouse	20	23-24 months (old)	Intact	Plasma from retro-orbita sinus plexus	Inflammation/Oxidative stress
29	Jazbutyte, 2008[40]	Mouse	71	18 weeks	Ovariectomized	Descending aorta/Heart	• Vascular reactivity: Blood Pressure • Protein/Gene expression in cardiovascular system
30	Jesmin, 2010[29]	Mouse	36	16 weeks	Intact	Heart	Endothelial function and healing: • proliferation/apoptosis • protein/gene expression
31	Jesmin, 2010[15]	Mouse	36	15 weeks	Intact	Cerebral artery	Endothelial function and healing: • proliferation/apoptosis • protein/gene expression
32	Jobe, 2010[69]	Ovine	8	120-147 days	Intact	Uterine artery endothelial cells	Inflammation

Table 1A. General Characteristics of the Animal Studies Included in this Review (continued)

No	Author	Animal Type	N	Age	Intact/ Ovariectomized	Tissue	Outcome
33	Kararigas, 2014[75]	Mouse	8	2 months	Intact	Heart (Transverse aortic constriction)	• Protein/Gene expression in cardiovascular system
34	Kararigas, 2011[76]	Mouse	16	9 week	Intact	Heart (Transverse aortic constriction)	Protein/Gene expression in cardiovascular system
35	Lee, 2014[70]	Wistar rat	27	8 weeks	ovariactomized	Left anterior descending artery	Inflammation
36	Li, 2011[44]	Sprague Dawley Rat	ND	3 months	Intact	Smooth muscle cells from aorta	Endothelial function and healing: Smooth muscle cell proliferation/apoptosis
37	Liang, 2001[50]	Mouse	ND	3 months	ovariactomized	Thoracic aorta	Endothelial function and healing: protein/gene expression
38	Lin, 2009[57]	Mouse	15	8 weeks	Ovariectomized	Heart	Protein/Gene expression in cardiovascular system
39	Liu, 2008[77]	Mouse	12	11-12 weeks	Ovariectomized	Heart	Protein/Gene expression in cardiovascular system
40	Luksha, 2005[39]	Mouse	47	14-22 weeks	Intact	Femoral arteries	Vascular reactivity: adrenergic control
41	Luksha, 2006[24]	Mouse	47	14-22 weeks	Intact	Femoral arteries	Vascular reactivity: Vasodilatation
42	Ma, 2010[124]	Mouse	7	12 weeks	Intact	Thoracic aorta	Vascular reactivity: Vasodilatation
43	Makela, 1999[61]	Wistar Rat	ND	7 day	Ovariectomized	Smooth muscle cells of Carotid artery	Endothelial function and healing: Smooth muscle cell proliferation/apoptosis
44	Mishra, 2006[125]	Rhesus Macaque Monkey	12	11-20 years old	Ovariectomized	Coronary smooth muscle cells	Endothelial function and healing: protein/gene expression

Table 1A. General Characteristics of the Animal Studies Included in this Review (continued)

No	Author	Animal Type	N	Age	Intact/ Ovariectomized	Tissue	Outcome
45	Nikolic, 2007[78]	Mouse	44		3 months	Ovariectomized	Heart (with ischemia reperfusion injury)	Protein/Gene expression in cardiovascular system
46	Nilsson, 2000[25]	Mouse	6		6-7 months	Intact	Abdominal aorta	Vascular reactivity: Vasodilatation
47	Nudelling , 2001[28]	Rat	ND		1-2 day	Intact	Cardiomyocite	Protein/Gene expression in cardiovascular system
48	O'Lone, 2007[88]	Mouse	ND		2,5-11,5 months	Ovariectomized	Total aortic RNA	Protein/Gene expression in cardiovascular system
49	Otsuki, 2003[79]	Mouse	29		11 weeks	Intact & ovariectomized	Total aortic RNA	Protein/Gene expression in cardiovascular system
50	Pedram, 2010[80]	Mouse	ND		ND	Ovariectomized	Cardiac fibroblasts	Protein/Gene expression in cardiovascular system
51	Pedram, 2013[81]	Mouse	ND		10-12 weeks	Intact & ovariectomized	Heart	Protein/Gene expression in cardiovascular system
52	Pellegrini, 2013[62]	Sprague Dawley Rat				Intact	Vascular smooth muscle cells of descending aorta	Endothelial function and healing: Smooth muscle cell proliferation/apoptosis
53	Pelzer, 2005[43]	Mouse	89		ND	Ovariectomized	Left coronary artery/ Heart	• Vascular reactivity: Blood Pressure • Protein/Gene expression in cardiovascular system
54	Queiros, 2013[82]	Mouse	ND		9-10 weeks	Intact	Murine Cardiomyocite cell line HL-1 and fibroblast	Protein/Gene expression in cardiovascular system
55	Raffetto, 2010[13]	Sprague Dawley Rat	6		12 week	Intact	Inferior vena cava	Vascular reactivity: Vasodilatation

Table 1A. General Characteristics of the Animal Studies Included in this Review (continued)

No	Author	Animal Type	N	Age	Intact/ Ovariectomized	Tissue	Outcome
56	Rayner, 2009[85]	Mouse	42-54	6 weeks	Ovariectomized	Aorta/ THP-1 human macrophages culture	Protein/Gene expression in cardiovascular system
57	Reslan, 2013[22]	Sprague Dawley Rat	ND	12 weeks	Intact	Cephalic, thoracic and abdominal arteries	Vascular reactivity: Vasodilatation
58	Schrepfer, 2006[17]	Fisher Rat	ND	ND	Ovariectomized	Abdominal aorta	• Vascular reactivity: Vasodilatation • Endothelial function and healing: proliferation/apoptosis protein/gene expression
59	Sebag, 2011[73]	Mouse	26	7-8 months	Intact & ovariectomized	Heart/Cardiac cell line	Protein/Gene expression in cardiovascular system
60	Shin, 2013[53]	Mouse	6	10-11 weeks	Ovariectomized	Transient middle cerebral artery	• Protein/Gene expression in cardiovascular system • Endothelial function and healing: protein/gene expression
61	Skavdahl, 2005[83]	Mouse (transverse aortic constriction)	49	7 weeks	Intact	Heart (Transverse aortic constriction)	• Protein/Gene expression in cardiovascular system
62	Stygar, 2007[86]	Sprague Dawley Rat	32	8 weeks	Ovariectomized	Blood from vena cava	• Protein/Gene expression in cardiovascular system
63	Traupe, 2007[16]	Swine	8-20	ND	Intact	Left anterior descending coronary artery/ endothelium	• Vascular reactivity: Vasodilatation • Endothelial-derived relaxing factor
64	Wang, 2008[84]	Mouse	20	12-20 weeks	Intact	Heart	• Protein/Gene expression in cardiovascular system • Inflammation
65	Wang, 2009[126]	Mouse	20	12-20 weeks	Intact	Heart	Protein/Gene expression in cardiovascular system

Table 1A. General Characteristics of the Animal Studies Included in this Review (continued)

No	Author	Animal Type	N	Age	Intact/ Ovariectomized	Tissue	Outcome
66	Wang, 2015[87]	Mouse	60	3 weeks	Ovariectomized	Heart (Transverse aortic constriction)	Protein/Gene expression in cardiovascular system
67	Xiang, 2010[18]	Sprague Dawley Rat	8	ND	Intact	Aorta (collected from a region 1.5 cm left of the aortic arch)	Vascular reactivity: Vasodilatation
68	Xing, 2007[67]	Sprague Dawley Rat	ND	10 weeks	Intact	Aortic smooth muscle cell	Inflammation
69	Xing, 2012[68]	Sprague Dawley Rat	ND	10 weeks	Intact	Aortic smooth muscle cell	Inflammation
70	Zhang, 2012[54]	Ewe	ND	145 days	Intact	Uterine artery endothelial cells	Endothelial function and healing: protein/gene expression
71	Zhao, 2013[127]	Mouse	ND	10 weeks	Ovariectomized	Aortic smooth muscle cell	Endothelial function and healing: protein/gene expression

Table 1B. General Characteristics of the Human Studies Included in this Review

No	Author	N	Age	Menopausal Status	Tissue	Outcome
1	Baruscotti, 2010[45]	ND	ND	ND	Endothelial progenitor cells	Endothelial function and healing: • Angiogenesis • protein/gene expression
2	Chakrabarti, 201031	ND	ND	Pre-menopausal	Umbilical vein endothelial cells	• Endothelial function and healing: protein/gene expression • Endothelial-derived relaxing factor
3	Christian, 200694	55	>18 years old	Pre and post-menopausal	Coronary artery	• Atherosclerosis

Table 1B. General Characteristics of the Human Studies Included in this Review

No	Author	N	Age	Menopausal Status	Tissue	Outcome
4	Corcoran, 2013	NA	ND	Premenopausal	Chorionic plate arteries and myometrial arteries	Vascular reactivity: Vasodilatation
5	Eid, 200735	2	23 and 44 years old	Pre-menopausal	Cutaneous vascular smooth muscle cell	Vascular Reactivity: adrenergic control
6	Giddabasappa, 201246	ND	ND	ND	Human retinal microvascular endothelial cell and a mouse model of oxygen induced retinopathy (n=18-30, intact)	Endothelial function and healing: angiogenesis
7	Greaves, 201347	NA	ND	ND	Endometrium endothelial cells, uterine myometrial microvascular endothelial cells, umbilical vein endothelial cells	Endothelial function and healing: • Angiogenesis • protein/gene expression Protein/Gene expression in cardiovascular system
8	Leung, 200748	ND	ND	Pre-menopausal	Umbilical vein endothelial cell	Endothelial function and healing: • Angiogenesis • protein/gene expression
9	Leung, 2009128	ND	ND	Pre-menopausal	Umbilical vein endothelial cell	Endothelial function and healing: • Angiogenesis • protein/gene expression
10	Nakamura, 200466	22	ND	Postmenopausal	Smooth muscles cells of abdominal aorta	Endothelial function and healing: Smooth muscle cell proliferation /apoptosis
11	Novella, 201293	68	58 ± 13	Postmenopausal	Uterine arteries	Inflammation
12	Novella, 201333	N	ND	Pre-menopausal	Umbilical vein endothelial cell	Endothelial-derived relaxing factor
13	Ortmann, 201164	ND	ND	Pre-menopausal	Smooth muscle cells from Aorta	Endothelial function and healing: • Smooth muscle cell proliferation/ apoptosis • protein/gene expression

Table 1B. General Characteristics of the Human Studies Included in this Review

No	Author	N	Age	Menopausal Status	Tissue	Outcome
14	Simoncini, 200534	ND	ND	Pre-menopausal	Umbilical vein endothelial cells	Endothelial-derived relaxing factor
15	Sitges, 200532	20	67,8 ± 8.8	Postmenopausal	Internal mammary artery	Endothelial function and healing: • protein/gene expression
16	Su, 200957	ND	ND	ND	Villous endothelial cells	Endothelial function and healing: • protein/gene expression
17	Xu, 200965	ND	ND	Pre-menopausal	Umbilical vein endothelial cells	Endothelial function and healing: Smooth muscle cell proliferation /apoptosis

Vascular reactivity

Vasoconstriction and vasodilatation

Seventeen studies focused on the role of ERβ in vasoconstriction or vasodilatation (16 animal studies and 1 human study) **(Supplemental Table 1A)**.

ERβ ligands: Eight studies (7 animal model studies and 1 human model study) showed that activation of ERβ via its agonists (diarylpropionitrile (DPN), Biochanin A and Genistein) was associated with increased relaxation of the vessels (thoracic aorta, inferior vena cava, descending coronary artery, aorta, mesenteric artery; and chorionic and myometrial arteries)[12-19], whereas two studies showed no effect of ERβ on vascular reactivity (thoracic aorta and mesenteric artery)[20,21]. Furthermore, two studies showed that the effects of ERβ in vasodilatation are tissue-dependent: one study showed that DPN did not have any effect on relaxation of cephalic and thoracic arteries but provided a significant relaxation in abdominal arteries[22] while the second study showed that the effect of DPN was more potent in the aorta than in the mesenteric artery[14].

Studies looking at the mechanism of action of ERβ agonists on vasodilatation were inconsistent. Three studies showed an endothelium-dependent effect of ERβ on vessel relaxation[16,17,19] while just one study reported that the relaxation caused by ERβ actions was largely endothelium independent[14]. Three studies showed that relaxation of the vessel due to ERβ activation was NO-dependent[12,18,19] whereas three other studies showed that vasodilatation due to ERβ activation was not dependent on NO [13,14,16]. A single study reported that the vessel relaxation induced by DPN was not mediated via the thromboxane receptor pathway[16].

Knockout mouse model: Six studies looked at the effect of ERβ gene deletion on vasodilatation and showed no consistent results. Three studies reported no effect of ERβ on endothelium-dependent relaxation in thoracic aorta, the mesenteric artery and femoral arteries[23-25]. One study reported a role of ERβ on vessel relaxation via extracellular signal-regulated kinase/ mitogen activated protein kinase (ERK/MAPK) and phosphatidylinositol-3 kinase (PI3K) activation[26]. Two studies reported a direct involvement of ERβ in mediating vasodilatation exerted by estrogen[25,27]; one study reported that ERβ may exert a hitherto unrecognised inhibitory influence on the acute dilatory response to estrogen achieved through down regulation of ERα-mediated NO release[27].

Endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS), nitric oxide synthase (NOS) and nitric oxide (NO)

Ten studies overall (6 animal model studies[15,16,23,28-30] and 4 human model studies [31-34]) focused on the role of ERβ on eNOS (6 studies), nNOS (1 study), iNOS (2 studies), NOS (1 study) and NO expression (6 studies) **(Supplemental Table 1B)**.

eNOS: Two studies using ERβ agonists [31,34] reported an increase in eNOS expression due to the activation of ERβ whereas another study[28] using ERβ antagonist showed a decrease in eNOS expression. Three knockout mouse model studies reported a decrease in eNOS expression [15,30] and phosphorylation [29] due to the deletion of the ERβ gene.

iNOS, nNOS and NOS: Activation of ERβ via an ERβ agonist did not have any effect on iNOS or nNOS expression[31] but the use of an ERβ antagonist was associated with lower iNOS expression[28]. One study examined the correlation between ERβ expression in the internal mammary artery and NOS but found none[32].

NO: Increase in NO expression with activation of ERβ via ERβ agonist was reported in two studies[16,34] whereas two other studies showed no change in NO expression[31,33]. One study[15] reported a decrease in NO production due to the deletion of ERβ gene whereas another study reported no effect [23].

Adrenergic control of arteries

ERβ ligands: One study (using human tissue) (**Supplemental Table 1B**) investigated the role of ERβ agonist in α-2C adrenoreceptors expression and reported an increased expression of these receptors[35] (α-2C adrenoreceptors are located on the vascular smooth muscle cells of certain blood vessels and binds both norepinephrine and epinephrine[36]. Its activation causes vasoconstriction of certain arteries, including the coronary artery[37,38]).

Knockout mouse model: One study examined the effect of ERβ gene deletion on adrenergic control of arteries (mice femoral tissue) and showed no effect[39] (**Supplemental Table 1B**).

Blood pressure

ERβ ligands: Three studies (animal models) (**Supplemental Table 1C**) examined the effect of ERβ agonists 8β-VE2 and DPN on blood pressure: of these, two studies showed a decrease in systolic and diastolic blood pressure in the aorta and one study showed a non-significant decrease in arterial pressure in the coronary arteries[40-42].

Knockout mouse model: There were two studies that examined the role of ERβ gene deletion on blood pressure regulation or hypertension (**Table 2 and Supplemental Table 1B**); one study reported that deletion of the ERβ gene was associated with hypertension (aorta tissue)[11] whereas the other did not report any effect on systolic blood pressure (coronary artery tissue)[43].

*Overall, the studies looking at ERβ ligands suggest that activation of ERβ via ERβ agonist may cause relaxation, and therefore might prevent the pathological effect of excessive vasoconstriction; but this may depend on the type of vessel (**Table 2**). The studies from knockout mouse models are inconsistent in this respect (**Table 2**). The included studies suggest for both an endothelium-dependent and -independent action of ERβ on vascular reactivity; but the results are contradictory on the mechanisms involved. Nevertheless, the included studies that used ERβ ligands or knockout mouse models are consistent in showing a role of ERβ on eNOS production, which might suggest that ERβ is responsible solely for eNOS-derived NO production. The possible mechanisms of action of ERβ on vascular reactivity are depicted in **Figure 1** and may include eNOS, ERK/MAPK and PI3K signalling and adrenoreceptors. In line with the dilatory effects of ERβ, the included studies (although not conclusive) suggest that reduced blood pressure may be produced by the activation of ERβ.*

Table 2: Summarizing the evidence from the studies included in this systematic review on the role of estrogen receptor on Vascular reactivity, endothelial functioning and healing and inflammation by the method used to assess the role of estrogen receptor beta function

Vasodilation			
	Increased	No effect	Decreased
ERβ agonist			
No of studies using animal tissue	8	2	0
No of studies using human tissue	1	0	0
Knockout mice			
No of studies using animal tissue		3	3
Endothelial nitric oxide synthase			
	Increased	No effect	Decreased
ERβ agonist			
No of studies using animal tissue	1*	0	0
No of studies using human tissue	2	0	0
Knockout mice			
No of studies using animal tissue	0	0	3
Nitric oxide synthase			
	Increased	No effect	Decreased
ERβ agonist			
No of studies using animal tissue	1	0	0
No of studies using human tissue	1	2	0
Knockout mice			
No of studies using animal tissue	0	1	1
Blood pressure			
	Increased	No effect	Decreased
ERβ agonist			
No of studies using animal tissue	0	0	3**
No of studies using human tissue	0	0	0
Knockout mice			
No of studies using animal tissue	1	1***	0
Angiogenesis (arteriogenesis, capillary formation/density, re-endothelialisation)			
ERβ agonist	Increased	No effect	Decreased
No of studies using animal tissue	0	0	1
No of studies using human Tissue	0	1	3
Knockout mice			
No of studies using animal tissue		3	1

Affected protein and gene expression associated with angiogenesis		
	Yes	No
ERβ agonist		
No of studies using animal tissue	3	0
No of studies using human Tissue	3	1
Knockout mice		
No of studies using animal tissue	4	0
Affected smooth muscle cell proliferation		
	Yes	No
ERβ agonist		
No of studies using animal tissue	3	1
No of studies using human Tissue	0	1
Knockout mice		
No of studies using animal tissue	1	1****
Affected smooth muscle cell apoptosis and migration		
	Yes	No
ERβ agonist		
No of studies using animal tissue	4	0
No of studies using human Tissue	1	0
Knockout mice		
No of studies using animal tissue	1	0
Affected inflammation		
	Yes	No
ERβ agonist		
No of studies using animal tissue	2	3
No of studies using human Tissue	1	0
Knockout mice		
No of studies using animal tissue	0	0

*One study used ERβ antagonist and showed that deactivation of ERβ decreased eNOS expression.

**One study showed a non-significant decrease in arterial pressure in the coronary artery with the use of ERβ agonist

*** One study examined only systolic blood pressure

****One study investigated whether the effect of estradiol on smooth muscle cells proliferation was mediated via estrogen receptor beta and found that estrogen receptor beta does not mediate the effects of estradiol on smooth muscle cells proliferation.

Angiogenesis

Arteriogenesis, capillary formation/density, re-endothelialization

ERβ ligands: Five studies (1 animal model study[44] and 4 human model studies[45-48]) focused on the role of ERβ agonists (DPN, Ginsenoside Rb1) in angiogenesis. Four studies[44,46-48] showed anti-angiogenic effects of the ERβ agonist whereas one study [45] reported no effect on capillary formation **(Supplemental Table 2A)**.

Knockout mouse model: Three studies (animal models) investigated the role of the ERβ gene deletion on angiogenesis. Two of these studies[49,50] reported no effect and one study [51] reported a reduction in capillary density after the deletion of the ERβ gene **(Supplemental Table 2A)**. Also, one study[52] showed that ERβ did not mediate the effect of estrogen in angiogenesis **(Supplemental Table 2A)**.

Protein and gene expression associated with angiogenesis

ERβ ligands: Seven studies (4 animal model studies [17,53-55] and 3 human model studies[45,47,48]) focused on the role of ERβ agonists on the expression of proteins or genes associated with angiogenesis **(Table 2 and Supplemental Table 2B)**. One study[17] reported that activation of ERβ was associated with attenuation of vascular cell adhesion protein-1 (VCAM-1) and tumor necrosis alpha (TNF-α) on the rat abdominal aorta whereas no effect was shown on intracellular adhesion molecule (ICAM). In contrast, activation of ERβ affected the expression of ICAM in human tissue[47]. One study demonstrated that ERβ activation was associated with decrease of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1α (which has been shown to increase VEGF expression) in the murine middle cerebral artery[53]. Furthermore, one study reported that ERβ activation was associated with increased claudin-5 protein and mRNA content[55]. Two studies examining the role of ERβ activation on s-nitrosylation (SNO) showed contradictory results: one study [54] in animal tissue reported increase in SNO protein expression whereas the other study [45] using human tissue reported no effect. In tissue of premenopausal women, activation of ERβ was associated with activation of pigment epithelium-derived factor (PEDF)[48]. One study looked at genomic effects in human endothelial cells from the endometrium, myometrium and placenta. It reported that ERβ activation affected gene expression for 22 out of 99 genes associated with angiogenesis (**Growth factors:** GRWANG, CXL12, ECGF1, EDIL3, FGF2, GRN, MDK, PDGFB, TGFA, VEGF; **Inhibitors**: CXCL10, FLT1, IFNB1, SEMA3F, SERPINF1; **Markers**: CEACAM1, HEY1, **Target**: ENPP2, NRP2, TIE1, **Lymph-angiogenesis**: FIGF, FOXC2) [47]. However, this study also showed that there were very few ERβ-dependent genes common to more than one of the three cell lines used and that several of the shared ERβ-dependent genes identified were regulated in opposite directions in endometrium and myometrium cell lines[47].

Knockout mouse model: Four studies (3 animal model studies[15,56] and 1 human model [57]) looked at the effect of ERβ gene deletion on protein or gene expression associated with angiogenesis **(Supplemental Table 2B)**. Two animal studies showed that deletion of the ERβ gene was associated with a decrease in both VEGF and its kinase insert domain receptors (KDR); and with a reduction

in fibroblast growth factor-2 (FGF-2)[15]. One animal study showed an interaction between ERβ and specificity factor-1 (Sp1) site of the claudin-5 receptor, which consequently induced an increase in claudin-5 expression [56]. One human study showed an inverse association between ERβ gene deletion in cyclo-oxygenase-2 (COX-2) RNA and its protein expression[57].

*Collectively, these studies provide evidence for anti-angiogenic effects of ERβ agonists; whereas knock-out mouse models reported inconsistent results on the role of ERβ in angiogenesis (**Table 2**). Both studies using ERβ ligands or knockout mouse models were consistent in showing ERβ to affect VEGF, claudin-5 and FGF-2 in protein and gene expression level; all of which are associated with angiogenesis. However, similar to the findings on vascular reactivity, the role of ERβ on angiogenesis may be vessel-specific[47]. Angiogenesis is the physiological process of formation of new blood vessels from pre-existing ones and therefore may be a target for combating diseases characterized by either poor vascularization (e.g., ischemic diseases) or abnormal vasculature (e.g., atherosclerosis). ERβ antagonists, by enhancing capillary growth and increasing blood perfusion in ischemic tissue, can be a novel treatment in cardiac and peripheral ischemia. On the other hand, ERβ agonists may be used in the treatment of atherosclerosis. Since neovascularization is a key factor in the development of advanced plaques and is considered a risk factor for plaque rupture[58], ERβ agonists may be used to improve plaque stability and reduce plaque progression by inhibiting angiogenesis. In fact, targeting ERβ with agonists has been reported to be valuable in the treatment of cancer by supressing vascularization[59].*

Vascular smooth muscle cell proliferation, apoptosis, migration and inflammation

Vascular smooth muscle cell proliferation, apoptosis, migration

Eight studies overall (6 animal model [15,17,29,44,60-63] and 2 human model studies[64,65]) examined the role of ERβ on smooth muscle cell (SMC) proliferation (6 studies), apoptosis (2 studies) and migration (3 studies) **(Supplemental Table 2C)**.

ERβ ligands: An inhibitory effect of ERβ agonists on SMC proliferation were reported in 3 studies[17,44,61] whereas two studies reported no effect[60,64]. Two studies focused on the role of ERβ agonists on cell apoptosis and showed enhancement of this process with activation of ERβ [60,65]. Three studies reported an anti-migratory effect by ERβ [17,61,62]. Two studies showed this effect was mediated via ERβ playing a role on the phosphorylation of p38 [60,65] whereas two other studies reported no effect of ERβ on ERK phosphorylation in the mediation of anti-migration[60,64].

Knockout mouse model: One study showed that ERβ may repress proliferation of SMC by means other than NO signalling[63] while another study reported ERβ promoting the activation of pro-apoptotic molecules[15]. One study in humans focused on the role of ERβ on estradiol signaling in SMC proliferation and found no effect[66].

Vascular smooth muscle cell and inflammation

ERβ ligands: Two animal studies[67,68] focused on the role of ERβ agonist DPN on inflammation in vascular endothelium and SMC and reported an inhibitory effect of ERβ on TNF-α induced

inflammation **(Supplemental Table 3)**. Another study using human endothelial cells from the endometrium, myometrium and placenta found that ERβ activation affected gene expression in 14 of the 92 genes associated with inflammation (**Lipase:** PCLB2; **Kinase**: MAPK3; **Prostaglandin metabolism**: PTGS1, PTGS2, TBXAS1; **Factor**: NFKB1; **GPCR Receptor**: LTB4R, PTAFR, PTGIR, TBXA2R: **Interleukin receptor**: IL1RL1; **Adhesion Molecule**: ICAM and ITGB2)[47]. One study reported no effect of ERβ antagonist 2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a] pyrimidin-3-yl]phenol (PHTPP) on prostacyclin [69]**(Supplemental Table 3)**. Similarly, although not endothelium/SMC specific, two studies examining the role of DPN in the thoracic aorta and left anterior descending artery reported no effect of this agonist on TNF-α, interleukin-1 beta, C-reactive protein and p-cofilin levels [20,70]**(Supplemental Table 3)**. No studies were found to use a knockout mouse model to examine the role of ERβ in inflammation.

*Overall, the results from studies using either ERβ-ligands or knockout mouse models are consistent in supporting anti-migratory and pro-apoptotic effects of ERβ in SMC. Although no consistency was found on the role of ERβ in SMC proliferation and inflammation (**Table 2**), the included studies suggest a protective effect of ERβ against TNFα-induced inflammation. SMC migration and apoptosis are key factors in certain vascular diseases. Restenosis, a common adverse event of endovascular procedures to treat the vascular damage from atherosclerosis, is characterized by the sequence of inflammation and SMC proliferation and migration; whereas the role of SMC apoptosis in restenosis remains unclear[58,71]. Also, intimal SMC apoptosis in advanced atherosclerotic plaques may promote plaque rupture while medial apoptosis may promote aneurism formation[71]. Furthermore, SMC apoptosis may be directly pro-inflammatory by triggering the release of chemoattractant and cytokines from inflammatory cells[71]. Therefore, targeting ERβ through ERβ agonists may be valuable in the treatment of restenosis. Similarly, the use of ERβ antagonists may prove to be useful in the treatment of advanced atherosclerotic lesions and aneurisms.*

Proteins and genes in the cardiovascular system

Twenty seven studies (in animal models) focused on the role of ERβ signalling in cardiovascular targets **(Supplemental Table 4)**. Among them, 21 studies[10,30,43,49,57,60,72-87] investigated the molecular mechanism of ERβ cardioprotective actions against left ventricular hypertrophy and ischemia-reperfusion injury. ERβ in the heart tissue was shown to play multiple roles such as: calcium homeostasis[43,72,73,81], regulation of lipoprotein lipase activity[77,83] and regulation of fatty acid metabolism gene expression[30], S-nitrosylation protein expression[57], activation of the functional estrogen response element of lipocalin-type prostanglandin D synthase[79], bone morphogenetic protein-4 expression[87], levels of ventricular atrial natriuretic peptide[43,74] and heat shock protein 27 as well as expression of 70 proteins[78,82,85]. Also, ERβ was reported to play a role in cardiac fibrosis and inflammation via genomic and non-genomic actions[10,49,76,78,80,82,84,86]. Furthermore, ERβ was shown to exert cardioprotective effects through regulation of apoptotic proteins and gene expression[9,10,60,74,75,78], modulation of genes involved in mitochondrial modelling[9,76,88], cytoskeletal regulation[75,78,86,88], oxidative stress[76,88], glucose and insulin pathways [75,78] and NO synthesis[78]. Two studies reported no role of ERβ on stromal cell-derived factor-1 production[89] or on corticotrophin-releasing hormone receptor 2[90] whereas one study, by using plasma from retro-orbital sinus plexus,

showed that deficiency of ERβ may affect the energy metabolism of platelets resulting in greater production of thrombogenic microvesicles in the circulation [91]. In contrast to the study performed in hearts [78], in which ERβ selective agonist upregulated 122 genes and down-regulated 23 genes, a gene array study comparing gene expression in ovariectomized mice aorta treated with estrogen showed that 90% of the genes showing an estrogen-mediated decrease, were ERβ dependent[88].

Role of age and menopause status

Ovariectomy was reported to decrease relaxant response of ERβ-agonist DPN in the rat thoracic aorta; and induction of both ovariectomy and diabetes had synergistic effects on decreasing this relaxant response[12]. Also, two studies (one animal [92] and one human study[93]) focused on the role of ERβ in inflammation as a function of age and menopausal status. One study showed that estrogen through ERβ may inhibit inflammation in young but not in old mice[92], whereas another human study showed an increased linear trend in ERβ expression with ageing and years since menopause, as well as a positive correlation between expression of ERβ and inflammatory markers such as interleukin-8 and TNF-α[93]. In contrast, one (cross-sectional) population-based study found that ERβ correlated with coronary calcification and atherosclerosis and tended to be greater correlated in women who reported no use of hormone replacement therapy. However, this correlation did not differ by menopausal status[94].

Collectively, these results support the notion that the actions of ERβ may depend on age, menopausal status and diabetes.

DISCUSSION

Based on the findings from 88 studies, the present review shows that ERβ has multiple functions that may contribute to protect the cardiovascular system in women. However, the majority of studies have been conducted in animals and none has evaluated the role of ERB function and treatment-related strategies on cardiovascular outcomes. The cardiovascular effects of ERβ signaling could occur through multiple mechanisms including a direct effect on the vessel wall. ERβ signaling may cause vasodilation (**Figure 2**), and therefore prevent the pathological effect of excessive vasoconstriction by regulating the bioavailability of nitric oxide, altering membrane ionic permeability in vascular smooth muscle cells and by regulating adrenergic control of the arteries. Also, ERβ may have angiogenic properties that may protect against vascular injury by inhibiting vascular smooth muscle cell migration. (**Figure 2**). Furthermore, the data from this systematic review suggest that ERβ may have anti-inflammatory properties mainly by suppressing TNF-α-induced inflammation. Supporting the role of ERβ in vasodilatation, wound healing, and inflammation, several population-based studies have reported an association between ERβ gene polymorphisms with blood pressure, inflammatory markers (e.g., TNF-α, fibrinogen) and venous ulceration in women[95-101]. Furthermore, various studies have shown tht activation of ERβ has resulted in suppression of IL-6 promoter activity in human endothelial cancer cells in vitro and to play a role in regulation of neutrophil infiltration following acute injury[102,103].

Figure 2. Possible mechanism of estrogen receptor beta actions in endothelial and vascular smooth muscles cells. eNOS, endothelial nitric oxide synthase; ERβ, estrogen receptor beta; ERK, extracellular signal-regulated kinase; MAPK, mitogen activated protein kinase; NO, nitric oxide; PI3K, phosphatidylinositol-3 kinase.

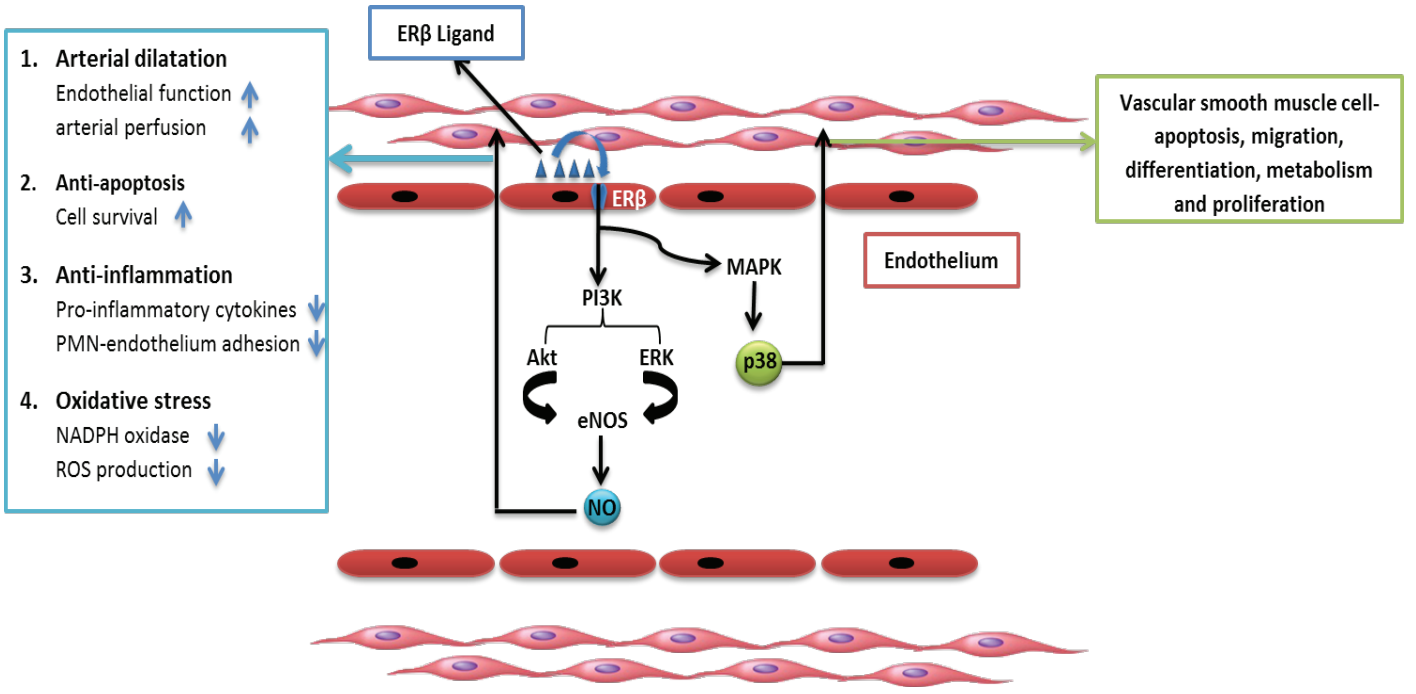
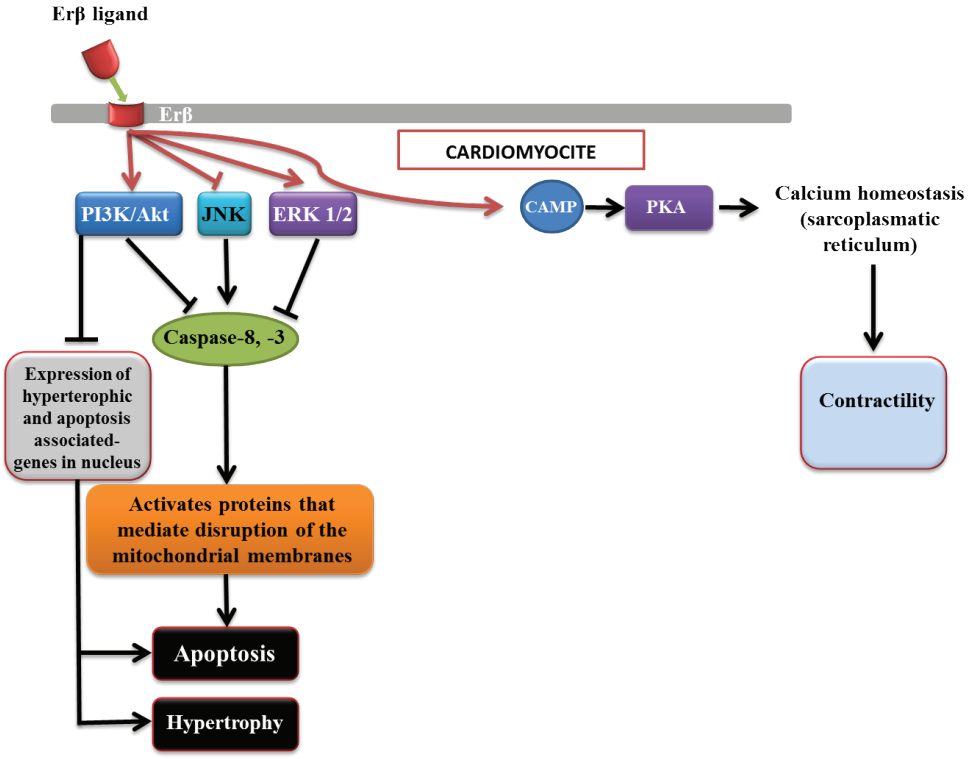


Figure 3. Possible mechanisms of estrogen receptor beta actions in cardiomyocytes. CAMP, cyclic adenosine monophosphate; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol-3 kinase, PKA, protein kinase A.



Vessel-specific actions of ERβ

The vascular effects of ERβ may be vessel-specific, and therefore ERβ may play a role in site-specific vascular diseases such as carotid intimal hyperplasia, peripheral artery disease and thoracic and abdominal aortic aneurysm as well as in localized atherosclerotic disease. Targeting ERβ with selective receptor agonists or antagonists may be of additional benefit in the treatment of localized vascular disease. For instance, specific ERβ agonists might be a therapeutic option in occlusive artery disease, whereas ERβ antagonist may be useful in vascular disease characterized by arterial wall distention and aneurysm. The combination of ERβ ligands with specific targeting or drug delivery techniques such as drug-eluting stents and perivascular gel might be effective in modulating the activity of ERβ in a specific blood vessel without altering other vessels in the systemic circulation.

ERβ in left ventricular hypertrophy and ischemic vascular diseases

The selected studies included in this systematic review indicate a possible protective effect of ERβ signaling against left ventricular hypertrophy and ischemia/reperfusion injury via genomic and non-genomic pathways (**Figure 3**). ERβ may play a role in the phosphorylation of p38-MAPK, activation of atrial natriuretic factor, inflammatory response, cardiac fibrosis, nitric oxide synthesis and in the regulation of several genes involved in cell metabolism and mitochondrial modelling. Additionally, ERβ was shown to exert the role of gatekeeper in the genomic response of the heart to pressure overload, and may thus play a protective role against cardiac hypertrophy[76]. These results are in line with the population-based studies showing an association between polymorphisms of ERβ gene and homocysteine, nitric oxide, ATP-binding cassette transporter A1 gene expression, left ventricular mass and wall thickness and with the risk of myocardial infarction in women[104-108]. Based on these experimental data from animal and human models, subsequent clinical studies are warranted to test if the results of these experimental investigations can be reproduced in interventions conducted in humans and to explore whether isoform-selective ERβ ligands could constitute an effective target for cardiovascular disease treatment. ERβ might confer the cardioprotective effects of estrogen while avoiding its specific adverse effects on other tissues such as those of the uterus and breast. In fact, targeting ERβ with agonists has be shown to be valuable in the treatment of certain types of cancers, diminishing several aspects of the malignant process such as proliferation, vascularization, and dissemination[109-111].

Role of age, menopause and diabetes in the actions of ERβ

Despite the evidence put forth, the effect of age on the regional distribution of ERs in the heart vessels remains unclear. The expression of ERβ nay be vulnerable to the effects of age not only in the cardiovascular system but also in other systems such as the nervous system[112]. It has been suggested that the change in the expression of ERs with ageing could be due to variation in methylation-associated inactivation of ER gene promoter in various types of tissues, including those of the cardiovascular system[113,114]. Similarly, loss of locally synthesized estrogen mainly after menopause can affect the expression of ERβ[112]. As also noted in this systematic review, ERβ-mediated vascular actions may be affected by age, menopause status and diabetes. In line with this, it has been reported that diabetes undermines estrogen control of inducible NO synthase function in aortic smooth muscle cells through overexpression of ERβ[115]. In this respect, studying the differences in ERβ distribution and activity in the cardiovascular system of young versus old animals, in intact versus ovariectomized females or in non-diabetic and diabetes-induced females could provide further evidence on the potential usefulness of a specific estrogen receptor agonist/antagonist to be used during and after menopause. Furthermore, it may be of interest to investigate the mechanisms regulating the localization of ERβ; for instance, future research may focus on whether age-related alterations in NO signaling can contribute to the age-related differences in ERβ expression and signaling.

Animal models: ERβ ligands versus knockout mouse models

Notably, the protective role of ERβ in the cardiovascular system was consistently shown in studies using ERβ agonist/antagonist whereas some discrepancies with the results of the studies using knockout mice were observed (**Table 2**). These differences could be related to the animal model used and/or different tissue examined. In knockout mice studies, since ERβ has a lower affinity for estradiol, estradiol concentrations may not be sufficiently high to affect ERβ signaling. In contrast, local administration of ERβ agonists can result in more potent activation of ERβ signaling. Furthermore, the intervention at a certain time point in the knockout mice models may result in potential compensatory mechanisms which may ultimately affect ERβ signaling. Also, since the discovery of ERβ, certain mice strains have been genetically modified to delete their ERβ gene, which have shown markedly different phenotypes[116,117] This may also add to the discrepancies found in the reported results[116]. Therefore, further research is required to better elucidate the differences between studies using animal models and ERβ agonists/antagonists.

Limitations

To our knowledge, this is the first systematic review on the subject that critically appraised the literature following an *a priori* designed protocol with clearly defined inclusion and exclusion criteria. After a systemic search in major electronic databases, few narrative or expert reviews evaluating the role of ERβ in the cardiovascular system were found[8,118-120]. They were all expert or narrative reviews without a systematic search. Nevertheless, some limitations from the included studies in this review merit careful consideration. Some of the results should be interpreted with cautions. For instance, six of 15 human studies included in this review used umbilical vein endothelial cells for which the sex of the donor was not reported. [121]This may explain some of the discrepancies in the reported results. Although every effort has been made to undertake a comprehensive search of the published and unpublished literature, we cannot exclude the possibility of publication bias from underreporting of negative findings. Furthermore, a meaningful quantitative pooling of the existing data was unfeasible due to different animals and tissues used as samples in the included studies, heterogeneity in the input parameters, assumptions and the study designs.

Conclusion

In summary, this systematic review summarizes all the available evidence on the role of ERβ signaling in the female cardiovascular system through multiple mechanisms. Although these findings contribute with the generation of hypotheses and the identification of potential therapeutic targets, the majority of the studies were conducted in animals and further evaluations in humans are warranted.

Supplementary Material can be found online: http://www.maturitas.org/article/S0378-5122%2816%2930009-3/pdf

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4.3 Vasomotor Symptoms in Women and Cardiovascular Risk Markers: a Systematic Review and Meta-Analysis

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ABSTRACT

We performed a systematic review and meta-analysis of the observational or interventional studies assessing the association of vasomotor symptoms (hot flushes and night sweats) with various cardiovascular risk markers (systolic (SBP) and diastolic blood pressure (DBP), hypertension, total cholesterol, body mass index (BMI), and measures of subclinical atherosclerosis), in peri-menopausal, menopausal, or postmenopausal women. Eleven unique studies were identified with data available on 19,667 non-overlapping participants.Pooled analysis showed that women with hot flushes, compared to those without, tended to have significant higher levels of SBP (mean difference (MD): 1.95 mmHg (95%CI, 0.27 to 33.63)), and DBP (MD 1.17 mmHg (95%CI, -0.21 to 2.54)) and higher odds of having hypertension (OR: 1.18, 95%CI: 0.93 to 1.51), albeit non-significant. Similarly, women who reported night sweats compared to those who did not, had significant higher levels of SBP, (MD: 1.33 mmHg (95%CI, 0.63 to 2.03)), DBP (MD: 0.55 mmHg (95%CI, 0.19 to 0.91)), total cholesterol (MD: 0.17 mmHg (95%CI, 0.03 to 0.31)) and BMI (MD: 0.64 mmHg (95%CI, 0.47 to 0.80)).Vasomotor symptoms in women were not associated with measures of subclinical atherosclerosis. Women with vasomotor symptoms may have an unfavorable cardiovascular risk profile compared to women without vasomotor complaints.

INTRODUCTION

Menopause has been linked to increased risk of cardiovascular disease (CVD) among older women [1-3]. During their menopausal transition, women commonly report vasomotor symptoms (VMS), which typically include hot flushes along with night sweats[4]. VMS are known to impair the quality of life[5,6] principally owing to irritability, fatigue, a generally depressed mood[4,7] and disturbed sleep [8]. Up to 80% of women experience hot flashes and night sweats, mainly in the peri-menopausal and early post-menopausal period [9]. VMS remain the leading reason menopausal women seek menopause-related healthcare [8,10,11].

In addition to a decreasing quality of life in menopausal women, a growing body of evidence suggests a link between VMS and cardiovascular risk profile [12-17]. Although many studies have reported associations between VMS and adverse cardiovascular risk profile, [15,16] others either failed to show such associations [18-21] or point towards a favorable link between VMS and cardiovascular parameters [22-24]. Although the relation between VMS and cardiovascular risk is an area of active inquiry, interpretation of the often conflicting findings remains a challenge. As CVD remains the leading cause of death for women worldwide, [25-27] assessing the published evidence on VMS and cardiovascular risk markers in a systematic manner is of particular importance.

In the present study, we have synthesised all available evidence of VMS in relationship with various conventional cardiovascular risk factors such as systolic (SBP) and diastolic blood pressure (DBP), hypertension, total cholesterol, body mass index (BMI) and measures of subclinical atherosclerosis.

METHODS

Data sources, search strategy and eligibility criteria

This review was conducted using a predefined protocol and in accordance with the PRISMA and MOOSE guidelines[28,29] (**eAppendix A** and **B**). Relevant studies, published before February 12th, 2015 (date last searched) were identified by two independent authors, through electronic searches without language restriction in MEDLINE, EMBASE, and Web of Science databases. Electronic searches were supplemented by scanning reference lists of articles identified for all relevant studies (including review articles), by hand searching of relevant journals and by correspondence with study investigators and experts in the field. The computer-based searches combined terms related to the exposure (e.g., *hot flushes, night sweats*) and outcomes (e.g., *blood pressure, cholesterol, coronary artery calcification (CAC)*). Details on the search strategy are provided in **Appendix C**. Studies were sought that had reported on associations of vasomotor symptoms (defined as hot flushes and/or night sweats) with cardiovascular risk markers, including SBP, DBP, hypertension, serum total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, BMI, CAC and carotid intima-media thickness (IMT).

Study selection

Observational or interventional studies in humans with relevant data were eligible for inclusion if they reported on associations of any VMS (defined above) with cardiovascular risk factors in peri-

menopausal, menopausal, or postmenopausal women. Two independent reviewers working in pairs screened the titles and abstracts of all initially identified studies according to the selection criteria. A third reviewer was available in case of disagreements. Full texts were retrieved from studies that satisfied all selection criteria. Reference lists of selected studies and relevant reviews identified on the topic were searched for additional publications.

Data extraction

Data were extracted by two independent authors and a consensus was reached with involvement of a third. A predesigned data extraction form was used to collect relevant information. This included questions on study size; study design; baseline population; location; age at baseline; duration of follow-up (for cohort studies); reported degree of adjustment (defined as '+'when risk estimates were adjusted for age and/or sex; '++' further adjustment for established vascular risk factors (eg, age, sex, BMI, smoking status, lipids, hypertension, history of cardiometabolic disease); type of outcome and reported risk estimates (mean differences for continuous outcomes and odds ratios for categorical outcomes). Additionally, in the case of multiple publications, the most up-to-date or comprehensive publication was included.

Assessing the risk of bias

Bias within each individual study was evaluated using the validated Newcastle-Ottawa Scale[30], a semi-quantitative scale designed to evaluate the quality of nonrandomized studies. Study quality was judged by two independent reviewers 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.

Statistical analysis

The inverse variance weighted method was used to combine mean differences (for continuous outcomes) and odds ratios (for categorical outcomes) to produce pooled respective estimates using random effects models to allow for between-study heterogeneity. Heterogeneity was assessed using the Cochrane $\chi 2$ statistic and the I^2 statistic. Publication bias was evaluated through a funnel plot and Egger's test. All tests were two-tailed and p-values of 0.05 or less were considered significant. STATA release 12 (Stata Corp, College Station, Texas) was used for all statistical analyses.

RESULTS

Literature search

A total of 13,636 potentially relevant citations were retrieved from electronic searches **(Supplemental Figure 1).** After the initial screening based on titles and abstracts, 103 articles in full text were extracted for further evaluation. In the full-text assessment, 16 of these articles and 11 unique studies [12-15,31-37] met our inclusion criteria and were included in the current systematic review and meta-analysis. Of the 87 articles excluded following full text evaluation, 60 were based on unrelated exposures, 14 did not report relevant risk estimates and 13 were duplicate publications.

Table 1. General characteristics of prospective studies of cardiovascular disease outcomes included in review

Lead Author, Publication Date	Name of study	Location	Baseline survey	Baseline age range, yr	Population source	Average follow up, yr	No of total participants	No. of cases	Outcome	Covariates adjusted for
Ahto, 2007[24]	-	Finland	1991-1991	≥64	Population register	12[c]	378	21	CHD mortality	Age, marital status, social status, number of medicines.
Penninx, 1998[23]; Mendes de Leon, 1998[28]	EPESE	USA	1988	≥65	Healthcare register.	4[a]	2812	557	Incident CVD , CHD and CHD mortality	Age, sex, smoking, alcohol intake, body mass index, blood pressure, history of stroke, diabetes, cancer and physical disability.
Chandola, 2010[27]	WHII	USA	1985-1988	35-55	Healthcare register	15	3234	450	Incident CHD	No adjustment
Ferketich, 2000[25]	NHANES I	USA	1982-1984	≥30	Healthcare register	8.3	5006	187	Non-fatal CHD and CHD mortality	Age, poverty index, smoking, hypertension, diabetes and body mass index.
Gast, 2011[17]	EPOS/ WHILA	Netherlands & Sweden	1995-2000	46-64	Screening program	10.3	10,787	606	Incident CHD	Age, education, smoking, physical activity, ovariectomy, hormone therapy use, oral contraceptive use, menopausal state, BMI, SBP, DBP, total cholesterol
Sands-Lincoln, 2013[18]	WHI	USA	1993-1998	50-79	Healthcare register	10.3	86,329	7,257	CVD and incident CHD	Age, race, education, income, smoking, BMI, physical activity, alcohol intake, depression, diabetes, high BP, hyperlipidemia, comorbid conditions

Table 1. General characteristics of prospective studies of cardiovascular disease outcomes included in review (continued)

Lead Author, Publication Date	Name of study	Location	Baseline survey	Baseline age range, yr	Population source	Average follow up, yr	No of total participants	No. of cases	Outcome	Covariates adjusted for
Smoller, 2007[19]	MIMS	USA	1997-2000	51-83	Healthcare register	5.3	3,369	81	Incident CHD and Stroke.	Age, race, income, BMI, alcohol, hormone use, high cholesterol requiring medication, history of DM treatment, smoking, depression, history of AF, hypertension status, moderate to strenuous activity for longer than 20 minutes 3 or more times a week, history of MI, CABG, PTCA, stroke before questionnaire administration
Svartberg, 2009[21]	Rancho Bernado	USA	1984-1987	50-89	Population register	11.5	867	194	CHD mortality	Past or current estrogen and/or progestin use, BMI, exercise, smoking
Whooley, 1998[26]	SOF	USA	1988-1990	≥67	Healthcare register	6	7518	127	CHD mortality	Age, history of myocardial infarction, stroke, chronic obstructive pulmonary disease, hypertension, diabetes, smoking, perceived health and cognitive function.
W-Smoller, 2004[20]; Szmuilowicz 2011[22]	WHI-OS	USA	1994-1998	50-79	Healthcare register	9.7	93,676	2,557	CVD, incident CHD and Stroke.	Age, race, education, income, BMI, cholesterol, smoking, hormone therapy, physical activity, and hypertension status
Total							*213,976*	*12,037*		

[a] median

[c] total follow-up AF, atrial fibrillation; BMI, body mass index; CABG, coronary bypass graft surgery; CHD, coronary heart disease; CVD, cardiovascular disease; DM, diabetes mellitus; DBP, diastolic blood pressure; EPESE, Established Populations for Epidemiological Studies of the Elderly; EPOS, Eindhoven Perimenopausal Osteoporosis Study; MI, myocardial infarction; MIMS, Myocardial Ischemia and Migraine Study; PTCA, percutaneous transluminal coronary angioplasty; BP, systolic blood pressure; SOF, Study of Osteoporotic Fractures; WHILA, Women's Health in the Lund Area; WHI-OS, Women's Health Initiative Observational Study.

Of the 11 unique studies included in this review, 8 studies were solely cross-sectional and 2 were based solely on a prospective cohort design, whereas one remaining study published both a cross-sectional and a separate prospective cohort report.

Characteristics of the included studies

Characteristic details of the included studies are summarised in **Table 1**. Overall, data were available on 19,667 unique participants. The age of the participants at baseline across the included studies ranged from 18 to 79 years. Three studies were based in Europe, seven in North America and one in Asia-Pacific region. The year of baseline survey ranged in these studies from 1983 to 2012. Overall, seven studies recruited subjects from healthcare registers, two from clinical trial registers, one involved participants from academic institutions and one from various marketing registers. All three cohort studies were judged as high-quality, and the quality varied across the cross-sectional studies.

Vasomotor symptoms, blood pressure and hypertension

Five studies with a total of 13,195 participants reported on the association of VMS (presence of hot flushes or night sweats) with levels of SBP. Pooled analysis showed that women who experienced hot flushes or night sweats compared to those who did not, tended to have a significant higher overall level of SBP [mean difference for hot flushes: 1.95 mmHg (95% CI, 0.27 to 3.63); mean difference for night sweats: 1.33 mmHg (95% CI, 0.63 to 2.03); **Figure 1**]. The pooled analysis for DBP outcome, based on four studies and including data from 13,041 total participants, showed a non-significant increase of 0.83 mmHg (95% CI, -0.12 to 1.78) in women reporting hot flushes compared to those without, and a significant higher DBP for women experiencing night sweats compared to those who did not (mean difference: 0.55 mmHg (95% CI, 0.19 to 0.91) **(Figure 2)**.

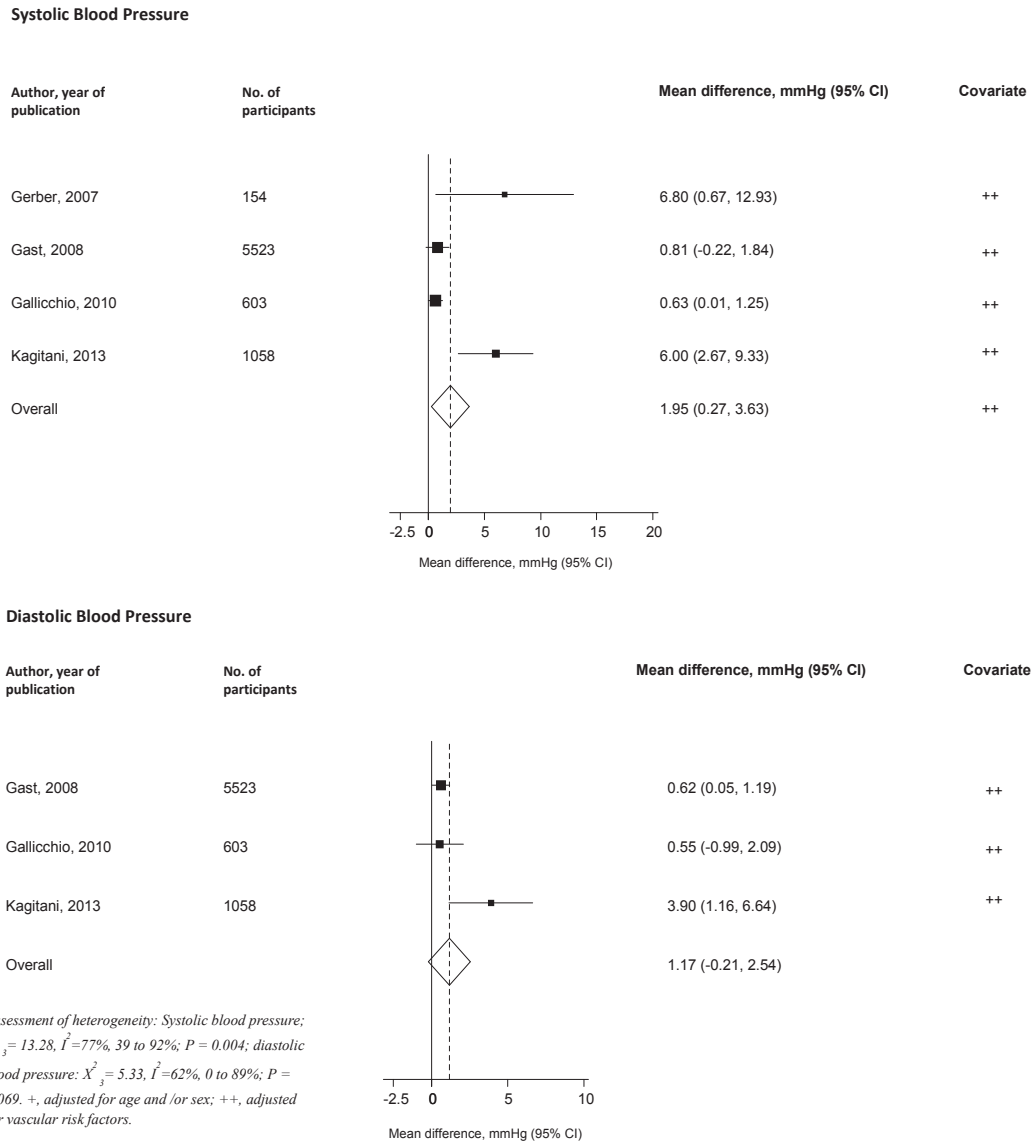
In the pooled analysis (based on available data from two studies with 6,126 total participants) women who reported to have hot flashes tended to have higher risk of having hypertension (OR: 1.18, 95% CI: 0.93 to 1.51, **Figure 3)**, albeit non-significant. The association between night sweats and hypertension was focus of only one paper which showed higher odds of having hypertension for women reporting night sweats compared to those without (OR: 1.17, 95% CI: 1.04 to 1.31)**.**[15]

Vasomotor symptoms and blood lipids

Only one study reported on the association between presence of hot flushes and serum total cholesterol and showed significant higher levels of total cholesterol in women who experienced hot flashes compared to those who did not [mean difference: 0.27 mmol/L (95% CI, 0.15 to 0.39)].[15] Similarly, there was only one study to examine the association between presence of hot flushes and LDL-cholesterol, HDL-cholesterol and triglycerides reporting higher levels of these blood lipids in women experiencing hot flushes compared to those without[35]. Relevant data on night sweats and total cholesterol were available in two European studies comprising of 11,380 total participants **(Figure 4).** In these studies, symptoms of night sweats were associated with significant increased total cholesterol level of 0.17 mmol/L (95% CI, 0.03 to 0.31) compared to women with no symptoms of night sweats**(Figure 4)**. Furthermore, two studies, comprising 9,159 total participants, examined

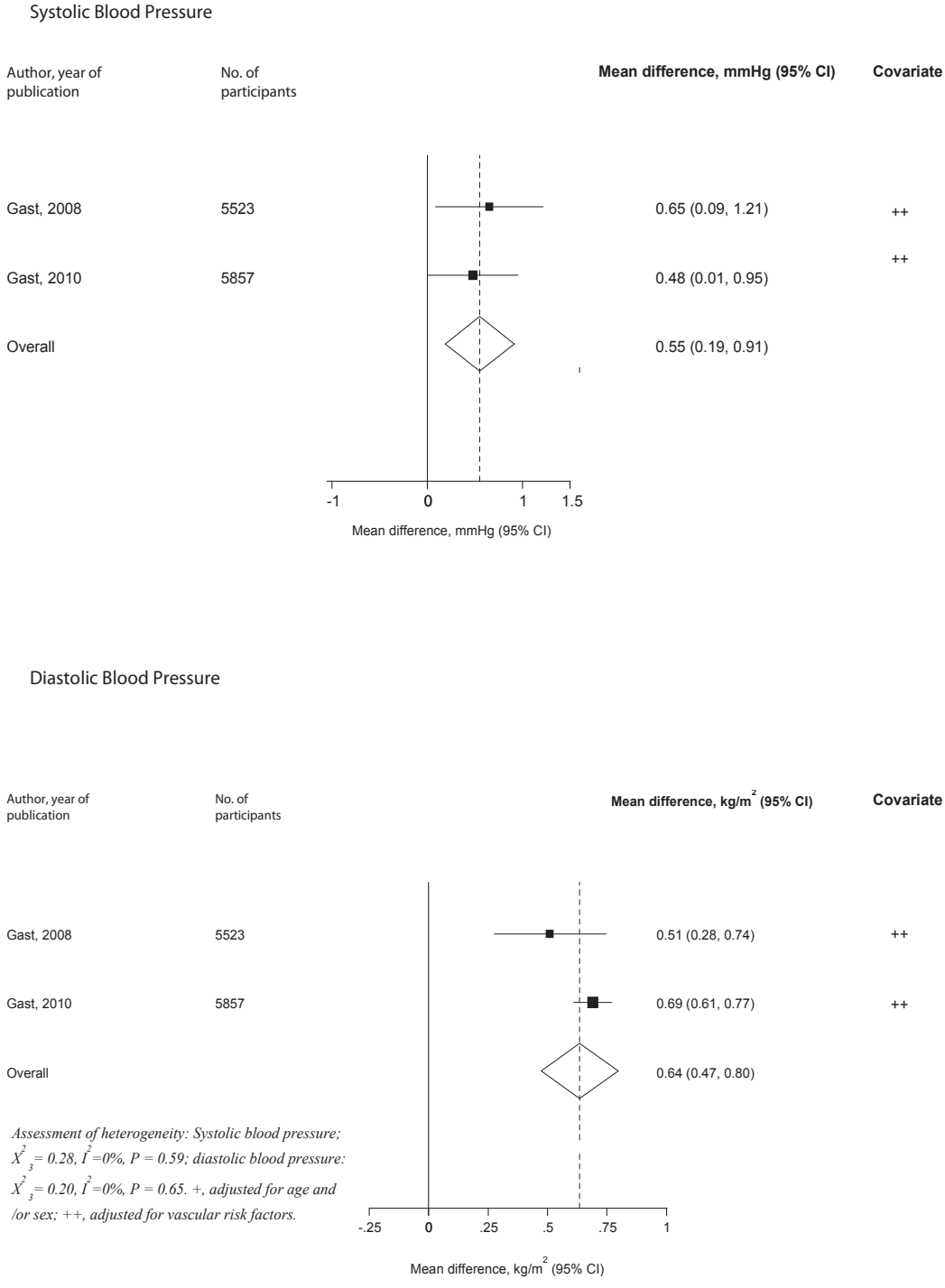
the association between night sweats and LDL-cholesterol, HDL-cholesterol and triglycerides **(Supplemental Figure 2-4)**. Women who reported night sweats compared to those who did not, had a significant higher levels of LDL-cholesterol [mean difference: 0.07 mmol/L (95% CI, 0.01 to 0.13); **(Supplemental Figure 2)**] and tended to have higher overall levels of HDL [mean difference: 0.33 mmol/L (95% CI, -0.42 to 1.08); **(Supplemental Figure 3)**] and triglycerides [mean difference: 3.09 mmol/L (95% CI, -3.01 to 9.19); **(Supplemental Figure 4)**], albeit non-significant. The severity of VMS combined (hot flushes and night sweats) in relation to blood lipids was focus of only one study, which reported positive associations between the severity of VMS, LDL and triglycerides.[38]

Figure 1. Association of hot flushes with levels of blood pressure



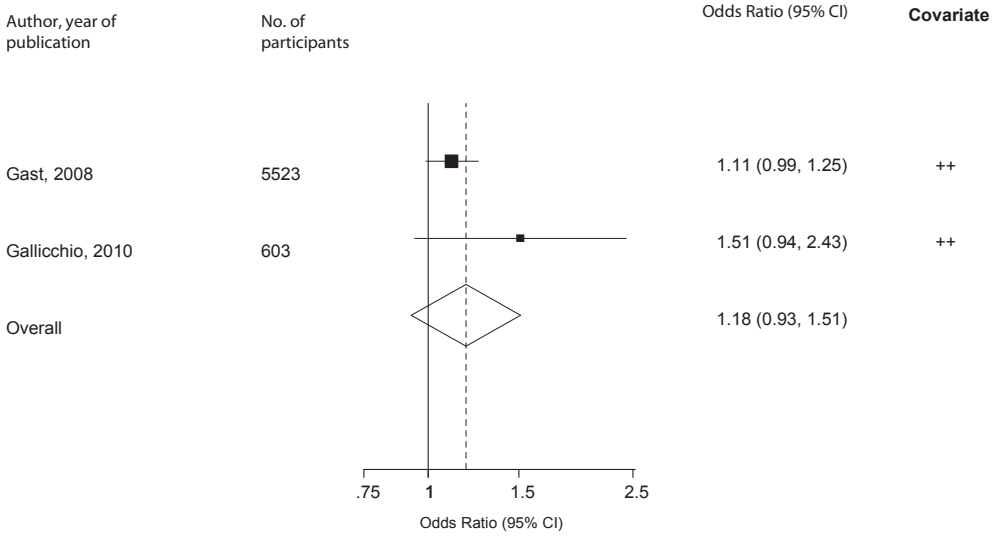
Assessment of heterogeneity: Systolic blood pressure; X^2_3 = 13.28, I^2=77%, 39 to 92%; P = 0.004; diastolic blood pressure: X^2_3 = 5.33, I^2=62%, 0 to 89%; P = 0.069. +, adjusted for age and /or sex; ++, adjusted for vascular risk factors.

Figure 2. Association of night sweats with levels of blood pressure



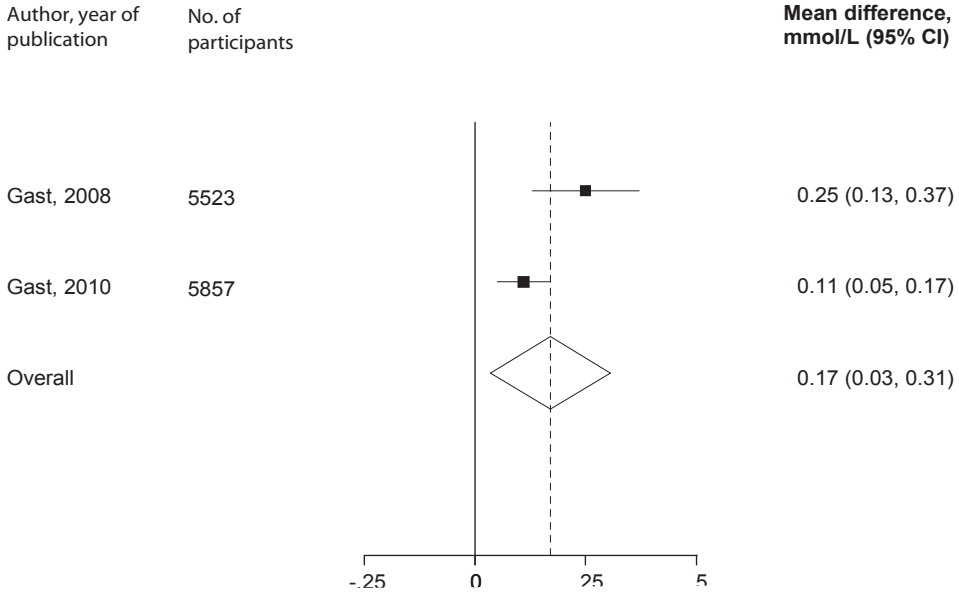
Assessment of heterogeneity: Systolic blood pressure; $X^2_3 = 0.28$, $I^2 = 0\%$, $P = 0.59$; diastolic blood pressure: $X^2_3 = 0.20$, $I^2 = 0\%$, $P = 0.65$. +, adjusted for age and /or sex; ++, adjusted for vascular risk factors.

Figure 3. Association of night sweats with hypertension



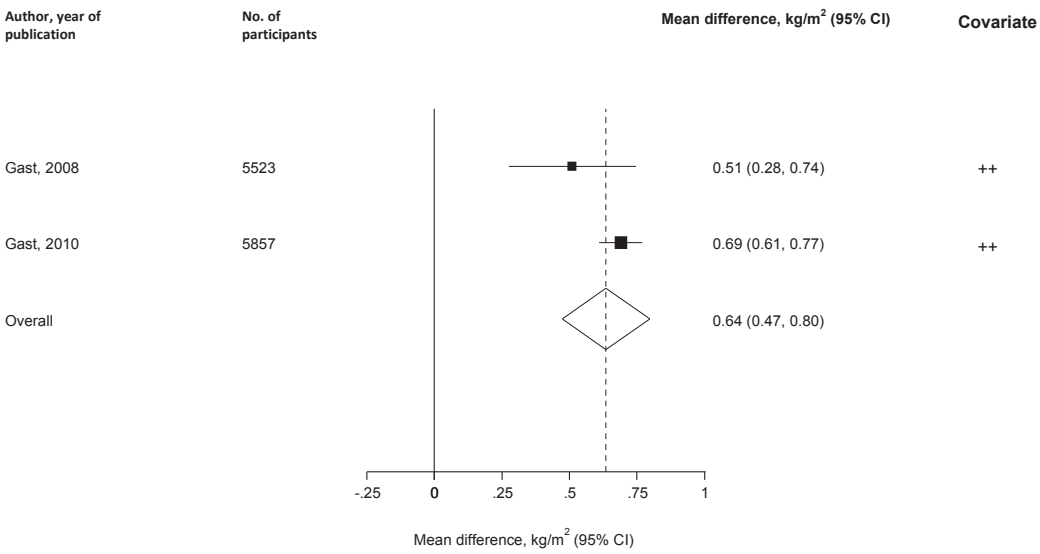
Assessment of heterogeneity, $X^2_3 = 1.52$, I^2=34%, $P = 0.13$. +, adjusted for age and /or sex; ++, adjusted for vascular risk factors.

Figure 4. Association of night sweats with circulating total cholesterol levels.



Assessment of heterogeneity, $X^2 = 4.18$, I^2=76%, 0 to 95%, P = 0.65. +, adjusted for age and /or sex; ++, adjusted for vascular risk factors.

Figure 5. Association of night sweats with body mass index



Assessment of heterogeneity, $X^2 = 2.02$, I2=50%, 0 to 87%, P = 0.16. +, adjusted for age and /or sex; ++, adjusted for vascular risk factors.

Vasomotor symptoms and BMI

One study examined the association between presence of hot flushes and BMI and showed women who experienced hot flushes to have higher BMI [mean difference: 0.62 kg/m^2 (95% CI, 0.04 to 1.19)].[15] Furthermore, in the pooled analysis of night sweats and BMI reported in two studies, there was a significant increase in the levels of body mass index of women experiencing night sweats compared to women without [mean difference: 0.64 kg/m^2 (95% CI, 0.47 to 0.80); **Figure 5**]. Furthermore, there was only one study to report on the severity of VMS combined in relation to BMI and showed that the severity of VMS was associated with higher levels of BMI.[38]

Vasomotor symptoms and surrogate CVD markers

All studies reporting on the association of hot flushes, night sweats or combined (presence, history, duration or severity) and CAC were consistent in showing no association[14,31,36,37]. One study showed no cross-sectional association between severity of hot flashes or night sweats and IMT [37]. Another study showed a positive cross-sectional association between number of days hot flashes or night sweats were experienced and IMT but no association with the progression of IMT [39]. Because of different exposure definition and outcome assessments used in these studies, pooling on CAC or IMT as outcome was not feasible.

Assessment of heterogeneity and publication bias

There was evidence of between-study heterogeneity across the studies (I^2 estimates ranged from 0% to 96% in these meta-analyses). Furthermore, visual examination of Begg's funnel plots for

studies that examined associations with levels of blood pressure **(Supplemental Figure 5)** were moderately symmetrical, therefore providing little evidence for publication bias. This was further supported by the results of Egger's test which were non-significant for both SBP, p=0.06, and DBP, p=0.45.

DISCUSSION

Findings of this review indicate that women with vasomotor symptoms including flushing and/or night sweats have an unfavorable cardiovascular risk profile compared to women without vasomotor complaints. Women experiencing VMS have significantly higher SBP and DBP, higher circulating total cholesterol levels and higher BMI than their counterparts with no symptoms. There is also a positive, albeit weak, association of VMS with hypertension. However, no signification association between VMS in women and risk of subliclinical atherosclerosis measures was observed in this analysis.

The associations of VMS with some cardiovascular risk factors observed in this quantitative review may indicate a vulnerability of women to hormonal fluctuations that occur during menopause. Women with VMS tend to have lower levels of endogenous estrogen, which have been linked to cardiovascular risk [40,41]. On the other hand, the associations reported in this review may indicate a cause-effect relationship. A possible mechanism linking women with VMS with increased risk of hypertension is a potential up-regulation in sympathetic nervous activity,[15,32,42] particularly associated with an increasing age at a higher rate than in men [32,43]. Epinephrine and Nor-epinephrine (NE) secreted from the sympathetic nervous system are possible mediators for various vascular and metabolic abnormalities including hypertension and increased cholesterol levels [44-46]. Additionally, VMS are associated with increased catecholaminergic activity and modification in calcitonin related peptide, which on the other hand are associated with increased cardiovascular risk [47,48]. Furthermore, the hypothalamic-pituitary-adrenal axis and an increase in cortisol production observed in women who present VMS may represent another mechanism linking VMS with insulin resistance and alterations in lipid metabolism [49]. The significant increase in the levels of BMI in older women with VMS, as observed in the current meta-analysis, may also be due to nor-epinephrine secretion from sympathetic nerves [15,42]. Gast et al. have replicated findings of positive associations between VMS and BMI and Cholesterol levels as well as with systolic and diastolic blood pressures[15,33] through the EPOS and WHILA studies. Recent evidence does not support the role of an unfavorable CVD risk profile as a risk factor for VMS later in life [50]. However, whether vasomotor symptoms precede the risk for cardiovascular disease or are symptomatic manifestations of underlying adverse changes in a woman's vasculature remains to be investigated.

Our findings do not support an association between VMS and CAC. Furthermore, no consistent associations were found between VMS and IMT. However, there is evidence that these associations between VMS and measures of subclinical atherosclerosis may be modified by use and duration of hormone therapy, suggesting a positive association between VMS and subclinical measures of atherosclerosis mainly in women who report use of hormone therapy and who start hormone

therapy not shortly after menopause [31,36]. Evidence of interactions by hormone replacement therapy is consistent with previous studies, reporting women with VMS and randomized to hormone replacement therapy to have the greatest CVD risk [51]. Similarly, women who start hormone therapy later after menopause, especially if they also report VMS, have an increased risk of coronary heart disease [52]. Experimental and clinical evidence suggest that the effects of estrogen on arteries vary with the stage of reproductive life, time since menopause, and extent of subclinical atherosclerosis [53]. In line with this concept, data from the Women's Health Initiative suggest that the predictive value of vasomotor symptoms for cardiovascular risk may vary with onset of vasomotor symptoms at different stages of menopause [54]. The relations between VMS, hormone replacement therapy use and cardiovascular risk are not well understood and require greater elucidation. Moreover, examining the relationships between VMS and surrogate CVD risk markers among women at different menopausal stages warrants investigation.

The strengths and limitations of the findings from this study merit careful consideration. The present analysis, involving data from nearly 20,000 individuals, is the first-ever quantitative synthesis of these associations thus far. However, as the present study was based on principally cross-sectional assessments, it does not establish causative associations between VMS and cardiovascular risk factors due to the possibility of reverse causation. Furthermore, we observed a moderate to high level of heterogeneity across the included studies (e.g., the meta-analysis for the association of VMS with blood pressure pooled the results from the studies based on either office-based or ambulatory blood pressure measurements with varying magnitude of the mean differences in SBP or DBP). In this respect, literature-based meta-analyses are influenced by the level of diversity across the studies, and do not provide detailed information necessary for the reliable assessment of any independent association. Furthermore, since the number of available studies in each analysis was generally small, it precluded our ability to investigate the sources of the observed heterogeneity by subgroup analyses involving various study-level characteristics (such as age, ethnicity, location, education level, menopausal status and parity), While individual studies attempted to adjust for these variables, the levels of adjustment was inconsistent across the studies.

Nonetheless, our findings may have some important implications. First, they indicate a potential link between VMS and several cardiovascular risk factors. Second, these relations seem to be in line with available prospective evidence that show an association between VMS and clinical cardiovascular outcomes [16,54]. Finally, they stimulate future research using large-scale studies with individual-level data to further reliably quantify these associations in greater detail and to identify potential mediators (or confounders) in associations of VMS with disease risk.

In conclusion, findings of this review indicate that women with menopausal complaints including flushing and/or night sweats may have an unfavorable cardiovascular risk profile compared to women without vasomotor complaints. However, the available studies are generally limited and somewhat diverse – highlighting the need for further larger-scale investigations to reinforce these findings.

Supplementary Material can be found online: http://www.sciencedirect.com/science/article/pii/S0378512215006702

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4.4 Association of Vasomotor and Other Menopausal Symptoms with Risk of Cardiovascular Disease: a Systematic Review and Meta-Analysis

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ABSTRACT

Importance: Vasomotor symptoms (hot flushes and night sweats) and other symptoms, including depression, anxiety and panic attacks, are commonly experienced by menopausal women and have been associated with an unfavourable cardiovascular risk profile.

Objective: To investigate whether presence of menopausal symptoms is associated with the development of cardiovascular disease (CVD).

Data Sources: Medical databases (Medline, EMBASE and Web of Science) until February 17th, 2015.

Study Selection: Observational cohort studies or randomised intervention studies were eligible for inclusion if they followed participants prospectively (at least 1 year of follow-up), and reported relevant estimates on the association of any vasomotor symptoms, or other menopausal symptoms, with risk of CVD, coronary heart disease (CHD), or stroke in perimenopausal, menopausal, or postmenopausal women.

Data Extraction and Synthesis: Data were extracted by two independent reviewers using a pre-designed data collection form. Separate pooled relative risks (RRs) for age and non-established cardiovascular risk factors (e.g., education, ethnicity) adjusted data and for established cardiovascular risk factors and potential mediators-adjusted data (e.g., smoking, body mass index, hypertension) were calculated.

Main Outcomes: Composite CVD, CHD and stroke.

Results: Out of 9,987 initially identified references, ten studies were selected, including 213,976 women with a total of 10,037 cardiovascular disease outcomes. The age and non-established cardiovascular risk factors adjusted RRs) [95% confidence intervals] for development of CHD, Stroke and CVD comparing women with and without any menopausal symptoms were 1.34 [1.13-1.58], 1.30 [0.99 -1.70], 1.48 [1.21-1.80] respectively, and the corresponding RRs adjusted for cardiovascular risk factors and potential mediators were 1.18 [1.03-1.35], 1.08 [0.89 -1.32], 1.29 [0.98-1.71]. However, these analyses were limited by potential unmeasured confounding and the small number of studies on this topic.

Conclusion: Presence of vasomotor symptoms and other menopausal symptoms are generally associated with an increased risk of cardiovascular disease, which is mainly explained by cardiovascular risk factors.

Relevance: The findings from this study might indicate the potential usefulness of menopausal symptoms in assessing cardiovascular disease risk among women.

INTRODUCTION

Hot flushes (also known as hot flashes) along with night sweats are collectively known as vasomotor symptoms (VMS). These well-known symptoms of the menopause are commonly experienced by menopausal women[1]. 50.3%[2] to 82.1%[3] of menopausal women experience VMS, and the intensity and duration of these symptoms also vary[3]. In addition to VMS, menopausal women often experience a range of other symptoms including anxiety, depression, irritability, fatigue, decreased libido, insomnia, and panic attacks, among others[4-7].

Menopausal transition represents a critical period in women's lives that marks an increase in cardiovascular disease (CVD) risk. Traditional CVD risk factors, such as reduced glucose tolerance, increased blood pressure and endothelial dysfunction [8,9], tend to become very present during menopausal transition and post-menopausal years which can partly explain the augmentation in CVD risk among women. The natural decline in oestrogen levels during menopause has been associated with the increase in CVD risk among post-menopausal women [10]. Similarly, VMS are postulated to originate from changes in brain neurotransmitters and instability in the hypothalamic thermoregulatory center brought about by decline in oestrogen levels [11].

Oestrogen supplementation therefore has been considered as the most effective treatment for these symptoms [12]. However, it remains unclear whether presence of menopausal symptoms is associated with the development of CVD[13]. Since menopausal symptoms can vary greatly between women, they might help identify women at greater CVD risk that could benefit from effective preventative strategies.

In the present study, we aimed to synthesise all available observational evidence of cardiovascular disease risk in relation to either VMS or non-vasomotor symptoms of menopause, including depression, insomnia, and panic attacks, to determine the association between VMS and non-vasomotor symptoms of menopause with risk of cardiovascular disease.

METHODS

Data Sources, Search Strategy and Eligibility Criteria

This review was conducted in accordance with the PRISMA[14] and MOOSE[15] guidelines (**eAppendix 1** and **2**). Two independent authors, in duplication, sought studies published before February 17, 2015 (date last searched) using Medline, EMBASE and Web of Science databases. The computer-based searches combined terms related to the exposure (eg, *hot flashes, night sweats*) and outcomes (eg, *cardiovascular disease, coronary disease*), without any language restriction. Details on the search strategy are provided in **eAppendix 3**. Studies were sought that had reported on associations of vasomotor symptoms (defined as hot flashes and/or night sweats), or menopausal symptoms such as depression, insomnia, or panic attacks, with vascular outcomes defined as composite CVD (combination of CHD and stroke), fatal or non-fatal coronary heart disease (CHD), or stroke.

CHD events included myocardial infarction, coronary artery bypass graft, ischemic heart disease or sudden cardiac death if caused by myocardial infarction and CHD deaths. Stroke included both haemorrhagic and ischemic cerebrovascular events.

Study Selection

Observational cohort studies or randomised intervention studies were eligible for inclusion if they followed participants prospectively (at least 1 year of follow-up), and reported relevant estimates on the association of any vasomotor symptoms, or other menopausal symptoms (defined above), with risk of composite CVD, fatal or non-fatal CHD, or stroke in perimenopausal, menopausal, or postmenopausal women. Two independent reviewers, working in pairs, screened the titles and abstracts of all initially identified studies according to the selection criteria. In case of disagreement, decision was reached through consensus or consultation with a third independent author. Full texts were retrieved from studies that satisfied all selection criteria.

Data Extraction

Data were extracted by two independent authors. A predesigned data extraction form was used to collect relevant information. This included questions on study size; study design; baseline population; location; age at baseline; duration of follow-up; reported degree of adjustment (defined as '+' when RRs were adjusted for age and non-established cardiovascular risk factors (e.g., education, income, ethnicity) and "++"further adjustment for established vascular risk factors and potential mediators (e.g., body mass index, smoking status, lipids, hypertension); type and numbers of vascular outcomes and reported RRs. Additionally, in the case of multiple publications, the most up-to-date or comprehensive information was included.

Assessing the Risk of Bias

Bias within each individual study was evaluated using the validated Newcastle-Ottawa Scale, a semi-quantitative scale designed to evaluate the quality of nonrandomized studies[16]. Study quality was judged 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.

Data Synthesis and Analysis

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. Separate summary estimates were obtained for age and potential cardiovascular risk factors adjusted data (e.g., education, income, ethnicity) and for established risk factors and potential mediators-adjusted data (e.g., body mass index, smoking status, lipids, hypertension). Heterogeneity was assessed using the Cochrane $\chi 2$ statistic and the I2 statistic. Publication bias was evaluated through a funnel plot and Egger's test. All tests were two-tailed and p-values of 0.05 or less were considered significant. STATA release 13 (Stata Corp, College Station, Texas) was used for all statistical analyses.

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

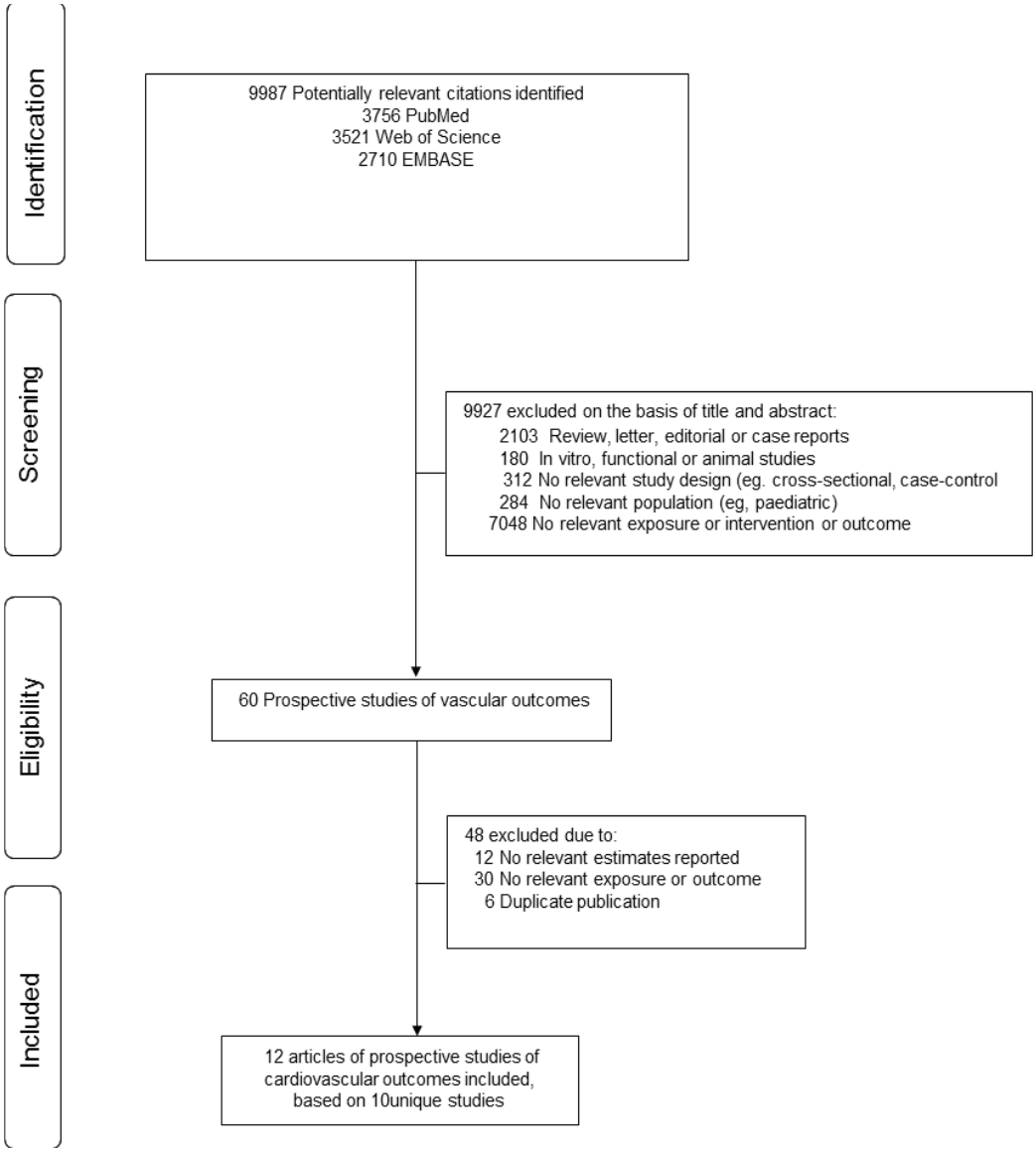


Table 1. General characteristics of prospective studies of cardiovascular disease outcomes included in review

Lead Author, Publication Date	Name of study	Location	Baseline survey	Baseline age range, yr	Population source	Average follow up, yr	No of total participants	No. of cases	Outcome	Covariates adjusted for
Ahto, 2007[24]	-	Finland	1991-1991	≥64	Population register	12[c]	378	21	CHD mortality	Age, marital status, social status, number of medicines.
Penninx, 1998[23]; Mendes de Leon, 1998[28]	EPESE	USA	1988	≥65	Healthcare register.	4[a]	2812	557	Incident CVD , CHD and CHD mortality	Age, sex, smoking, alcohol intake, body mass index, blood pressure, history of stroke, diabetes, cancer and physical disability.
Chandola, 2010[27]	WHII	USA	1985-1988	35-55	Healthcare register	15	3234	450	Incident CHD	No adjustment
Ferketich, 2000[25]	NHANES I	USA	1982-1984	≥30	Healthcare register	8.3	5006	187	Non-fatal CHD and CHD mortality	Age, poverty index, smoking, hypertension, diabetes and body mass index.
Gast, 2011[17]	EPOS/ WHILA	Netherlands & Sweden	1995-2000	46-64	Screening program	10.3	10,787	606	Incident CHD	Age, education, smoking, physical activity, ovariectomy, hormone therapy use, oral contraceptive use, menopausal state, BMI, SBP, DBP, total cholesterol
Sands-Lincoln, 2013[18]	WHI	USA	1993-1998	50-79	Healthcare register	10.3	86,329	7,257	CVD and incident CHD	Age, race, education, income, smoking, BMI, physical activity, alcohol intake, depression, diabetes, high BP, hyperlipidemia, comorbid conditions

Table 1. General characteristics of prospective studies of cardiovascular disease outcomes included in review (continued)

Lead Author, Publication Date	Name of study	Location	Baseline survey	Baseline age range, yr	Population source	Average follow up, yr	No of total participants	No. of cases	Outcome	Covariates adjusted for
Smoller, 2007[19]	MIMS	USA	1997-2000	51-83	Healthcare register	5.3	3,369	81	Incident CHD and Stroke.	Age, race, income, BMI, alcohol, hormone use, high cholesterol requiring medication, history of DM treatment, smoking, depression, history of AF, hypertension status, moderate to strenuous activity for longer than 20 minutes 3 or more times a week, history of MI, CABG, PTCA, stroke before questionnaire administration
Svartberg, 2009[21]	Rancho Bernado	USA	1984-1987	50-89	Population register	11.5	867	194	CHD mortality	Past or current estrogen and/ or progestin use, BMI, exercise, smoking
Whooley, 1998[26]	SOF	USA	1988-1990	≥67	Healthcare register	6	7518	127	CHD mortality	Age, history of myocardial infarction, stroke, chronic obstructive pulmonary disease, hypertension, diabetes, smoking, perceived health and cognitive function.
W-Smoller, 2004[20]; Szmuilowicz 2011[22]	WHI-OS	USA	1994-1998	50-79	Healthcare register	9.7	93,676	2,557	CVD, incident CHD and Stroke.	Age, race, education, income, BMI, cholesterol, smoking, hormone therapy, physical activity, and hypertension status
Total							*213,976*	*12,037*		

[a] median

[c] total follow-up

AF, atrial fibrillation; BMI, body mass index; CABG, coronary bypass graft surgery; CHD, coronary heart disease; CVD, cardiovascular disease; DM, diabetes mellitus; DBP, diastolic blood pressure; EPESE, Established Populations for Epidemiological Studies of the Elderly; EPOS, Eindhoven Perimenopausal Osteoporosis Study; MI, myocardial infarction; MIMS, Myocardial Ischemia and Migraine Study; PTCA, percutaneous transluminal coronary angioplasty; BP, systolic blood pressure; SOF, Study of Osteoporotic Fractures; WHILA, Women's Health in the Lund Area; WHI-OS, Women's Health Initiative Observational Study.

RESULTS

The search strategy identified 9,954 unique citations. Following initial screening based on titles and abstracts, 54 articles were retrieved and evaluated further. Of these articles, 48 were excluded for reasons shown in **Figure 1**. The remaining 12 articles[17-28] (based on 10 distinct studies) were included in the review and meta-analysis (**Table 1**). Consequently, 213,976 individuals were included within the meta-analysis, with a total of 10,037 CVD outcomes. The average follow-up ranged from 5.3 to 15 years (**Table 1**). Eight studies included women from the USA, whilst the remaining study included Dutch, Swedish and Finish participants. The age of participants ranged from 30 to 89 years. Three studies assessed VMS whereas the rest of the included studies assessed other menopause symptoms; 4 studies assessed depression, 2 sleep disturbance and insomnia, and 1 study panic attacks (**eTable 1**). All available studies were prospective cohorts in design which were judged as medium or high-quality studies, with few potential source of bias coming from participant selection (**Table 2**).

Association of Menopausal Symptoms and CHD

*Menopausal symptoms, either VMS or other symptoms, in relation to risk of developing CHD were reported in ten prospective observational studies, three of which assessed VMS and seven assessed other menopausal symptoms. Pooled RRs for subsequent development of CHD, adjusted for age along with other potential cardiovascular risk factors, were 1.34 (95%CI: 1.13 -1.58) when comparing women with and without VMS (70,814 participants, 1,860 events), and 1.48 (95% CI: 1.20-1.83) when comparing women with and without non-VMS (190,503 participants, 6,789 events) (***Figures 2***). Whilst the pooled RR, adjusted for age and other potential cardiovascular risk factors, comparing women with and without any menopausal symptoms, using data from 261,317 women with 8,649 CHD outcomes, was 1.34 (95%CI: 1.13-1.58) (***Figures 2***). Further adjustment for established cardiovascular risk factors and potential mediators attenuated but did not abolish the associations of VMS (RR=1.28; 95%CI=1.08; 1.52) and of any menopausal symptoms (RR=1.18; 95%CI=1.03; 1.35) with CHD. In contrast, there was no significant association between non-VMS and the risk of CHD after adjustment for established cardiovascular risk factors and potential mediators (RR=1.14; 95%CI=0.98; 1.34) (***Figure 2***). There was evidence of between-study heterogeneity for non-VMS and any for menopausal symptom analyses (I^2=92% (95%CI: 86%, 96%) and χ^2: p<0.001 for non-vasomotor symptoms and I^2=91% (95%CI:85%, 95%) and χ^2: p<0.001 for all menopausal symptoms). There was no evidence of heterogeneity for the VMS analysis (I^2 =35% and χ^2: p=0.21 for VMS) (***Figure 2***). Restricting the CHD outcome to fatal CHD events only, no associations were found between any menopausal symptoms, VMS or non-VMS, with fatal CHD in both models (***eFigure 1***).*

Association of Menopausal Symptoms and Stroke

Menopausal symptoms in relation to subsequent stroke risk were reported in three prospective observational studies; VMS was evaluated by one study and two studies assessed non-VMS. From

the single study evaluating stroke risk in women with and without VMS, the age and potential risk factors- adjusted RR was 1.43 (95% CI: 1.07-1.92) (60,027 participants, 1226 stroke events) (**Figure 3**). Age and potential cardiovascular risk factors- adjusted summary estimate of the RR was 1.34 (95%CI: 0.73-2.46) when comparing women with and without non-VMS (97,045 participants, 504 stroke events) (**Figure 3**). The pooled RR for subsequent stroke, adjusted for age and potential cardiovascular risk factors, was 1.30 (95% CI: 0.99-1.70) when comparing the presence and absence of any menopausal symptoms (157,072 participants, 1,727 stroke events) (**Figure 3**). After multiple adjustments for cardiovascular risk factors and potential mediators, the overall summery estimates of the RRs decreased to 1.14 ((95% CI: 0.82-1.59), 1.19 (95% CI: 0.68-2.11) and 1.08 (95% CI: 0.89-1.32) for VMS, non-VMS and any menopausal symptoms respectively (**Figure 3**). There were some evidence of between study heterogeneity, however, in either meta-analysis: I^2=51% and χ^2: p=0.15 for non-vasomotor symptoms and I^2=39% and χ^2: p=0.20 for all menopausal symptoms.

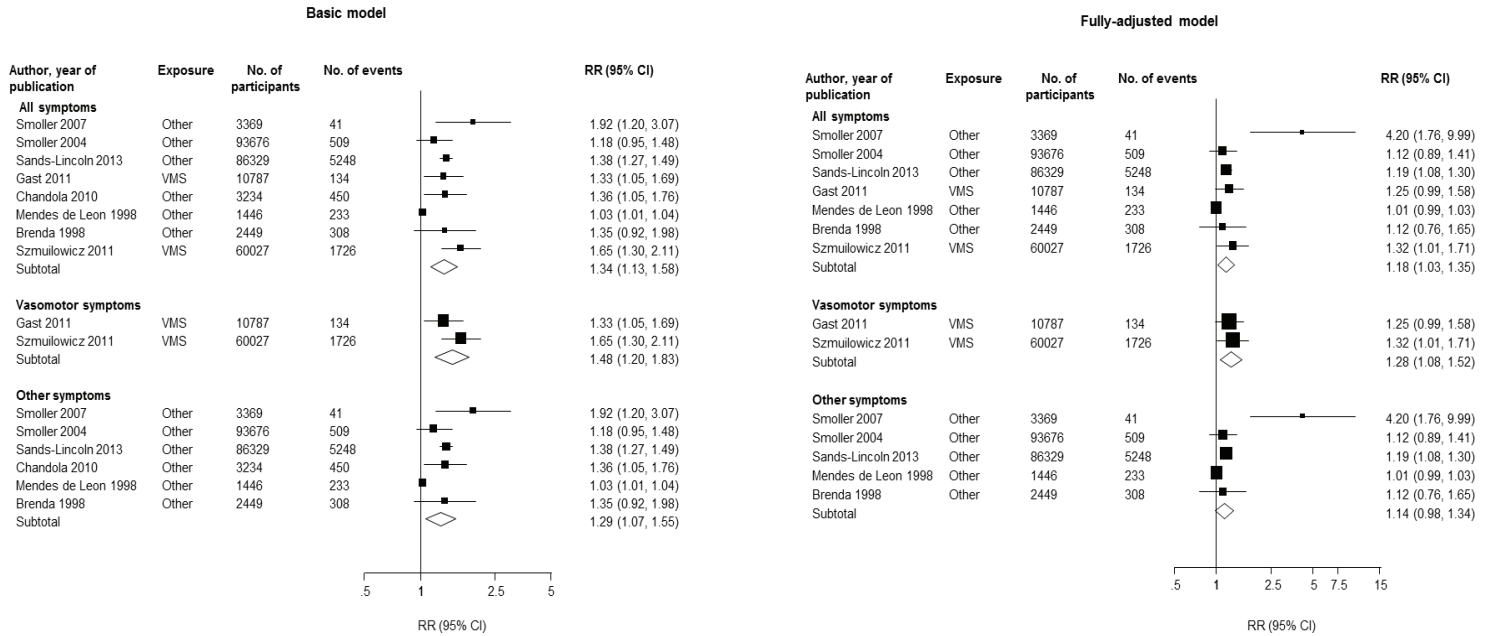
Association of Menopausal Symptoms and CVD

Four cohort studies reported menopausal symptoms in relation to subsequent risk of combined CVD outcomes. All of these studies included American participants. Only one study examined the association between VMS and the risk of CVD and showed a RR adjusted for age and potential risk factors of 1.54 (1.27-1.86) (60,027 participants, 2,812 CVD events) for women experiencing VMS compared to women who did not (Figure 4). Pooled RRs for subsequent development of CVD, adjusted for age along with other potential established cardiovascular risk factors was 1.51 (95% CI: 1.12-2.02) (92,147 participants, CVD 7,758 events) when comparing women with and without non-VMS (**Figure 4**). The pooled RR for CVD, adjusted for age but not for conventional cardiovascular risk factors, comparing women with and without any menopausal symptoms was 1.48 (95%CI: 1.21-1.80) (152,174 participants with 10,570 CVD events, **Figure 4**). Multiple adjustment for established cardiovascular risk factors attenuated the overall RRs: 1.23 (95% CI: 1.00-1.52) for VMS, 1.40 (95% CI: 0.88 -2.25) for non-VMS and 1.29 (95% CI: 0.98-1.71) for any menopausal symptom (**Figure 4**). There was evidence of between-study heterogeneity in these meta-analysis: I^2 =72% (95%CI: 4%, 92%) and χ^2: p=0.045 for non-VMS; I^2=7% (95%CI: 12%, 92%) and I^2=70% (95%CI: 13%, 90%) and χ^2: p=0.02 for all menopausal symptoms combined.

Publication Bias

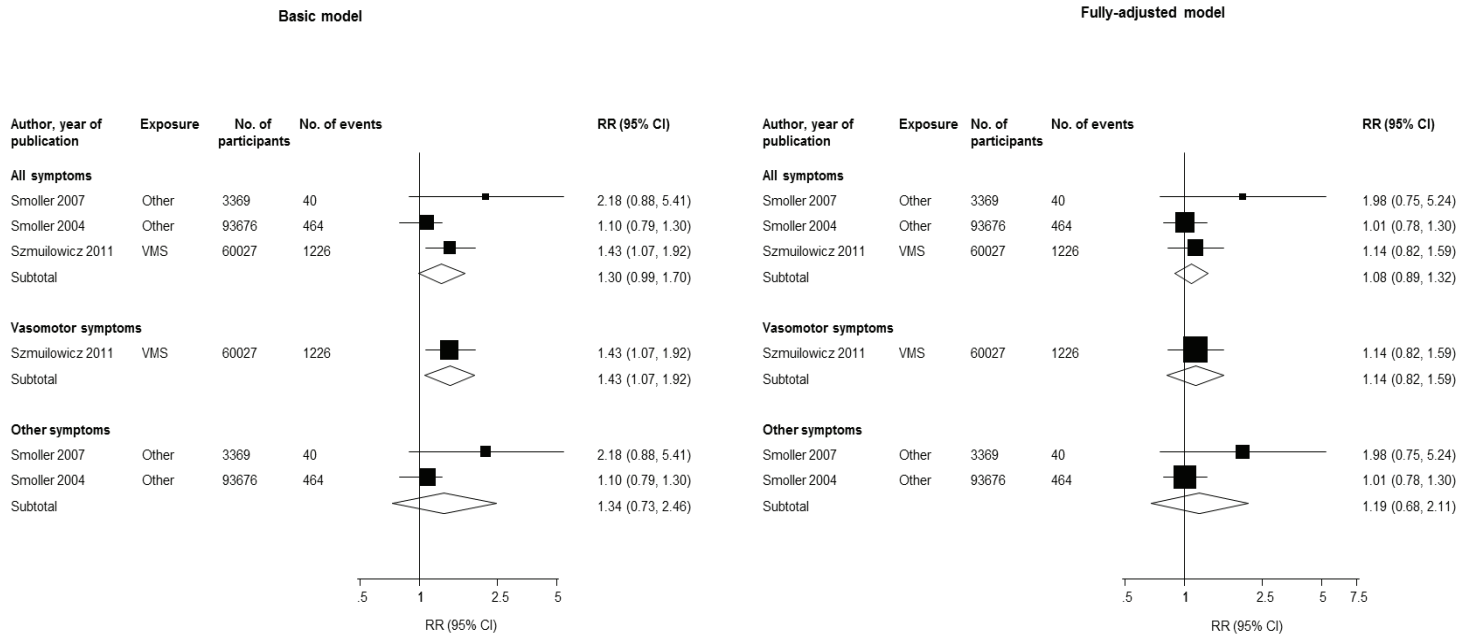
Under visual examination, Begg's funnel plots for studies assessing the risk of CHD (**eFigure 2**) or combined cardiovascular outcomes (**eFigure 2**) were not symmetrical, and therefore, providing evidence for publication bias. This was further supported by the results of Egger's test which were significant for both CHD and composite CVD, in particular for the established cardiovascular risk factors and potential mediators-adjusted data (**eFigure 2)**. No evidence of publication bias was observed for fatal CHD analysis, either graphically from the funnel plot (**eFigure 2)** or quantitatively (P=0.60 for Egger's test asymmetry).

Figure 2. Relative risks of coronary heart disease associated with menopausal symptoms



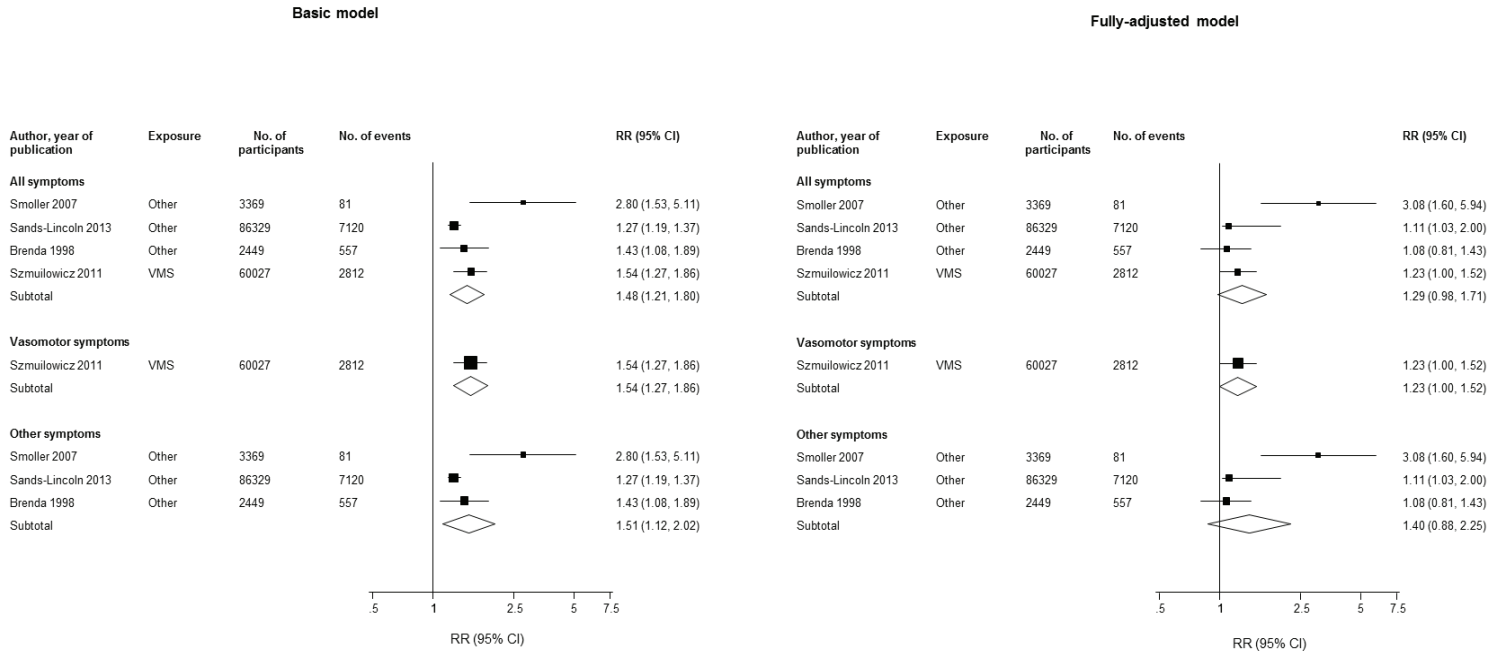
Basic model: adjusted for age and non-established cardiovascular risk factors; Fully-adjusted model, adjusted for established cardiovascular risk factors and potential mediators. VMS, vasomotor symptoms; vasomotor symptoms include hot flashes and /or night sweats; All, includes vasomotor and other menopausal symptoms; Other, includes menopausal symptoms such as depression, insomnia, and panic attacks. Assessment of heterogeneity for basic model: All symptoms, $X^2_7 = 81.22$, I^2=91%, 85 to 95%; $P < 0.001$; vasomotor symptoms , $X^2_1 = 1.55$, I^2=35%; $P = 0.213$; other symptoms, $X^2_5 = 63.48$, I^2=92%, 86 to 96%; $P < 0.001$. Assessment of heterogeneity for fully-adjusted model: All symptoms, $X^2_6 = 29.20$, I^2=79%, 58 to 90%; $P < 0.001$; vasomotor symptoms , $X^2_1 = 0.09$, I^2=0%; $P = 0.762$; other symptoms, $X^2_4 = 22.63$, I^2=82%, 59 to 92%; $P < 0.001$

Figure 3. Relative risks of stroke associated with menopausal symptoms



Basic model: adjusted for age and non-established cardiovascular risk factors; Fully-adjusted model, adjusted for established cardiovascular risk factors and potential mediators. VMS, vasomotor symptoms; vasomotor symptoms include hot flashes and /or night sweats; All, includes vasomotor and other menopausal symptoms; Other, includes menopausal symptoms such as depression, insomnia, and panic attacks. Assessment of heterogeneity for basic model: All symptoms, X^2_2= 3.26, I^2=39%, 0 to 81%; P = 0.196; other symptoms, X^2_1= 2.03, I^2=51%, 0 to 87%; P = 0.154. Assessment of heterogeneity for full-adjusted model: All symptoms, X^2_2= 1.86, I^2=0%, 0 to 90%; P = 0.395; other symptoms, X^2_1= 1.72, I^2=42%; P = 0.189

Figure 4. Relative risks of cardiovascular disease associated with menopausal symptoms



Basic model: adjusted for age and non-established cardiovascular risk factors; Fully-adjusted model, adjusted for established cardiovascular risk factors and potential mediators. VMS, vasomotor symptoms; vasomotor symptoms include hot flashes and /or night sweats; All, includes vasomotor and other menopausal symptoms; Other, includes menopausal symptoms such as depression, insomnia, and panic attacks. Assessment of heterogeneity for basic model: All symptoms, $X^2_3 = 9.95$, I^2=70%, 13 to 90%; $P = 0.019$; other symptoms, $X^2_2 = 7.05$, I^2=72%, 4 to 92%; $P = 0.045$. Assessment of heterogeneity for full-adjusted model: All symptoms, $X^2_3 = 8.68$, I^2=65%, 0 to 88%; $P = 0.034$; other symptoms, $X^2_2 = 8.67$, I^2=77%, 25 to 93%; $P = 0.013$

Table 2: Assessment of study quality through the Newcastle-Ottawa Scale

Lead Author, Publication Date	Selection	Comparability	Outcome
W-Smoller, 2004; Szmuilowicz 2011	**	**	***
Smoller, 2007	**	**	**
Svartberg, 2009	**	*	***
Gast, 2011	***	**	***
Sands-Lincoln, 2013	***	**	***
Penninx, 1998, Mendes de Leon, 1998	***	**	**
Ahto, 2007	**	*	***
Ferketich, 2000	**	**	***
Whooley, 1998	**	*	***
Chandola 2010	**		***

DISCUSSION

This is the first meta-analysis investigating the association between the presence of menopausal symptoms and subsequent development of cardiovascular disease. Overall we found that VMS and other menopausal symptoms are associated with increased risk of CHD, stroke or CVD. While the associations observed for menopausal symptoms with stroke and overall CVD could be explained, at least in part, by established cardiovascular risk factors; the association between menopausal symptoms and CHD was only partially attenuated, however, remained significant once corrected for cardiovascular risk factors and potential mediators.

Our study showed that VMS are associated with an increased risk of CVD, which was attenuated after adjustment for cardiovascular risk factors. These results are in line with the notion that menopausal symptoms may reflect an increased risk of CVD[13]. As well as other menopausal symptoms, VMS might serve as a CVD risk marker, as women with VMS may have a higher prevalence of other CVD risk factors [9,13,29,30], with conventional CVD risk factors (e.g., hypertension and total cholesterol) being possible mediators in the association between VMS and CVD. Although epidemiological studies such as those included in this review cannot provide causal evidence, some previous findings indicate that menopausal women with VMS have a higher prevalence of CVD risk factors including higher blood pressure and cholesterol levels [9,29].

On the other hand, mental health disorders, including depression, anxiety and panic attacks are already known to be CVD risk factors in both men and women of any age [31-33]. Short and long sleep duration, indicative of poor physical and mental health, has also been associated with CVD in the general population [34]. These different risk factors may work in tandem, as for instance depression has been implicated in the association between sleep quality and CVD [35]. It has also been suggested that these symptoms affect CVD risk through established risk factors such as raised BMI [36,37].

Moreover, depression and anxiety may result in autonomic activation, leading to increased blood pressure and heart rate and decreased endothelial function [38,39] or through affecting coagulation, by increasing platelet activity and plasma viscosity among others [38]. The sympathetic nervous activation following the panic attacks could also mediate an increased CVD risk [40].

Any results regarding the association between menopausal symptoms and CVD should be cautiously interpreted, as women with symptoms are more likely to commence hormone therapy than women without symptoms. Hormone therapy is taken to ameliorate menopausal symptoms, but might adversely affect the development of CVD [8]. Although several of the included studies adjusted the associations for hormone therapy use, the possibility of residual confounding cannot be entirely eliminated. However, the most common reason for using hormone therapy is VMS [41]; therefore if there was residual confounding by hormone therapy use, an association with VMS over and above other menopausal symptoms would be expected.

Our results might indicate the potential usefulness of menopausal symptoms in assessing CVD risk among women. As up to 20% of CHD events in women occur in the absence of conventional risk factors [42], better risk assessment among women is warranted. The magnitude of the observed association between menopausal symptoms and CVD (RR of 1.54) is similar to the estimates reported for other female-specific CVD risk factors such as gestational diabetes[43] and pregnancy-induced hypertension[44], which have recently been implicated for CVD risk classification among women by the American Heart Association[45]. Considering this potential, subsequent studies to further evaluate these associations are warranted.

This review underscores a number of gaps in the literature concerning CVD risk and menopausal symptoms. For instance (i) not all available studies assessed the severity or duration of menopausal symptoms, (ii) interaction with the age at which women experienced these symptoms was not consistently evaluated, and (iii) VMS were often grouped together, impeding the assessment of individual symptoms. Experiencing menopausal symptoms in isolation or in combination might carry different cardiovascular risk[17,21].

This review involved aggregate data from 213,613 women from the general population with over 10,000 cardiovascular events. A number of limitations, however, need to be considered. A number of studies did not adjust for several CVD risk factors; including alcohol consumption[17,20-22] and socioeconomic status[17,19-23]. Moreover, the results might also be subject to residual confounding. Due to using self-reported exposure and not specifying whether women with CVD at baseline were excluded, most studies failed to score well on selection as indicated by the Newcastle-Ottawa Scale. Although there is a risk of reverse-causation, one study excluded events that occurred in the first 6 months of follow-up, which had no material effect on the association[20]. Between-study heterogeneity was found in a number of analyses, which may have resulted from different levels of adjustment and differing methods of case ascertainment (ranging from death certificates to self-report validated by record linkage). Also, another potential source of bias include possible misclassification of exposure. The age-range of women included in our analysis varies across the included studies, with three studies including women age 64 and above, and therefore the

symptoms can occur beyond the menopausal transition. However, two of these studies examined the association of menopausal symptoms with CHD-mortality, and therefore, may have had little or no impact on the other results reported in this systematic review. Furthermore, VMS and other menopausal symptoms were assessed by questionnaire which is subject to some measurement error. However, because the outcome in all studies included in this systematic review was assessed prospectively, the subjective measure of menopausal symptoms 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. Also, a major limitation of the studies included in our systematic review is the reliance on retrospective self-report of menopausal symptoms, particularly of VMS, which are subject to fault memory and reporting bias. Assessing VMS physiologically with, for example, an ambulatory hot flash monitor to measure skin conductance, would be an alternative objective measure of vasomotor symptoms. Also, the studies varied on the time when they assessed the presence of menopausal symptoms, which may be an important factor in determining the risk for CVD. Data from the Women's Health Initiative suggest that the predictive value of vasomotor symptoms for cardiovascular risk may vary with onset of vasomotor symptoms at different stages of menopause[22]. Furthermore, studies usually combined assessment of VMS frequency and severity, constructs which are not interchangeable. The majority of studies were based in a single country (USA). Given that CVD rates, menopausal symptoms and age at menopause[46] all vary between countries, it is possible that the strength or existence of the associations also varies by population. Therefore, to improve the generalizability of the findings, these associations would need to be evaluated in other populations. Finally, we found evidence of reporting bias in our systematic review, and therefore, some relevant studies may have been overlooked in the current systematic review.

CONLSUSION

The evidence from observational studies indicate that women who experience VMS and other menopausal symptoms, including depression, anxiety and panic attacks during menopause are at greater risk of developing CVD. Further studies are needed to reliably establish which menopausal symptoms are independently associated with CVD outcomes and further clarify the potential mechanisms behind these associations.

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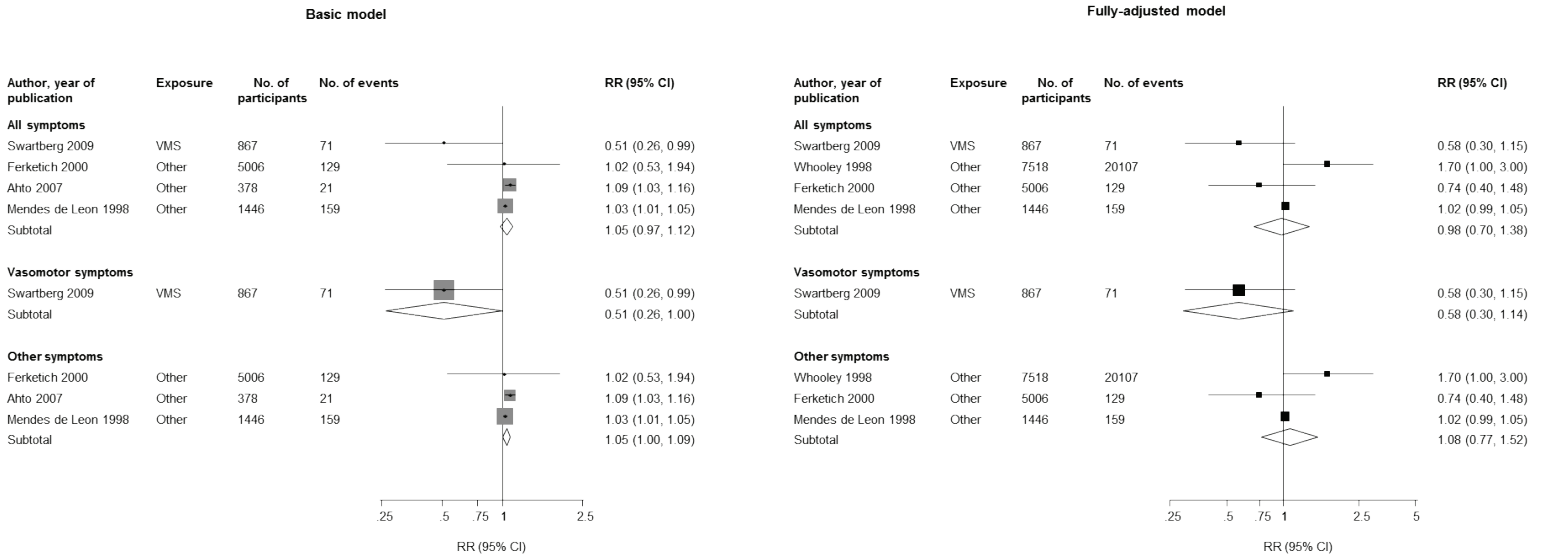
Supplemental material

Supplemental Table 1: Exposure and methods of assessment used in the studies included in the review

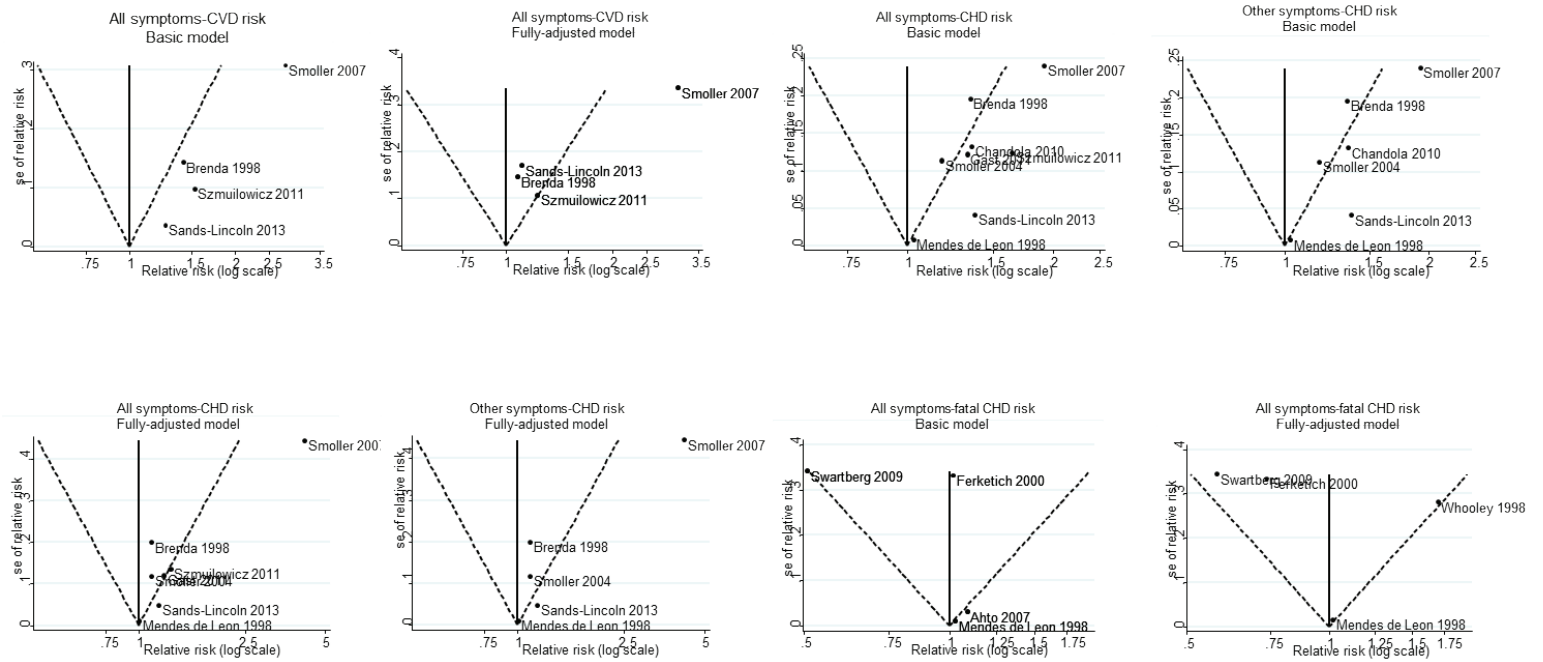
Lead Author, Publication Date	Exposure	Method used to assess the exposure
Ahto, 2007	Depression	Zung Self-Rating Depression Scale
Penninx, 1998; Mendes de Leon, 1998	Depression	The Center for Epidemiologic Studies Depression Scale
Chandola, 2010	Sleep disturbance	General Heath Questionnaire
Ferketich, 2000	Depression	CES-D Scale- a Self-report depression scale
Gast, 2011	Vasomotor Symptoms Presence in the previous week or during the preceding 3 months	Questionnaire
Sands-Lincoln, 2013	Insomnia	WHI Insomnia Rating Scale (WHIIRS)
Smoller, 2007	Panic Attacks in the previous 6 months	Questionnaire
Svartberg, 2009	Vasomotor Symptoms	Self-administered questionnaire
Whooley, 1998	Depression	The Geriatric Depression Scale short form- a self-report questionnaire
W-Smoller, 2004; Szmuilowicz 2011	Late Vasomotor Symptoms (also early and persistent vasomotor symptoms)	Self-administered questionnaire

Supplemental Figure 1. Relative risks of fatal CHD associated with menopausal symptoms



Legend for Supplemental Figure 1. Basic model: adjusted for age and non-established cardiovascular risk factors; Fully-adjusted model, adjusted for established cardiovascular risk factors and potential mediators. VMS, vasomotorA symptoms; vasomotor symptoms include hot flashes and /or night sweats; All, includes vasomotor and other menopausal symptoms; Other, includes menopausal symptoms such as depression, insomnia, and panic attacks.

Supplemental Figure 2. Egger's test in studies of menopausal symptoms and the risk cardiovascular disease.



Legend for Supplemental Figure 2. The dotted lines show 95% confidence intervals around the overall summary estimate calculated using a fixed effect model; P-values for bias calculated using Egger's test was 0.070 for all menopausal symptoms-CVD risk in basic adjusted analysis; 0.041 for all menopausal symptoms-CVD risk in fully- adjusted analysis ; 0.016 for all menopausal symptoms-CHD risk in basic adjusted analysis; 0.083 for other menopausal symptoms-CHD risk in basic adjusted analysis; 0.014 for all menopausal symptoms-CHD risk in fully-adjusted analysis; 0.098 for other menopausal symptoms-CHD risk in fully-adjusted analysis; 0.599 for all menopausal symptoms-fatal CHD risk in basic adjusted analysis; and 0.824 for all menopausal symptoms-fatal CHD risk in fully-adjusted analysis respectively e Appendix 1. PRISMA 2009 check-list

eAppendix 3: Literature search strategy for prospective studies of cardiovascular outcomes

Relevant studies, published before February 17, 2015 (date last searched), were identified through electronic searches not limited to the English language using MEDLINE, EMBASE, and Web of Science databases. Electronic searches were supplemented by scanning reference lists of articles identified for all relevant studies (including review articles), by hand searching of relevant journals and by correspondence with study investigators. The computer-based searches combined search terms related to markers related to vasomotor symptoms, related to vascular outcomes and related to study design without language restriction.

(hot flashes[MeSH Terms] OR "night sweat"[Text Word] OR "hot flash"[All Fields] OR "sweat"[All Fields] OR "flush"[All Fields] OR "hot flush"[All Fields] OR "ember flash"[All Fields] OR "vasomotor symptom"[All Fields] OR menopause[MeSH Terms] OR "menopause"[All Fields] OR perimenopause[MeSH Terms] OR postmenopause[MeSH Terms])

AND

("Cardiovascular Diseases" [Mesh] OR "Coronary Artery Disease" [MeSH] OR "Atherosclerosis" [MeSH] OR "Coronary Disease" [MeSH] OR "Myocardial Infarction" [MeSh] OR "Myocardial Ischemia" [MeSH] OR "Stroke" [MeSH])

AND

("longitudinal studies"[MeSH Terms] OR "prospective"[All Fields] OR "cohort"[All Fields] OR "follow up"[All Fields] OR ("Clinical Trials as Topic"[Mesh]) OR "Randomized Controlled Trial" [Publication Type])

4.5 Association of Age at Menopause and Duration from Onset of Menopause with Cardiovascular Outcomes, Intermediate Vascular Traits and All-Cause Mortality: a Systematic Review and Meta-Analysis of Observational Studies

Taulant Muka, Clare Oliver-Williams*, Setor Kunutsor, Joop S.E. Laven, Bart CJM Fauser, Rajiv Chowdhury, Maryam Kavousi*, Oscar H. Franco*.*

** Authors contributed equally*

ABSTRACT

Background*:* Early menopause and a longer duration since menopause, due to the early cessation of the protective effect of endogenous oestrogen, are suggested to result in a greater cardiovascular risk.

Methods and Results*:* Three medical databases were searched for studies (observational cohort, case-control or cross-sectional) that assessed age at menopause or duration since onset of menopause as exposures, and risk of cardiovascular outcomes (composite cardiovascular disease (CVD), fatal and non-fatal coronary heart disease (CHD) and overall stroke and stroke mortality), CVD-mortality, all-cause mortality and intermediate CVD endpoints, in perimenopausal, menopausal, or postmenopausal women. Out of initially identified references, 32 studies were selected, including 310,329 non-overlapping women. Women with a menopausal age of younger than 45 years compared to women with a menopausal age of 45 years or older, the relative risk (RR) [95% confidence intervals (CI)] was 1.50(1.28-1.76) for overall CHD, 1.11(1.03-1.20) for fatal CHD, 1.23 (0.98-1.53) for overall stroke, 0.99 (0.92-1.07) for stroke mortality, 1.19(1.08-1.31) for CVD mortality and 1.12(1.03-1.21) for all-cause mortality. For women with a menopausal age between 50-54 years compared to women with a menopausal age of younger than 50 years, there was a decreased risk of fatal CHD (RR:0.87(95%CI:0.80-0.96)), and no effect on stroke. Duration since menopause in relation to risk of developing intermediate cardiovascular traits or CVD outcomes was reported in 4 observational studies, reporting no consistent results.

Conclusions: The findings of this review indicate an excess CHD, CVD-mortality and overall mortality risk in women who have an early or premature menopause.

INTRODUCTION

Risk of cardiovascular disease (CVD) increases with age and, as women tend to live longer than men, the absolute number of women living with and dying from CVD is greater than men[1]. Therefore, early recognition of women at high risk for CVD and timely implementation of appropriate lifestyle or therapeutic interventions are of tremendous public health importance.

Adverse changes in cardiovascular risk factors occur around the menopausal transition[2-4], highlighting the need of CVD risk assessment during this period and of introduction of appropriate preventive or treatment modalities. While the average age at menopause is 51 years[5], menopausal age varies significantly among women with a range between 40-60 years of age. Women who have a premature or early menopause may not only be at risk from a younger age, but may also live more years of their lives at an increased risk of adverse outcomes[6-8]3–5. This highlights the need to evaluate the role of both menopausal age and time since onset of menopause as risk factors for CVD.

Age at menopause may be a marker for not only reproductive ageing but also for general health and somatic ageing[9]. Menopause has been proposed as the first step in a causal pathway that, due to hormonal changes, eventually results in organ dysfunction[10]. A hormonal change that is often cited as an important determinant in post-menopausal CVD development is the decrease in endogenous estrogen synthesis[11]. Estrogens are involved in the relaxation and expansion of blood vessels, helping to accommodate blood flow, and consequently, decreased levels of oestrogen would result in stiffer blood vessels [12]. Furthermore, loss of the ovarian function through menopause is associated with the activation of the renin-angiotensin-aldosterone system, leading to downstream endothelial dysfunction, inflammation and immune dysfunction[13]. These processes are associated with obesity, diabetes and hypertension[13]. Thus, early menopause has been hypothesized to be detrimental to cardiovascular health, due to the early cessation of the protective effect of endogenous oestrogen. Longer duration since menopause would therefore result in a greater risk of intermediate and hard CVD outcomes. However, whether this is indeed the case remains unclear given that there is not yet a comprehensive assessment of menopause in association with subsequent adverse cardiovascular outcomes.

Hence, we conducted a systematic review and meta-analysis of all available observational evidence to quantify the associations of age at menopause and duration since onset of menopause with (i) primarily clinical CVD outcomes and intermediate vascular traits; and (ii) all-cause mortality.

METHODS

Data sources and search strategy

This review was conducted using a predefined protocol and in accordance with the PRISMA and MOOSE guidelines (**eAppendix 1** and **2**). Two independent reviewers, in duplication, sought studies

published up to March, 2015 (date last searched) using Medline, EMBASE and Web of Science electronic databases. The computer-based searches combined terms related to the exposure (eg, *age at menopause, duration from onset of menopause*) and outcomes (eg, *endothelial dysfunction, inflammation, cardiovascular disease, mortality*), without any language restriction. Details on the search strategy are provided in **eAppendix 3**. Furthermore, in order to identify further studies, we checked reference lists and contacted authors and experts in the field

Study selection and eligibility criteria

Studies were sought if they (i) were observational cohort, case-control, or cross-sectional studies; (ii) had reported on age at menopause (for the natural menopause it is defined as occurring 12 months after the last menstrual period, and for surgical menopause it is the age at which ovaries are removed (by bilateral oophorectomy) or if the oophorectomy occurred before natural menopause), and/or duration from onset of menopause (defined as age of menopause onset until outcome event or diagnosis) as exposures; and (iii) had assessed associations with risk of CVD-related outcomes (composite CVD, fatal and non-fatal coronary heart disease (CHD) and overall and fatal stroke), CVD-mortality, all-cause mortality, or intermediate CVD endpoints (such as metabolic syndrome, obesity or BMI, hypertension, and markers of endothelial dysfunction and vascular inflammation) in menopausal or postmenopausal women. Two independent reviewers screened the titles and abstracts of all initially identified studies according to the selection criteria. Full texts were retrieved from studies that satisfied all selection criteria.

Data extraction and quality assessment

Data were extracted by two independent reviewers and a consensus was reached with involvement of a third. A predesigned data abstraction form was used to extract relevant information. This included questions on study size; study design; baseline population; location; age at baseline; duration of follow-up (for cohort studies); reported degree of adjustment (defined as '+' when risk estimates were adjusted for age only;'++' further adjustment for established CVD risk factors and other potential confounders (such as, smoking status, lipids, hypertension, body mass index (BMI), history of cardiometabolic disease, hormone therapy); type of outcome and reported risk estimates. Additionally, in the case of multiple publications, the most up-to-date or comprehensive information was included. Study quality (for cohort and case-control studies) was assessed based on the nine-star Newcastle–Ottawa Scale(NOS)[14] using three pre-defined domains namely: selection of participants (population representativeness), comparability (adjustment for confounders), and ascertainment of outcomes of interest. The NOS assigns a maximum of four points for selection, two points for comparability, and three points for outcome. Nine points on the NOS reflects the highest study quality. A score of ≥ 5 indicated adequate quality for inclusion in the review.

Data synthesis and analysis

The inverse variance weighted method was used to combine relative risks to produce a pooled relative risk using random-effects models to allow for between study heterogeneity. Heterogeneity was assessed using the Cochrane χ^2 statistic and the I^2 statistic. Publication bias was evaluated

through a funnel plot and Egger's test. All tests were two-tailed and p-values of 0.05 or less were considered significant. STATA release 12 (Stata Corp, College Station, Texas) was used for all statistical analyses.

RESULTS

Identification of relevant studies

The search strategy identified 9,444 citations. Following initial screening based on titles and abstracts, full-texts of 73 articles were evaluated further. Of these articles, 40 articles were excluded for reasons shown in **Figure 1**. The remaining 33 articles, based on 32 unique studies, met inclusion criteria, and therefore, were included in the review.

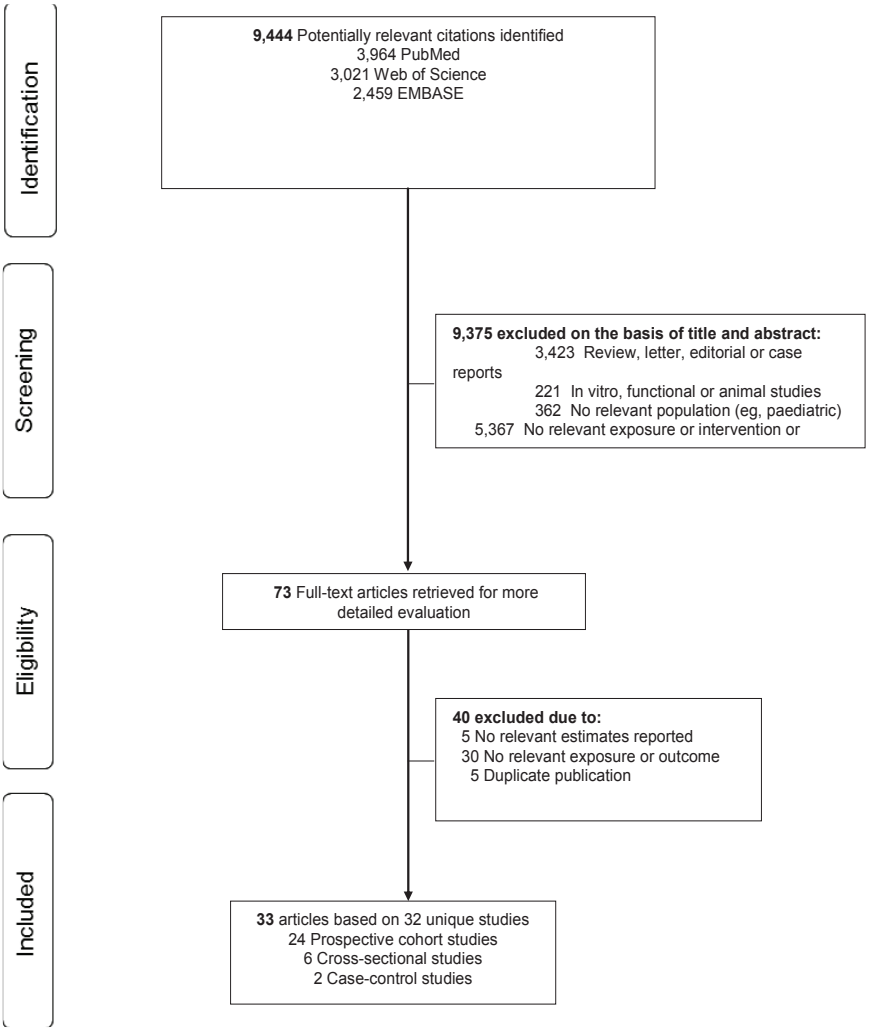
General characteristics of the included studies

Tables 1 and Supplemental Table S1 summarize the key characteristics of the studies that assessed age at menopause as an exposure and those which had duration since menopause as an exposure, respectively. All studies except one evaluated the risk in relation to the age at menopause, with 4 additionally evaluating the risk in relation to duration since menopause. In aggregate, in all included studies, 342,284 women were included in this review. However, not all studies provided relevant data that could be meta-analysed and it was not possible to combine any results related to duration since menopause, although an analysis of age at menopause was possible. Consequently, there were 297,496 participants in the age at menopause analysis, which included 44,962 cases. The average follow-up in these studies ranged from 4.0 to 51.8 years. The majority of studies (n=12) were based in Western European countries, 11 were from the United States, and the remaining 9 studies were based in East Asia. The baseline age of participants ranged from 28 to 89 years. The majority of studies (n=24) were prospective cohort studies, whilst the remaining studies were case-control (n=2) or cross-sectional studies (n=6). Overall, age at menopause in relation to risk of developing CVD intermediates or outcomes, or all-cause mortality was reported in 31 studies. Of these, 1 study provided risk estimates of overall CVD outcomes, 5 estimated risk of CHD, and 6 studies quantified risk of stroke. Additionally, 3 studies assessed risk of intermediate CVD endpoints, 9 estimated risk of all-cause mortality, 7 estimated risk of CVD-mortality, 8 estimated risk of CHD-mortality and 7 studies estimated risk of stroke-mortality. Among the prospective cohort and case-control studies, 14 studies were judged to be at low risk of bias, 10 were at medium risk, and two studied were evaluated to be at high risk of bias (**Table 1 and Supplemental Table S1**).

Association of age at menopause with incident (composite) cardiovascular disease, coronary heart disease and stroke

There was only one study examining the association between age of menopause and incidence of CVD and showed a pooled RR of 1.56 (1.08-2.26) for a menopausal age of younger than 45 years compared to a menopausal age of 45 years or older.[15] In the meta-analysis of 50,125 participants

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



and 1,217 CHD events, pooled RR (95%CI), comparing a menopausal age of younger than 45 years relative to a menopausal age of 45 years or older and adjusted for several established CVD risk factors and other potential confounders (such as, age, smoking status, lipids, hypertension, BMI, history of cardiometabolic disease, hormone therapy), was 1.50 (1.28-1.76) (**Figure 2**). Corresponding results, based on the meta-analysis of 49,246 participants and 770 stroke events, were 1.23 (0.98-1.53) for stroke risk (**Figure 3**). There was little evidence of between-study heterogeneity in these meta-analyses:CHD and stroke analyses: I^2=0% and χ^2: p=0.65, and I^2=51% and χ^2: p=0.09, respectively.

Table 1. Characteristics of the included studies that assessed the association of age at menopause with study outcomes

Lead Author, Publication Date	Name of study or source of participants	Location	Year of baseline survey	Baseline age range/ average age (years)	Study design	Follow up years	Total partici-pants	No. of study events	Outcome	Covariates adjusted for	Study quali-ty*
Palmer, 1992[36]	Massachusetts	USA	1986-1990	45-69	Case-control	N/A	1,716	858	Myocardial infarction	Smoking, hypertension, high cholesterol, family history of MI before 60 years, BMI, coffee drinking in year before interview, years of education, spouse's years of education, conjugated estrogen use, occupation	9
Lapidus, 1985[37]	Gothenburg	Sweden	1968-1969	38-60	Prospective cohort	12.0	1,462	25	Myocardial infarction	Age	8
van der Schouw, 1996[8]	DOM	Netherlands	1974-1977	50-65	Prospective cohort	20.0	12,115	824	CVD mortality	Age, year of birth, natural menopause, oral contraceptive use, parity/age at first delivery, BMI ≥ 30 kg/m^2, smoking, hypertension, diabetes, previous CVD	9
Jacobsen, 1997[38]	Vestfold, Nord-Trondelag, Aust- Agder	Norway	1961	32-74	Prospective cohort	29.0	19,309	2,767	CHD mortality	Age, county, occupational group	8
Cooper, 1998[39]	NHANES	USA	1971-1975	50-86	Prospective cohort	4.0	3,191	345	CHD	Age at baseline, years of follow-up, race, education, use of HRT	9
Cooper, 1999[27]	MRHS	USA	1934-1939	-	Prospective cohort	51.8	867	45	CHD	Age	6

Table 1. Characteristics of the included studies that assessed the association of age at menopause with study outcomes (continued)

Lead Author, Publication Date	Name of study or source of participants	Location	Year of baseline survey	Baseline age range/ average age (years)	Study design	Follow up years	Total participants	No. of study events	Outcome	Covariates adjusted for	Study quality*
Hu, 1999[40]	NHS	USA	1976-1994	30-55	Prospective cohort	18.0	35,616	757	CHD; Stroke	Age, BMI, parental history of MI, history of high BP, hypercholesterolemia, diabetes, smoking, parity	8
Jacobsen, 1999[41]	Adventist Health Study	USA	1974-1976	35-60	Prospective cohort	10.3	6,182	1,831	Mortality; CHD mortality	Diabetes, hypertension, parity, age at first birth, physical activity	8
Joakimsen, 1999[25]	Tromso	Norway	1994-1995	48.6	Cross-sectional	N/A	2,588	1,284	Carotid atherosclerosis	Age	N/A
Fioretti, 2000[14]	-	Italy	1983-1992	< 75	Case-control	N/A	1,292	429	Myocardial infarction	Age, study, education, smoking, BMI, diabetes, hypertension, hyperlipidemia, HRT	8
De Kleijn, 2002[11]	Nijmegen	Netherlands	1975	35-65	Prospective cohort	20.5	9,450	1,063	Mortality; CHD and stroke mortality	Age at entry, HRT use, hypertension, BMI, social economic class	9
Jacobsen, 2004[42]	Vestfold, Nord-Trondelag, Aust-Agder	Norway	1956-1959	32-74	Prospective cohort	37	19,731	3,561	Stroke mortality	Age, county, occupational group, birth cohort	8
Choi, 2005[43]	KEPEC	Korea	1993-1998	> 65	Prospective cohort	4.9	5,731	186	Stroke	Age, physical activity, history of hypertension	8

Table 1. Characteristics of the included studies that assessed the association of age at menopause with study outcomes (continued)

Lead Author, Publication Date	Name of study or source of participants	Location	Year of baseline survey	Baseline age range/ average age (years)	Study design	Follow up years	Total partici-pants	No. of study events	Outcome	Covariates adjusted for	Study quali-ty*
Mondul, 2005[44]	CPS-II	USA	1982	40-54	Prospective cohort	20	68,154	23,067	CHD, stroke, and all-cause mortality	Age, race, marital status, BMI, age at menarche, parity, education, alcohol consumption, oral contraceptive use, exercise	9
Ossewaarde, 2005[45]	DOM	Netherlands	1974-1977	48-68	Prospective cohort	17	12,134	2,607	CVD, CHD, stroke and all-cause mortality	Age, type of menopause, parity, BMI, hypertension, DM, previous CVD	9
Cui, 2006[46]	JACC	Japan	1988-1990	40-79	Prospective cohort	10	37,965	1,010	CVD, CHD, and stroke mortality	Age, BMI, DM, smoking status, ethanol intake, marital status, college or high school, type of menopause	9
Lokkegard, 2006[32]	DNA	Denmark	1993	> 44	Prospective cohort	5	10,533	286	CHD	Smoking, self-rated health, use of HRT, BMI, presence of hypertension, angina, diabetes	8
Amagai, 2006[47]; Baba, 2010[48]	JMS	Japan	1992-1995	36-89	Prospective cohort	10.8	4,683	215	Stroke	Age, SBP, TC, HDL-C, history of diabetes, BMI, smoking, alcohol, marital status, study area, type of menopause	9

Table 1. Characteristics of the included studies that assessed the association of age at menopause with study outcomes (continued)

Lead Author, Publication Date	Name of study or source of participants	Location	Year of baseline survey	Baseline age range/ average age (years)	Study design	Follow up years	Total participants	No. of study events	Outcome	Covariates adjusted for	Study quality*
Hong, 2007[49]	Kangwa Cohort Study	Korea	1985	≥ 55	Prospective cohort	15.8	2,658	1,193	Mortality; CVD, CHD and stroke mortality	Age, alcohol consumption, education, age at first birth, self-cognitive health level, chronic disease, marital partner, parity, age at menarche, oral contraceptive use, hypertension	9
Lisabeth, 2009[16]	FHS	USA	1948	28-62	Prospective cohort	22	1,430	234	Ischaemic stroke	Age, SBP, AF, current smoking, CVD, estrogen use	9
He, 2012[50]	Beijing	China	2009-2010	40-59	Cross-sectional	N/A	2,498	NS	CVD, CHD, stroke, and several cardiovascular intermediates	Age, BMI, smoking, marriage, education, occupation, lipid and blood pressure lowering therapy	N/A
Svejme, 2012[24]	Malmo	Sweden	1977	48	Prospective cohort	34	390	148	Mortality	Unadjusted	6
Tom, 2012[51]	Iowa cohort	USA	1980	≥ 65	Prospective cohort	24	1,684	1,477	CVD and all-cause mortality	Age, educational attainment, number of pregnancies, age at menarche, smoking, height, weight, use of estrogen therapy	9

Table 1. Characteristics of the included studies that assessed the association of age at menopause with study outcomes (continued)

Lead Author, Publication Date	Name of study or source of participants	Location	Year of baseline survey	Baseline age range/ average age (years)	Study design	Follow up years	Total partici-pants	No. of study events	Outcome	Covariates adjusted for	Study quali-ty*
Brand, 2013[52]	EPIC-InterAct	Multi-country in Europe	1991-2007	59.2	Prospective cohort	11.0	8,099	3,691	Diabetes	Age, BMI, smoking, alcohol intake, physical activity, education, number of full term pregnancies, ever oral contraceptive use, ever HRT use	9
Qiu, 2013[28]	Fujian	China	2011-2012	37-92	Cross-sectional	N/A	3,304	878	CVD and diabetes mellitus	Age, PA, parity, smoking, alcohol consumption, family history of diabetes, age at menopause, type of menopause, BMI, WC	N/A
Lee, 2013[31]	JNHS	Japan	2001	40-59	Cross-sectional	N/A	22,426	N/A	Diabetes; Hyperten-sion; Hyper-cholesterol-emia	Age, BMI, HRT, daily lifestyle	N/A
Li, 2013[53]	BWHS	USA	1995	NS	Prospective cohort	8	11,212	692	CVD and all-cause mortality	Age, time period, education, marital status, BMI, smoking, pack years of smoking, alcohol consumption, vigorous physical activity, vegetables/ fruits	9

Table 1. Characteristics of the included studies that assessed the association of age at menopause with study outcomes (continued)

Lead Author, Publication Date	Name of study or source of participants	Location	Year of baseline survey	Baseline age range/ average age (years)	Study design	Follow up years	Total participants	No. of study events	Outcome	Covariates adjusted for	Study quality*
Stockl, 2014[54]	KORA F4	Germany	2006-2008	50-81	Cross-sectional	N/A	800	NS	Carotid atherosclerosis	Age, BMI, education, family status, PA, smoking, alcohol consumption, total cholesterol, triglycerides, fasting blood glucose	N/A
Pfeifer, 2014[15]	NS	USA	1955-1979	≥45	Prospective cohort	11.9	600	205	CVD and CHD	Age and calendar year	6
Wellons, 2012[55]	MESA	IS	2000-2002	45-84	Prospective cohort	4.78	2509	87	CHD and Stroke	Age, ethnicity, MESA site, smoking status, total cholesterol, HDL-cholesterol, hypertension, diabetes, family history of CVD disease and hormone replacement therapy.	8
Wu, 2014[56]	SWHS	China	1997-2000	40-70	Prospective cohort	11.2	31,955	3,158	Mortality; CVD, CHD, stroke and diabetes mortality	Age, BMI, WHR, education, occupation, income, regular exercise, current smoking, alcohol consumption, marital status, age at menarche, and number of live births	9

Table 1. Characteristics of the included studies that assessed the association of age at menopause with study outcomes (continued)

Lead Author, Publication Date	Name of study or source of participants	Location	Year of baseline survey	Baseline age range/ average age (years)	Study design	Follow up years	Total partici-pants	No. of study events	Outcome	Covariates adjusted for	Study quali-ty*
Total							310,329	52,923			

*Based on Newcastle–Ottawa method for cohort and case-control studies. AF, atrial fibrillation; BMI, body mass index; CVD, cardiovascular disease; DM, diabetes mellitus; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; HRT, hormone replacement therapy; LDL, low-density lipoprotein; MI, myocardial infarction; NS, not stated; PA, physical activity; SBP, systolic blood pressure; WC, waist circumference. Study acronyms: NHANES, National Health and Examination Survey; MRHS, Menstruation and Reproductive History Study; N/A, Not applicable; NHS, Nurses' Health Study; KEPEC, Korean Elderly Pharmacoepidemiologic Cohort; CPS-II, Cancer Prevention Study II; DOM, Diagnostisch Onderzoek Mammacarcinoom; Japan Collaborative Cohort; DNA, Danish Nurses Association; JMS, Jichi Medical School Cohort Study; FHS, Framingham Heart Study; EPIC, European Prospective Investigation into Cancer and Nutrition; JNHS, Japan Nurse's Health Study; BWHS, Black Women's Health Study; SWHS, Shangai Women's Health Study; MESA: Multi-Ethnic Study Atherosclerosis.

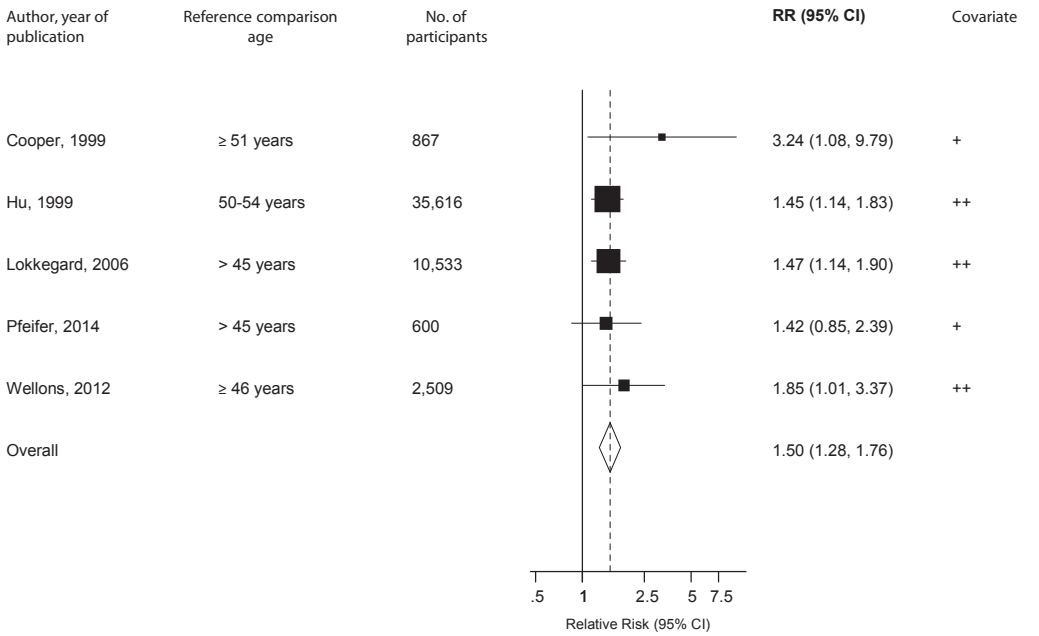
When women with a menopausal age between 45-49 years were compared to women with a menopausal age of 50 years or older, the corresponding adjusted pooled RRs (95%CI) were 1.12 (0.95-1.31; based on 36,483 participants and 784 CHD events) for CHD risk and 0.95 (0.74-1.23; based on 109,928 participants and 536 stroke events) for stroke risk (**Supplemental Figure S1 and S2**). There was no evidence of between-study heterogeneity in both these analyses: I^2 =0% and χ^2: p=0.37, I^2=0% and χ^2:p=0.78 for CHD and stroke analysis respectively.

Due to differences in age categories, it was not possible to include one study in these meta-analyses. Lisabeth and colleagues[16] evaluated risk of ischaemic stroke for women between 42-54 years old at menopause compared to women with a premature menopause (before 42 years of age). They found a decreased risk of ischaemic stroke (RR=0.50; 95%CI:0.29-0.89) for women who reported being between 42-54 years old at menopause relative to prematurely menopausal women. An additional lower risk was found for women who had menopause after 55 years of age (RR=0.31; 95%CI:0.13-0.76).

Association of age at menopause with all-cause, (composite) cardiovascular disease, coronary heart disease and stroke mortality

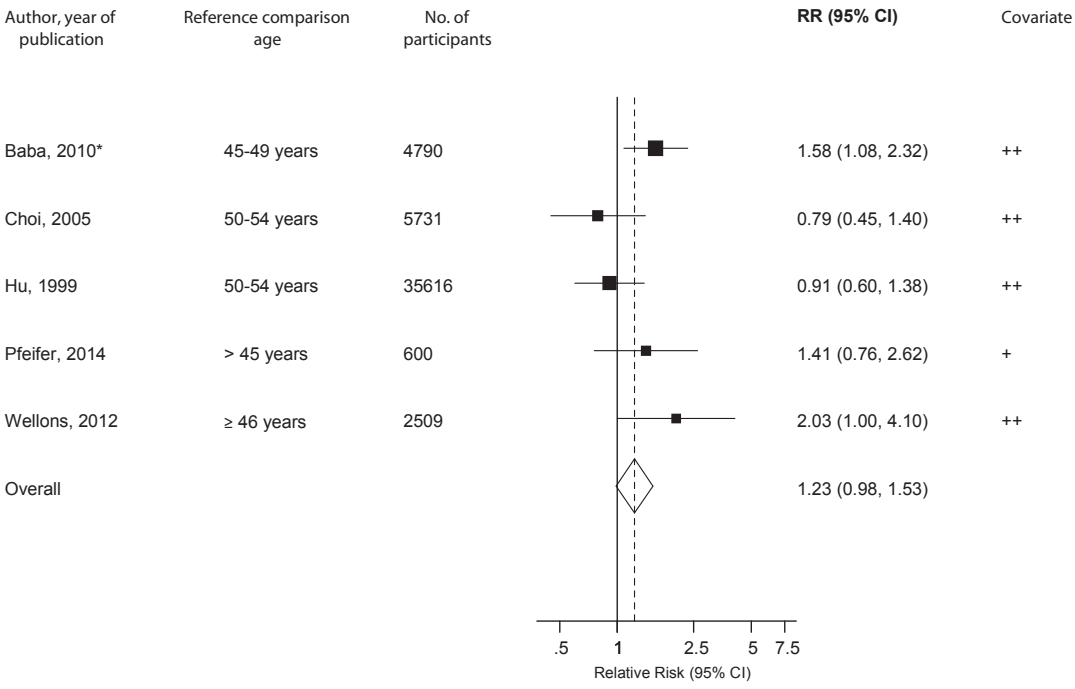
Pooling results of the risk for different mortality outcomes estimated for women with a menopausal age of younger than 45 years relative to a menopausal age of 45 years or older, adjusted for several

Figure 2. Age at menopause < 45 years and risk of incident CHD with menopausal age ≥ 45 years as reference category



+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, X^2 = 2.49, I^2=0%, 0 to 79%; P = 0.647.

Figure 3. Age at menopause > 45 years and risk of total stroke with menopausal age ≥ 45 years as reference category



+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, $X^2 = 8.12$, I^2=51%, 0 to 82%; P = 0.087.

established CVD risk factors and other potential confounders, yielded combined RRs (95% CI) of 1.12 (1.03-1.21) for all-cause mortality (109,898 participants and 31,427 all-cause deaths), 1.19 (1.08-1.31) for CVD mortality (65,653 participants and 6,979 CVD-deaths), 1.11 (1.03-1.20) for CHD mortality (118,150 participants and 8,737 CHD-deaths) and 0.99 (0.92-1.07) for stroke mortality (143,833 participants and 6,706 stroke-deaths) (**Figure 4-6 and Supplemental Figure S3**). There was evidence of between-study heterogeneity for all-cause mortality analysis (I^2=63% (95%CI: 20-83%) and χ^2: p=0.009) whereas little evidence of between-study heterogeneity was found for the other analyses (**Figure 4-6 and Supplemental Figure S3**). Corresponding pooled RR (95%CI) for all-cause mortality, CVD, CHD and stroke mortality comparing menopausal age of 45-49 years to menopausal age of 50 years or older were 1.03 (1.00-1.05) for all-cause mortality (90,691 participants, 28,188 all-cause deaths), 0.99 (0.92 -1.07) for CVD mortality (62,995 participants, 5,786 CVD-deaths), 0.98 (0.93 -1.04) for CHD mortality (121,444 participants, 5,954 CHD-deaths) and 1.03 (0.91 -1.16) for stroke mortality (141,175 participants, 6,320 stroke-deaths) **(Supplemental Figures S3-S7**. When comparing menopausal age between 50-54 years relative to menopausal age of younger than 50 years, the corresponding pooled RRs (95%CI) were 1.02 (0.89-1.15) for all-cause mortality (7,341 participants; 1,408 all-cause deaths), 0.96 (0.74-1.24) for CVD mortality (12,108 participants; 2,256 CVD-deaths), 0.87 (0.80- 0.96) for CHD mortality

(31,417 participants;3,279 CHD-deaths) and 1.19 (0.93-1.52) for stroke mortality (12,108 participants;623 stroke deaths) (**Supplemental Figures S8-S11**). There was little evidence of between-study heterogeneity in all these analyses (**Supplemental Figures S3-S11**). Due to differences in age categories, it was not possible to include one study in the pooled results[17]. Wu and colleagues reported an increased risk of all-cause mortality (RR=1.16, 95% CI:1.04-1.29) for women of menopausal age < 46.64 years compared to women aged between 48.80-50.15 years at menopause (**Supplemental Table S2**).

Association of age at menopause with intermediate cardiovascular traits

Only two studies were identified that evaluated risk for carotid atherosclerosis. Pooled RR (95% CI) for the risk of carotid atherosclerosis was 0.74 (0.63-0.87) when comparing women with a menopause age at or after 50 years to women with a menopausal of younger than 50 (3,388 participants) (**Supplemental Figure S12**). There was, however, evidence of between study heterogeneity: I^2 =69% (95%CI:0, 93%) and χ^2: p=0.073. Three studies could not be included in the meta-analysis of intermediate cardiovascular traits[11,12] (**Supplemental Table S2**). They all evaluated risk of diabetes depending on age at menopause. Two of the studies found no excess risk for Chinese women who experienced menopause before or after age 50, relative to women who underwent the menopause at age approximately 50 years. The same findings were observed in a study of European women; where relative to women who experienced menopause between the ages of 50-54, neither women who experienced an early menopause, between 45-49 years old, nor women who had their menopause after age 55 were at greater risk for diabetes.

Years since menopause with CVD outcomes and intermediate cardiovascular traits

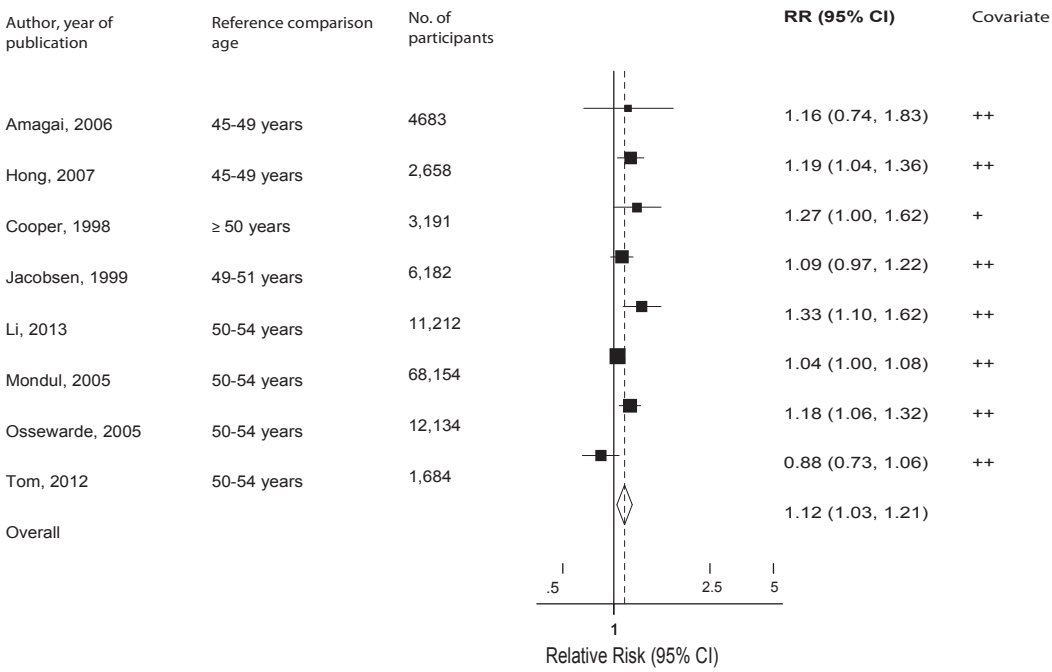
Duration since menopause in relation to risk of developing intermediate cardiovascular traits or CVD outcomes was reported in four observational studies. Of these, two studies reported risk of overall CVD outcomes, and three studies estimated risk of intermediate cardiovascular traits. The age at baseline ranged from 40 to 81. Two studies, conducted in Italy and China, evaluated risk of CVD outcomes, including CHD, stroke and composite CVD outcomes (**Supplemental Table S3**), and three studies assessed risk of intermediate cardiovascular traits, including metabolic syndrome, obesity or BMI, and hypertension (**Supplemental Table S4**) in Korean, Chinese and German populations. Owing to a substantial heterogeneity in the comparison groups used across these studies, no quantitative synthesis could be performed. The findings of these studies, therefore, were only qualitatively reviewed. In a Chinese population[13], relative to women less than 1 year post menopause, women 2-6 years post menopause were at a greater risk of CVD, with evidence for an increased risk after 6 years. In the same study, there was also evidence for a greater risk of CHD and stroke more than 1 year post menopause. However, these findings were in contrast to those in an Italian population[14], which found no increased risk of myocardial infarction in post-menopausal women less than 10 years, 10-20 years and more than 20 years post menopause compared with pre- or peri-menopausal women. Stockl and colleagues[15] found no association between time since menopause and the presence of carotid atherosclerosis, however time since menopause was dichotomised into broad groups, comparing women less than 15 years post menopause, to women more than 15 years post-menopause.

Relative to premenopausal women, postmenopausal Korean women were at greater risk of metabolic syndrome, and this risk increased with the time since menopause, with the greatest risk found in women between 10 and 14 years post-menopause[16]. Within the same population, there was evidence for increased risk of hypertension, abdominal obesity and high glucose levels, in postmenopausal women; however, risk did not vary with duration since menopause. Conversely, no increased risk of obesity, hypertension or diabetes was found in Chinese women who were more than 2 years post-menopause compared with women less than 1 year post-menopause[13]. Similarly, within the same study, systolic and diastolic blood pressure, BMI, WHR, and glucose levels did not vary with time since menopause.

Publication bias

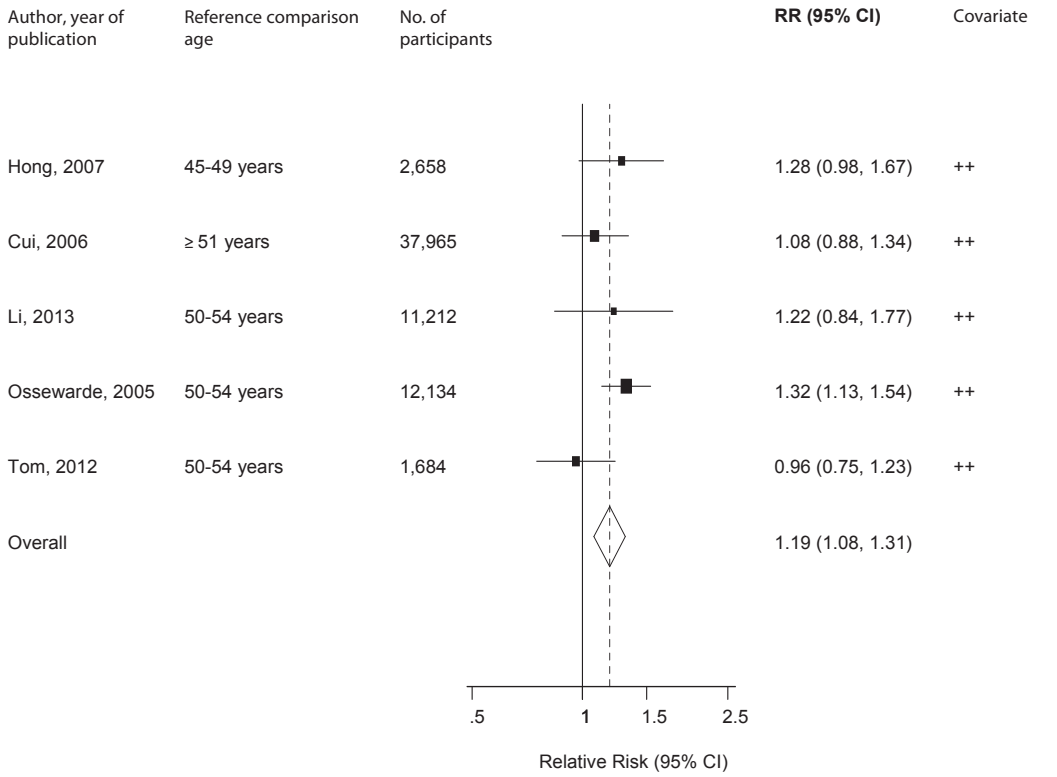
Under visual examination, Begg's funnel plots for those analyses that included a minimum of 5 studies were mostly symmetrical (**Supplemental Figure S13**), with possible exception of studies evaluating overall CHD risk and CHD mortality risk in women with menopausal age of less than 45 years. However, there was no statistical evidence of publication bias based on Egger's test, which was non-significant (P>0.05) for all analyses that involved 5 or more studies.

Figure 4. Age at menopause < 45 years and risk of mortality with menopausal age ≥ 45 years as reference category



+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, $X^2 = 18.80$, I^2=63%, 20 to 83%; P = 0.09.

Figure 5. Age at menopause < 45 years and risk of CVD mortality with menopausal age ≥ 45 years as reference category.



+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, $X^2 = 5.74$, I^2=30%, 0 to 73%; P = 0.219.

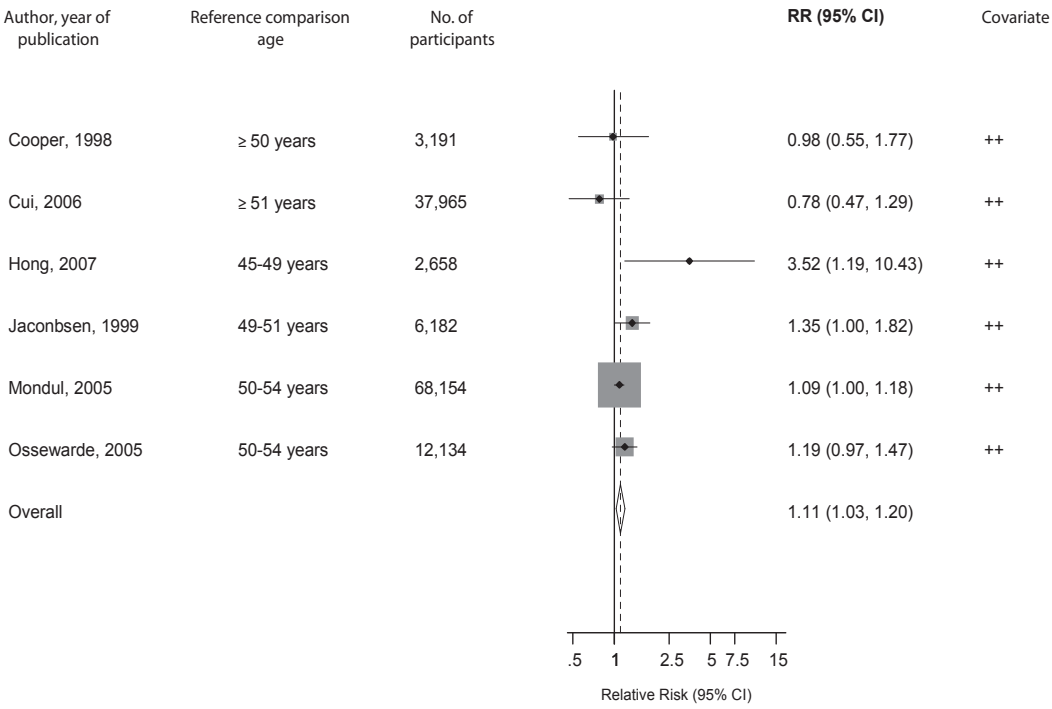
DISCUSSION

Overall, we found that women who experienced an early menopause (i.e. younger than 45 years) appear to have an excess risk of CHD, CVD-mortality and all-cause mortality and no association with stroke risk. By contrast, being 45-49 years at menopause compared to ≥50 years had no apparent association with adverse outcomes except for an increased risk of carotid atherosclerosis. Only a few studies evaluating risk in relation to duration since menopause could be found. Those that were identified had somewhat conflicting results, with some studies finding a greater risk of hypertension, abdominal obesity and high glucose levels, and others reporting essentially null associations.

Interpretation of findings

The current study supports previous findings that there is an increased risk of CVD with premature or early menopause, specifically identifying a greater risk with CHD, and potentially with carotid atherosclerosis. The findings of this systematic review on the risk of CVD associated with early menopause generally concur with and further extend a previous review[18], which reported an

Figure 6. Age at menopause < 45 years and risk of CHD mortality with menopausal age ≥ 45 years as reference category.



+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, $X^2 = 8.65$, I^2=42%, 0 to 77%; P = 0.124.

increased risk of CVD for women at menopausal age of 50 years or younger compared with menopausal age ≥51 years. However, the findings of the previous review were based only on few prospective studies (7 prospective cohort studies) and therefore the authors were not able to compare other age categories with the risk of CVD. Our finding on an association between premature or early menopause with carotid atherosclerosis needs to be interpreted with some caution, given the relatively small number of studies currently available and due to the presence of between-study heterogeneity. However, the number of participants in both studies were relatively large (12,108 participants in both studies).

The association between early menopause and CHD risk may have several mechanistic interpretations. Early loss of the ovarian function through menopause may lead to long term activation of the renin-angiotensin-aldosterone system, leading to endothelial dysfunction, inflammation and immune dysfunction, and therefore causing vascular damage[13,19]. This process may be partially mediated via the membrane G protein-coupled estrogen receptor[13]. Also, another common explanation is that menopause marks the start of a biological mechanism, led by hormonal changes, which causes tissue damage and organ dysfunction[10]. The multi-organ impact of the menopause proposed by this theory, and supported by findings of an increased risk of depression, dementia and osteoporosis[20-22], seems to be consistent with the increased risk of all-cause mortality found in the current analysis.

Alternatively, there may be shared risk factors, either genetic or environmental, that result in early menopause and also increase the risk of adverse health outcomes[10]. In this case, early menopause could be considered as only a marker of risk. In support of a shared genetic basis, a large-scale genome-wide association study of age at menopause identified a number of loci, including the ones relevant to cardiovascular disease, that were involved in inflammatory response, oxidative stress and genome stability[23]. Speculative environmental factors could include obstetric history, such as parity or having a stillbirth. These explanations are not necessarily mutually exclusive, however, and a combination of these mechanisms may be responsible for the observed increase in CVD and all-cause mortality risk.

Strengths and limitations

This is the first comprehensive quantitative review of observational evidence that assessed both the associations of age at menopause and duration since menopause with clinical CVD outcomes, intermediate cardiovascular traits and all-cause mortality. Our analyses included >300,000 women and evaluated the risk of a wide-range of outcomes in relation to various menopausal age-groups. However, strengths and limitations in the current study merit careful consideration. First, all systematic reviews are prone to reporting bias, owing to the possibility that studies with more extreme results are more likely to be published. Nonetheless, as demonstrated by Egger's test estimates, there was little evidence of publication bias in the current analyses. Additionally, all meta-analyses are limited by the quality of the individual published studies. However, the majority of studies included in the current analyses were of high quality, with a low risk of bias. In order to allow for a uniform analysis, it was necessary to combine some overlapping age-groups for some studies (eg, studies that reported estimates for menopausal age of 50-54 years and those based on >50 years). This might have introduced some heterogeneity into the analyses. Additionally, due to differential categorisation of menopausal age in various studies or only few studies evaluating a particular outcome (eg, carotid atherosclerosis), some of our analysis are based on a small number of studies. Therefore, these results need to be interpreted with caution, particularly those which yielded moderate between-study heterogeneity estimates. Furthermore, the lack of studies evaluating risk in relation to duration since menopause limited us from performing any meaningful quantitative synthesis using this exposure. Most studies that were identified adjusted for a range of relevant confounders, although one study was entirely unadjusted[24], and three only adjusted for age[25-27]. Therefore, the risk of residual confounding cannot be entirely ruled out. A specific concern is confounding from HRT use, which particularly may vary depending on the age at menopause. Women who experience menopause at a younger age may be more likely to start HRT than women who reach menopause in their 50s. Consequently, hormone therapy use may confound the relationship between age at menopause and CVD risk. Indeed, HRT was only adjusted for in a minority of identified studies[28-32]. However, the complex interplay between exogenous hormones and CVD risk is not fully understood, and the results regarding HRT and CVD risk are conflicting[33-35].

Clinical and scientific implications

This review underscores a potential increased risk of adverse cardiovascular outcomes in women who experience early or premature menopause, which may have important clinical and public health implications. This study has also identified a number of gaps in the literature concerning the relations between duration since menopause, age at menopause and intermediate cardiovascular traits and CVD outcomes. Few studies were identified that assessed duration since menopause as an exposure, limiting the review to four studies. Additionally, there were only few studies focusing on intermediate cardiovascular endpoints or risk factors leading to conflicting results and therefore impeding meaningful interpretation. These intermediate factors are of potential importance in interpretation of the observed excess cardiovascular risk, and thus, further research focusing on the intermediate cardiovascular traits is required. Finally, other areas of interest are the relation between age at menopause and duration since menopause and whether there are common determinants for premature menopause and CVD. The observed link between premature menopause and CVD risk may be modified by differing durations since the onset of the menopause among women. The excess CHD risk may be driven in part by a longer duration since the onset of the menopause in women with early menopause. Alternatively, the increased relative risk found with premature menopause may be present only in the first years after menopause, ameliorating over time. Thus far, the known risk factors for premature menopause, include genetics[21]; reproductive factors, i.e. parity and age at menarche[31]; as well as lifestyle factors such as smoking and BMI[31]. However, the role of these factors in mediating the association between premature menopause and CVD remains unclear and therefore warrants further research.

CONCLUSION

The findings of this review indicate an excess coronary heart disease, cardiovascular mortality and overall mortality risk in women who have an early or premature menopause below the age of 45 years. However, this review also highlights important gaps in the existing literature, calling for further research to reliably establish whether cardiovascular risk may vary in relation to the duration since menopause and the mechanisms leading early menopause to cardiovascular outcomes and mortality.

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Supplemental Table S1. Characteristics of included studies that assessed the association of years since menopause with study outcomes

Lead Author, Publication Date (Reference No.)	Name of study or source of participants	Location	Year of baseline survey	Baseline age range/ average age (years)	Study design	Follow up years	Total participants	No. of study events	Outcome	Covariates adjusted for	Quality*
Fioretti, 2000[1]	-	Italy	1983-1992	< 75	Case-control	N/A	1,292	429	Myocardial infarction	Age, study, education, smoking, BMI, diabetes, hypertension, hyperlipidemia, HRT	8
Cho, 2008[2]	Anam Hospital	Korea	Jan 2006 – Oct 2006	59	Cross-sectional	N/A	384	138	Metabolic syndrome; Abdominal obesity; Hypertension; High glucose	Age, BMI	N/A
He, 2012[3]	Beijing	China	2009-2010	40-59	Cross-sectional	N/A	2,498	NS	CVD, CHD, stroke, and several cardiovascular intermediates	Age, BMI, smoking, marriage, education, occupation, lipid and blood pressure lowering therapy	N/A
Stockl, 2014[4]	KORA F4	Germany	2006-2008	50-81	Cross-sectional	N/A	800	NS	Carotid atherosclerosis	Age, BMI, education, family status, PA, smoking, alcohol consumption, total cholesterol, triglycerides, fasting blood glucose	N/A
Total							4,974				

*Based on Newcastle–Ottawa method for cohort and case-control studies. BMI, body mass index; HRT, hormone replacement therapy; N/A, not applicable; NS, not stated; PA, physical activity

Supplemental Table S2. Associations of age at menopause with cardiovascular disease intermediates and outcomes where pooling was not possible

Lead Author, Publication Date	Study design	Outcome	Reference category (years)	Age at menopause (years)	Relative risks (95% confidence intervals)
Brand, 2013[5]	Prospective cohort	Diabetes mellitus	50-54	45-49	0.97 (0.86 to 1.10)
		Diabetes mellitus	50-54	≥ 55	0.85 (0.70 to 1.03)
Qiu, 2013[6]	Cross-sectional	Diabetes mellitus	50	≤ 46	0.96 (0.73 to 1.27)
		Diabetes mellitus	50	47-49	0.87 (0.67 to 1.13)
		Diabetes mellitus	50	51-52	0.97 (0.74 to 1.28)
		Diabetes mellitus	50	≥ 53	0.92 (0.70 to 1.20)
Wu, 2014[7]	Prospective cohort	Diabetes mellitus	48.80-50.15	< 46.64	1.06 (0.72 to 1.56)
		Diabetes mellitus	48.80-50.15	46.64-48.79	1.02 (0.70 to 1.50)
		Diabetes mellitus	48.80-50.15	50.16-52.03	0.90 (0.60 to 1.36)
		Diabetes mellitus	48.80-50.15	≥ 52.04	0.95 (0.63 to 1.42)
		CVD mortality	48.80-50.15	< 46.64	1.01 (0.83 to 1.22)
		CVD mortality	48.80-50.15	46.64-48.79	0.90 (0.74 to 1.09)
		CVD mortality	48.80-50.15	50.16-52.03	0.82 (0.67 to 1.00)
		CVD mortality	48.80-50.15	≥ 52.04	0.89 (0.73 to 1.08)
		CHD mortality	48.80-50.15	< 46.64	1.24 (0.83 to 1.86)
		CHD mortality	48.80-50.15	46.64-48.79	1.07 (0.71 to 1.62)
		CHD mortality	48.80-50.15	50.16-52.03	1.02 (0.66 to 1.56)
		CHD mortality	48.80-50.15	≥ 52.04	1.23 (0.82 to 1.84)
		Stroke mortality	48.80-50.15	< 46.64	0.86 (0.66 to 1.11)
		Stroke mortality	48.80-50.15	46.64-48.79	0.77 (0.60 to 1.00)
		Stroke mortality	48.80-50.15	50.16-52.03	0.76 (0.59 to 0.99)
		Stroke mortality	48.80-50.15	≥ 52.04	0.81 (0.63 to 1.04)
		All-cause mortality	48.80-50.15	< 46.64	1.16 (1.04 to 1.29)
		All-cause mortality	48.80-50.15	46.64-48.79	1.03 (0.92 to 1.15)
		All-cause mortality	48.80-50.15	50.16-52.03	1.11 (0.99 to 1.24)
		All-cause mortality	48.80-50.15	≥ 52.04	0.99 (0.88 to 1.11)
Lisabeth, 2009[8]	Prospective cohort	Ischaemic stroke	< 42	42-54	0.50 (0.29 to 0.89)
		Ischaemic stroke	< 42	≥ 55	0.31 (0.13 to 0.76)

Supplemental Table S3. Associations of years since menopause with cardiovascular disease outcomes where pooling was not possible

Lead Author, Publication Date	Study design	Outcome	Reference category	Years since menopause	Relative risks (95% confidence intervals)
Fioretti, 2000[1]	Case-control	Myocardial infarction	Pre/perimenopausal women	< 10 years	0.85 (0.55 to 1.32)
		Myocardial infarction	Pre/perimenopausal women	≥ 10 < 20 years	0.45 (0.25 to 0.83)
		Myocardial infarction	Pre/perimenopausal women	≥ 20 years	0.52 (0.24 to 1.14)
He, 2012[3]	Cross-sectional	Cardiovascular disease	0-1 years	2-3 years	1.95 (1.12 to 3.38)
		Cardiovascular disease	0-1 years	4-6 years	1.91 (1.00 to 3.65)
		Cardiovascular disease	0-1 years	7-27 years	2.34 (0.93 to 5.86)
		Coronary heart disease	0-1 years	2-3 years	2.13 (1.10 to 4.13)
		Coronary heart disease	0-1 years	4-6 years	1.74 (0.80 to 3.78)
		Coronary heart disease	0-1 years	7-27 years	2.71 (0.94 to 7.76)
		Stroke	0-1 years	2-3 years	1.37 (0.55 to 3.42)
		Stroke	0-1 years	4-6 years	1.96 (0.72 to 5.30)
		Stroke	0-1 years	7-27 years	1.17 (0.26 to 5.28)

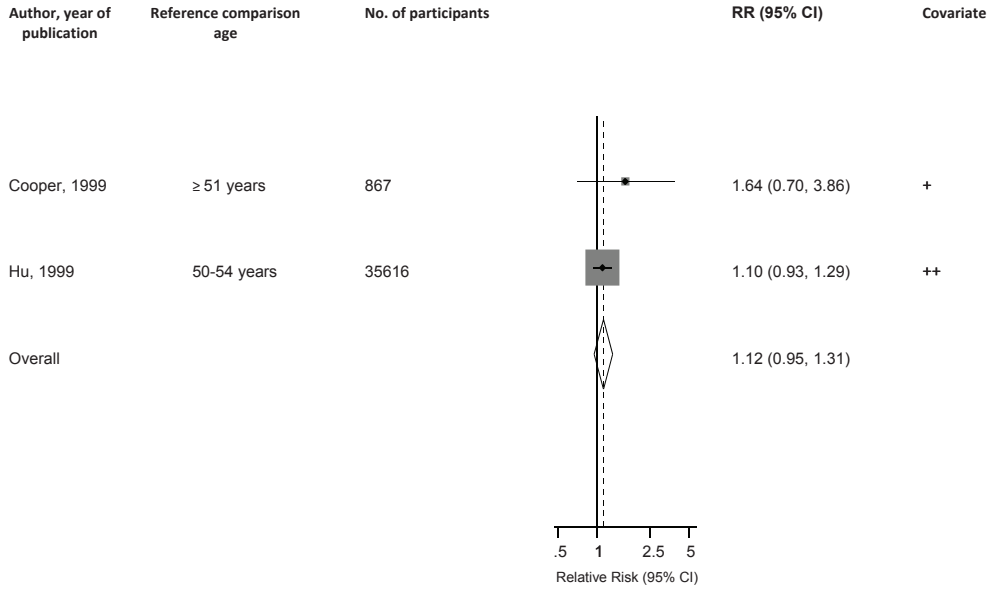
Supplemental Table S4. Associations of years since menopause with cardiovascular disease intermediates where pooling was not possible

Lead Author, Publication Date	Study design	Outcome	Reference category	Years since menopause	Relative risks/Beta (95% confidence intervals)
Cho, 2008[2]	Cross-sectional	Metabolic syndrome	Premenopausal women	< 5	2.59 (1.36 to 4.90)
		Metabolic syndrome	Premenopausal women	5-9	2.67 (1.25 to 5.68)
		Metabolic syndrome	Premenopausal women	10-14	4.03 (1.65 to 9.85)
		Metabolic syndrome	Premenopausal women	≥ 15	1.91 (0.66 to 5.52)
		Abdominal obesity	Premenopausal women	< 5	3.03 (1.10 to 8.30)
		Abdominal obesity	Premenopausal women	5-9	2.87 (0.88 to 9.37)
		Abdominal obesity	Premenopausal women	10-14	1.43 (0.20 to 10.00)
		Abdominal obesity	Premenopausal women	≥ 15	1.78 (0.34 to 9.24)
		Hypertension	Premenopausal women	< 5	3.09 (1.66 to 5.72)
		Hypertension	Premenopausal women	5-9	3.15 (1.51 to 6.57)
		Hypertension	Premenopausal women	10-14	2.59 (1.06 to 6.35)
		Hypertension	Premenopausal women	≥ 15	2.61 (0.92 to 7.41)
		High glucose	Premenopausal women	< 5	2.76 (1.05 to 7.26)
		High glucose	Premenopausal women	5-9	2.77 (0.92 to 8.35)
		High glucose	Premenopausal women	10-14	2.92 (0.79 to 10.77)
		High glucose	Premenopausal women	≥ 15	3.26 (0.71 to 14.91)
He, 2012[3]	Cross-sectional	Diabetes	0-1 years	2-3	0.94 (0.62 to 1.43)
		Diabetes	0-1 years	4-6	0.81 (0.50 to 1.32)
		Diabetes	0-1 years	7-27	0.65 (0.31 to 1.34)
		Hypertension	0-1 years	2-3	1.02 (0.76 to 1.38)
		Hypertension	0-1 years	4-6	1.01 (0.70 to 1.45)
		Hypertension	0-1 years	7-27	0.93 (0.53 to 1.63)
		Obesity	0-1 years	2-3	0.90 (0.66 to 1.23)
		Obesity	0-1 years	4-6	1.05 (0.72 to 1.54)

Supplemental Table S4. Associations of years since menopause with cardiovascular disease intermediates where pooling was not possible

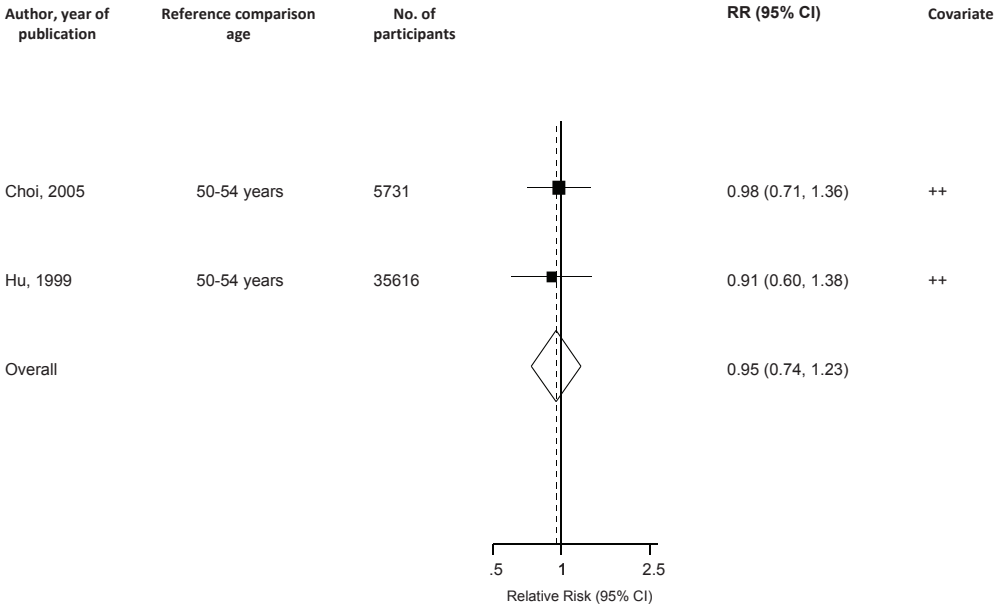
Lead Author, Publication Date	Study design	Outcome	Reference category	Years since menopause	Relative risks/Beta (95% confidence intervals)
		Obesity	0-1 years	7-27	1.08 (0.60 to 1.97)
		Systolic blood pressure	0-1 years	2-3	-0.71 (-2.79 to 1.30)
		Systolic blood pressure	0-1 years	4-6	-1.63 (-4.16 to 0.90)
		Systolic blood pressure	0-1 years	7-27	-1.51 (-5.45 to 2.42)
		Diastolic blood pressure	0-1 years	2-3	-0.16 (-1.33 to 1.02)
		Diastolic blood pressure	0-1 years	4-6	-0.04 (-1.49 to 1.42)
		Diastolic blood pressure	0-1 years	7-27	0.43 (-1.83 to 2.69)
		Body mass index	0-1 years	2-3	0.11 (-0.38 to 0.59)
		Body mass index	0-1 years	4-6	0.11 (-0.49 to 0.71)
		Body mass index	0-1 years	7-27	0.31 (-0.62 to 1.24)
		Waist-to-hip ratio	0-1 years	2-3	-0.01 (-0.01 to 0.01)
		Waist-to-hip ratio	0-1 years	4-6	-0.01 (-0.02 to 0.01)
		Waist-to-hip ratio	0-1 years	7-27	-0.02 (-0.03 to -0.01)
		Glucose	0-1 years	2-3	-0.03 (-0.38 to 0.32)
		Glucose	0-1 years	4-6	-0.4 (-0.83 to 0.04)
		Glucose	0-1 years	7-27	-0.28 (-0.95 to 0.4)
Stockl, 2014[4]	Cross-sectional	Carotid atherosclerosis	≤ 15 years	> 15	0.91 (0.54 to 1.53)

Supplemental Figure S1. Age at menopause 45-49 years and risk of total CHD with menopausal age ≥ 50 years as reference category



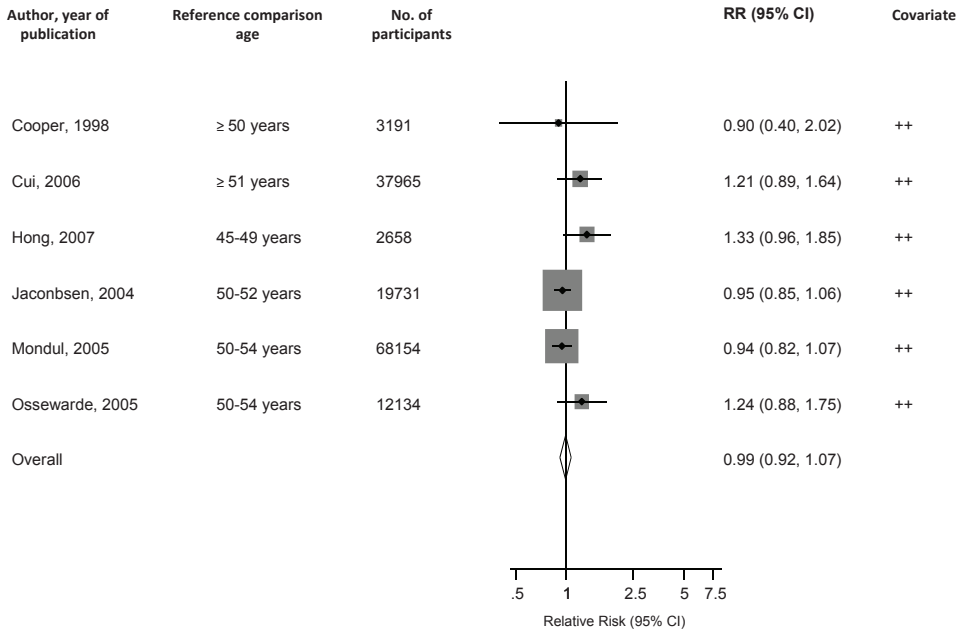
+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, X^2= 0.81, I^2=0%; P = 0.368

Supplemental Figure S2. Age at menopause 45-49 years and risk of total stroke with menopausal age ≥ 50 years as reference category



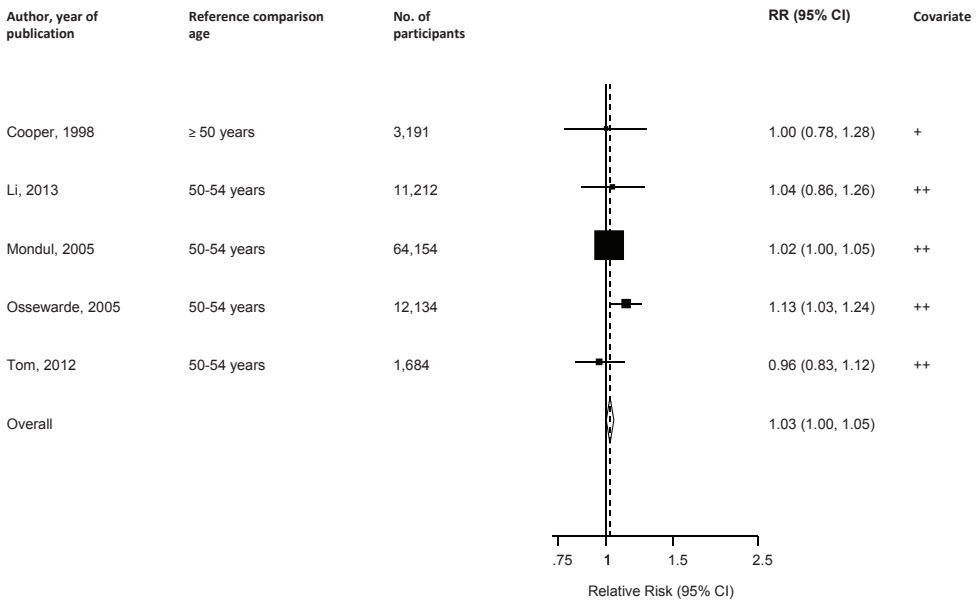
+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, X^2= 0.08, I^2=0%; P = 0.783

Supplemental Figure S3. Age at menopause < 45 years and risk of stroke mortality with menopausal age ≥ 45 years as reference category



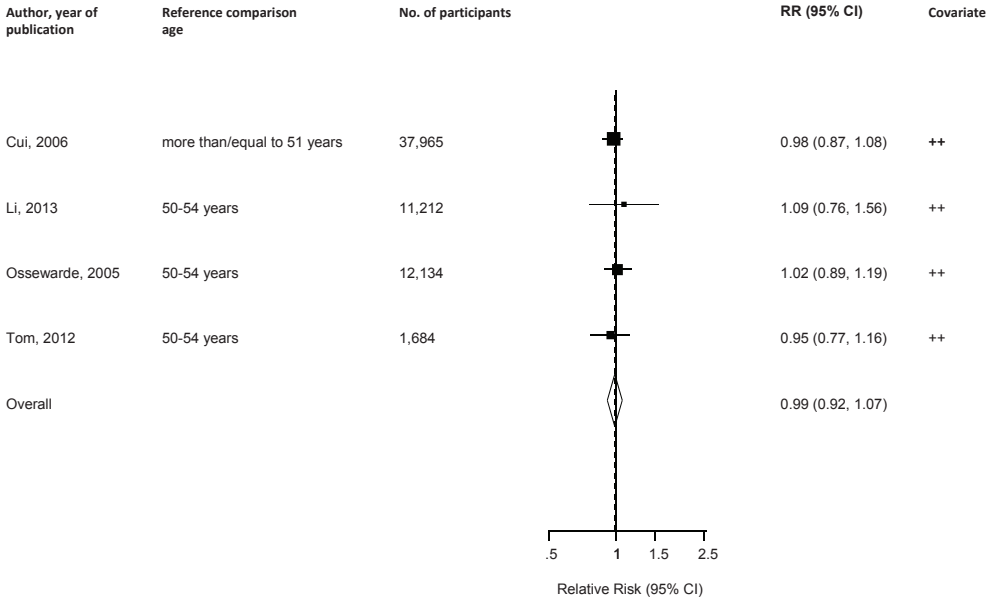
+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, X^2= 7.59, I^2=34%, 0 to 74%; P = 0.181

Supplemental Figure S4. Age at menopause 45-49 years and risk of mortality with menopausal age ≥ 50 years as reference category



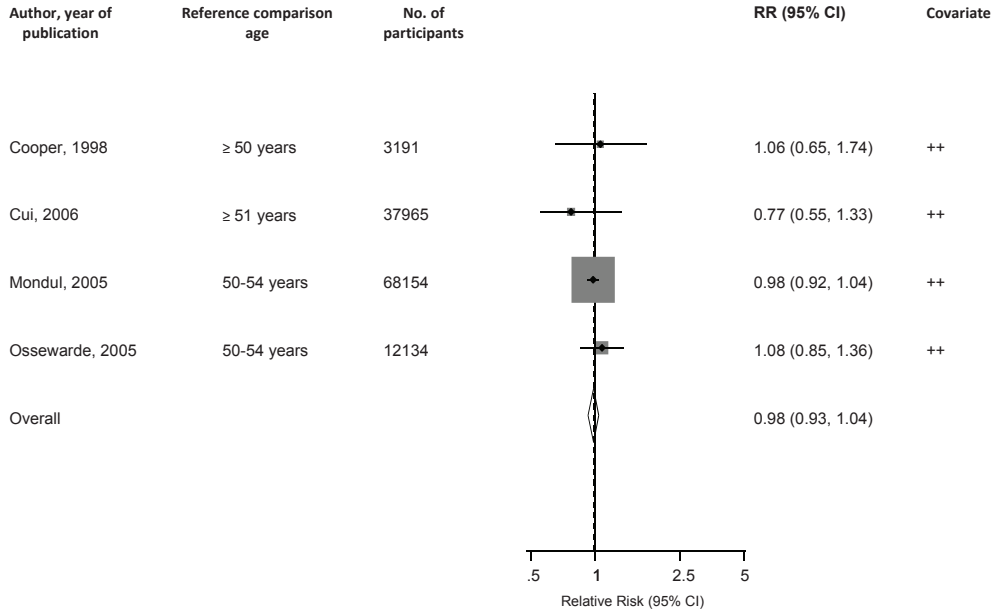
+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, X^2= 5.19, I^2=23%, 0 to 68%; P = 0.268

Supplemental Figure S5. Age at menopause 45-49 years and risk of CVD with menopausal age ≥ 50 years as reference category



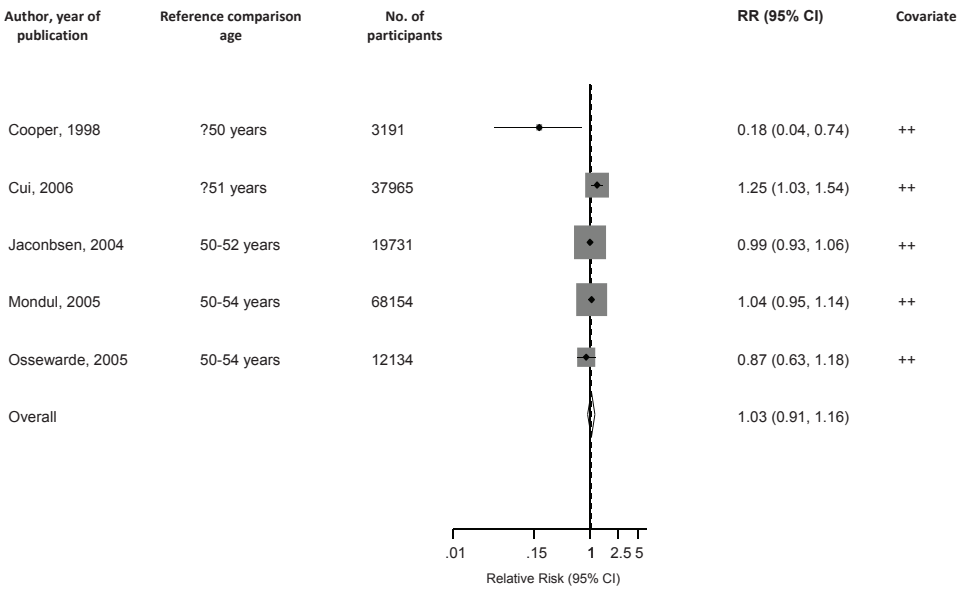
+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, X^2= 0.62, I^2=0%, 0 to 85%; P = 0.891

Supplemental Figure S6. Age at menopause 45-49 years and risk of fatal CHD with menopausal age ≥ 50 years as reference category



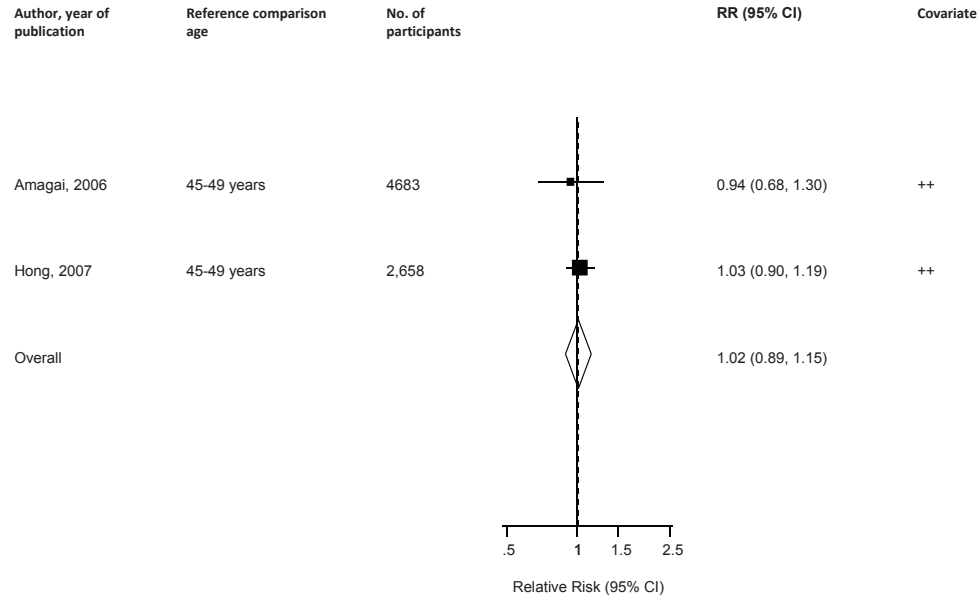
+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, X^2= 1.89, I2=0%, 0 to 85%; P = 0.595

Supplemental Figure S7. Age at menopause 45-49 years and risk of stroke mortality with menopausal age ≥ 50 years as reference category



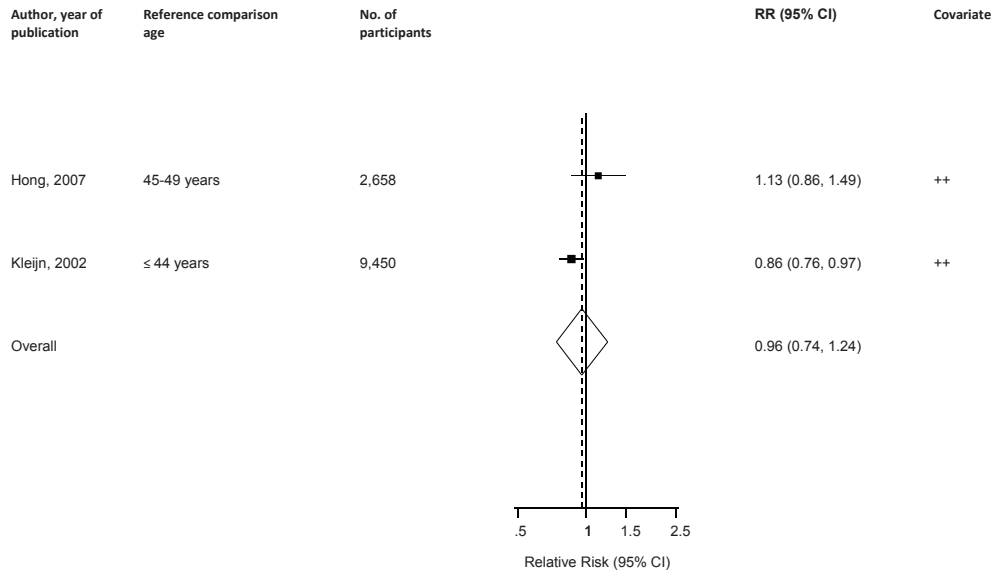
+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, X^2= 11.28, I^2=65%, 7 to 87%; P = 0.024

Supplemental Figure S8. Age at menopause 50-54 years and risk of mortality with menopausal age < 50 years as reference category



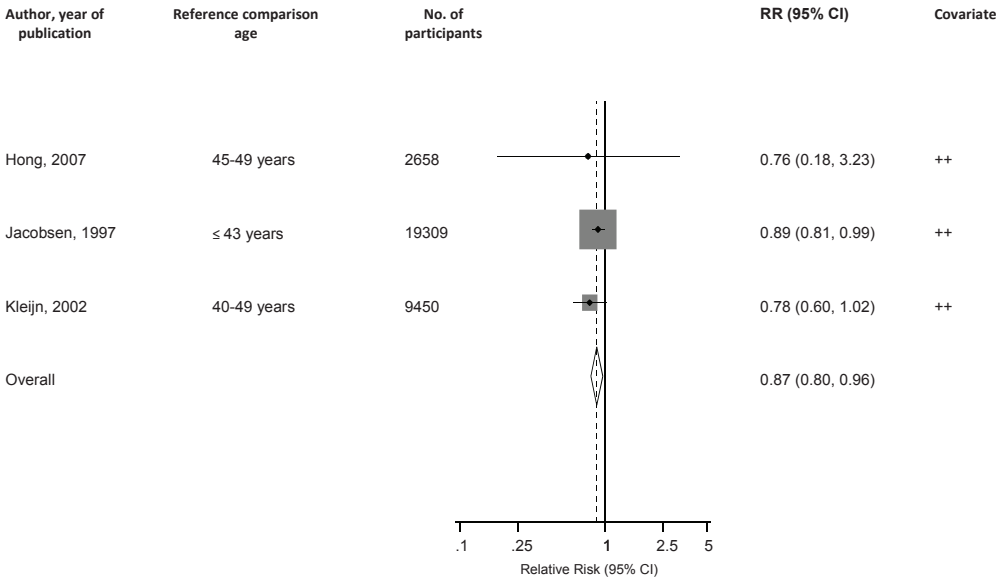
+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, X^2= 0.26, I2=0%, 0 to 0%; P = 0.612

Supplemental Figure S9. Age at menopause 50-54 years and risk of CVD with menopausal age < 50 years as reference category



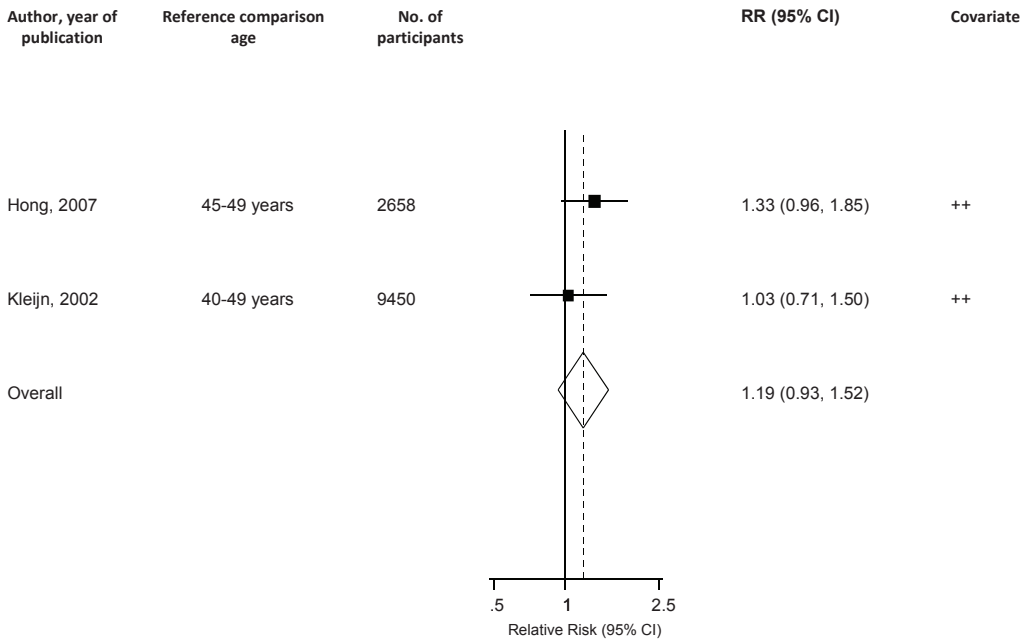
+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, X^2= 3.17, I^2=68%, 0 to 93%; P = 0.075

Supplemental Figure S10. Age at menopause 50-54 years and risk of fatal CHD with menopausal age < 50 years as reference category



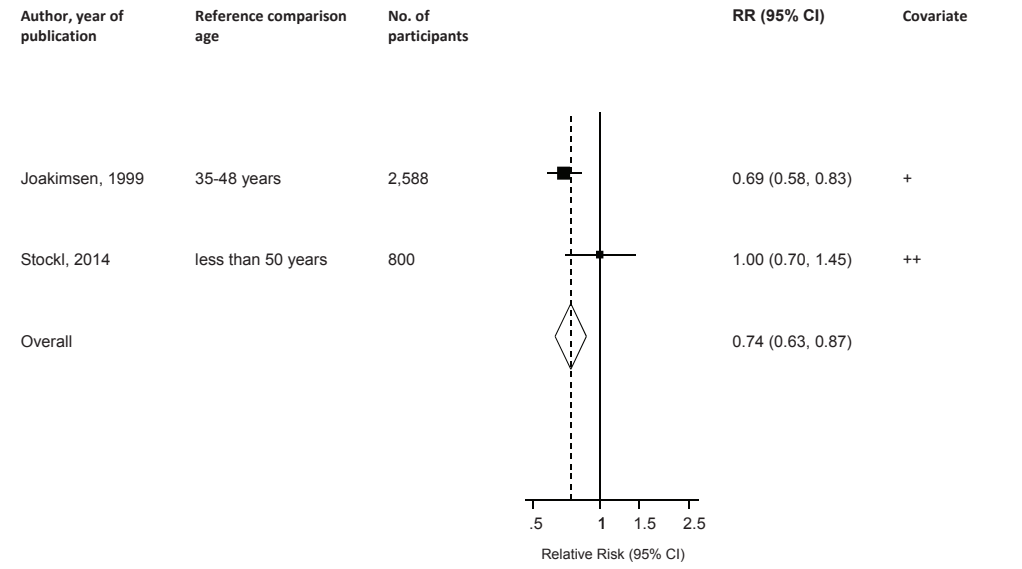
+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, X^2= 0.87, I^2=0%, 0 to 90%; P = 0.648

Supplemental Figure 11. Age at menopause 50-54 years and risk of stroke mortality with menopausal age < 50 years as reference category



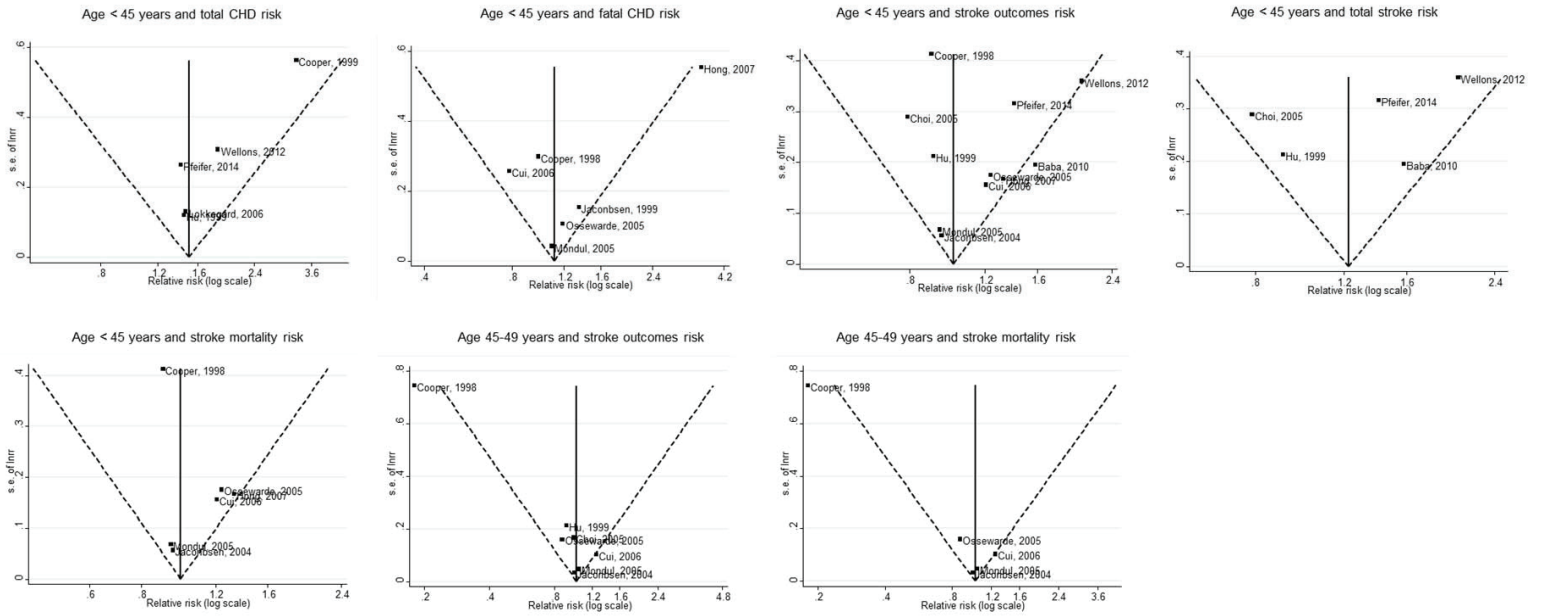
+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, X^2= 1.01, I^2=1%; P = 0.314

Supplemental Figure 12. Age at menopause ≥ 50 years and risk of carotid atherosclerosis with menopausal age < 50 years as reference category in cross-sectional studies



+, adjusted for age; ++, adjusted for vascular risk factors; Assessment of heterogeneity, X^2= 3.21, I^2=69%, 0 to 93%; P = 0.073

Supplemental Figure 13. Assessment of small study effects by funnel plots and Egger's test in prospective studies of age at menopause and cardiovascular disease outcomes



The dotted lines show 95% confidence intervals around the overall summary estimate calculated using a fixed effect model; P-values for bias calculated using Egger's test was 0.078; 0.493; 0.048; 0.831; 0.118; 0.453; and 0.599; for age at menopause < 45 years and total CHD risk; < 45 years and fatal CHD risk; < 45 years and stroke outcomes risk; < 45 years and total stroke risk; < 45 years and stroke mortality risk; 45-49 years and stroke outcomes risk; and 45-49 years and stroke mortality risk respectively; CHD, coronary heart disease

Appendix 3. Search strategy

Relevant studies, published before February 05, 2015 (date last searched), were identified through electronic searches not limited to the English language using MEDLINE, EMBASE, and Web of Science databases. Electronic searches were supplemented by scanning reference lists of articles identified for all relevant studies (including review articles), by hand searching of relevant journals and by correspondence with study investigators. The computer-based searches combined search terms related to age at menopause or duration from onset of menopause and endothelial dysfunction or inflammation or cardiovascular disease or mortality without language restriction.

(i) MEDLINE strategy to identify relevant exposures:

("age at menopause"[All Fields] OR "duration from onset of menopause"[All Fields] OR "Menopause"[Mesh] OR "menopause"[All Fields] OR "Climacteric"[Mesh] OR "climacteric"[All Fields] OR "Perimenopause"[Mesh] OR "perimenopause"[All Fields] OR "Postmenopause"[Mesh] OR "postmenopause"[All Fields])

(ii) MEDLINE strategy to identify relevant outcomes:

("Endothelial dysfunction"[Mesh] OR "endothelial dysfunction"[All Fields] OR "Inflammation"[Mesh] OR "inflammation"[All Fields] OR "Vasodilatation"[Mesh] OR "vasodilatation"[All Fields] OR "Nitric oxide"[Mesh] OR "nitric oxide"[All Fields] OR "Vasoconstriction"[Mesh] OR "vasoconstriction"[All Fields] OR "Hypertension"[Mesh] OR "hypertension"[All Fields] OR "Hypercholeserolemia"[Mesh] OR "hypercholesterolemia"[All Fields] OR "Diabetes mellitus"[Mesh] OR "diabetes"[All Fields] OR "Blood Coagulation"[Mesh] OR "Platelet Adhesiveness"[Mesh] OR "percentage flow mediated dilatation"[All Fields] OR "peripheral arterial tonometry"[All Fields] OR "Acetylcholine"[Mesh] OR "acetylcholine"[All Fields] OR "Angiotensin II"[Mesh] OR "angiotensin II"[All Fields] OR "Endothelin-1"[Mesh] OR "endothelin-1"[All Fields] OR "Hyperhomocysteinemia"[Mesh] OR "hyperhomocysteinemia"[All Fields] OR "Reactive Oxygen Species"[Mesh] OR "reactive oxygen species"[All Fields] OR "Interleukin"[Mesh] OR "interleukin"[All Fields] OR "Vasculitis"[Mesh] OR "vasculitis"[All Fields] "C-Reactive Protein"[Mesh] OR "C-reactive protein"[All Fields] OR "Atherosclerosis"[Mesh] OR "atherosclerosis"[All Fields] OR "P-Selectin"[Mesh] OR "p-selectin"[All Fields] OR "Tumor Necrosis Factor"[Mesh] OR "tumor necrosis factor"[All Fields] OR "Vascular Cell Adhesion Molecule-1"[Mesh] OR "vascular cell adhesion molecule-1"[All Fields] OR "intercellular adhesion molecule-1"[All Fields] "E-Selectin"[Mesh] OR "e-selectin"[All Fields] OR "mortality"[All Fields] OR "all cause mortality"[All Fields] OR "death"[All Fields] OR "survival"[All Fields] OR "Cardiovascular Diseases"[Mesh] OR "Coronary heart disease"[Mesh] OR "Coronary artery disease"[Mesh] OR "Cerebrovascular disease"[Mesh] OR "Stroke"[Mesh])

(iii) MEDLINE strategy to identify relevant population:

("humans"[MeSH Terms])

Parts i, ii and iii were combined using 'AND' to search the MEDLINE. Additionally, each part was specifically translated for searching alternative databases.

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4.6 Effects of Natural and Plant-Based Therapies on Menopausal Symptoms: a Systematic Review and Meta-Analysis

Oscar H. Franco, Rajiv Chowdhury, Jenna Troup*, Trudy Voortman, Setor Kunutsor, Maryam Kavousi, Clare Oliver-Williams*, Taulant Muka**

**indicates equal contribution*

ABSTRACT

CONTEXT: The proportion of women in Western countries who use complimentary therapies to manage menopausal symptoms is estimated to be 40-50%.

OBJECTIVE: To determine the impact of natural and plant-based therapies on the presence and severity of menopausal symptoms.

DATA SOURCES: The electronic databases MEDLINE, EMBASE, and Cochrane Central were systematically searched to identify appropriate studies published before December 16th 2015. Reference lists of the included studies were searched for further identification of relevant studies.

STUDY SELECTION: Randomized controlled trials (RCTs) and observational studies that assessed natural or plant-based therapies and the presence of one or more menopausal symptoms (e.g. hot flashes and night sweats).

DATA EXTRACTION: Data were extracted by two independent reviewers using a pre-designed data collection form.

MAIN OUTCOME AND MEASURES: Menopausal symptoms including, but not limited to, hot flashes, night sweats, and Kupperman Index.

RESULTS: In total, 119 articles based on 113 unique studies (101 RCTs and 12 prospective non-randomized intervention or observational studies) were identified, including a total of12,443 individual women. After meta-analyses of RCTs' estimates, use of phytoestrogens significantly reduced the reported number of daily hot flashes between the treatment groups [pooled mean

difference of changes: -1.26 (95%CI: -1.87, -0.65)] and significantly improved vaginal dryness score [pooled mean difference of changes: -0.31 (-0.52, -0.10)]. Individual phytoestrogen interventions such as dietary and supplemental soy isoflavones were beneficial for menopausal outcomes in general, and number of night sweats in 24 hours in particular. Additionally, behavioural therapies, acupuncture, and several herbal remedies in aggregate improved overall menopausal symptoms as measured by the Kupperman Index [pooled mean differences: -11.10 (-17.17, -5.03), -8.41 (-11.81, -5.0), and 6.72 (-8.10, -5.33), respectively]. There was substantial diversity in quality across the available studies.

CONCLUSIONS AND RELEVANCE: Findings indicate that composite and certain specific phytoestrogen supplementations may confer a benefit of reducing menopausal symptoms in women. Additionally, several other plant-based and natural therapies may also effectively improve menopausal symptoms. However, owing to a generally sub-optimal quality and the heterogeneous nature of the current evidence, further rigorous studies are needed to determine their impact on menopausal health and symptoms alleviation.

INTRODUCTION

Menopause is considered the end of a woman's reproductive life, generally indicated by the time when menstrual periods stop permanently[1]. While menopause is usually a natural change, the menopausal transition and its associated changes vary widely[2,3]. There is a range of symptoms that have been associated with menopause, including hot flashes, night sweats and vaginal dryness, with 50.3% to 82.1% of menopausal women reporting hot flashes or night sweats[4,5]. Medical treatments for these symptoms are available, including hormone replacement therapy (HT); however, given the potentially negative health consequence of HT on cardiovascular health and breast cancer[6,7], many women choose to use natural or plant-based therapies. The proportion of women in Western countries using complimentary therapies to manage menopausal symptoms is approximately 40-50%[8,9].

There is a broad range of natural and plant-based therapies that purport to have a positive effect on menopausal symptoms. These therapies include the oral use of phytoestrogens such as dietary isoflavones and soy extracts; herbal remedies such as red clover and black cohosh; and non-biological treatments, such as acupuncture and yoga. Although the potential impact of these treatments on menopausal symptoms has been assessed in many individual randomized trials[10-12], most of these intervention studies were limited by (i) inadequate power (i.e. limited sample size), (ii) short follow-up period, (iii) suboptimal quality (eg, high dropout rates), and (iv) inconsistent findings. While several attempts to summarize the available evidence have been conducted, they are also limited owing to (i) a focus on a specific therapy (eg, effects of phytoestrogens)[13], (ii) evaluation of a specific symptom (eg, hot flashes), and being (iii) non-quantitative[14] or largely non-systematic in nature[15]. Therefore, considering the increasingly wide-ranging use of natural and plant-based therapies by women to alleviate menopausal symptoms, an updated and comprehensive quantitative review on their efficacy is essential.

We present a systematic review and meta-analysis of all available intervention and observational studies to quantify the impact of a range of natural and plant-based therapies on menopausal symptoms.

METHODS

Data Sources and Search Strategy

This review was conducted using a predefined protocol and in accordance with PRISMA and MOOSE guidelines (**Appendices 1** and **2**). Three electronic databases (Medline Ovid, Embase and Cochrane Central) were searched until December 16th 2015 without language restriction. The computer-based searches combined terms related to (1) the exposures (or interventions, where appropriate) such as phytoestrogens, black cohosh, *ERr731 rhubarb raponticin*, and various alternative or complimentary natural therapies referred as to whole medical system (including *herbal medicine, traditional Chinese medicine, ayurveda, naturopathy, chiropractic, osteopathy,*

massage, yoga, relaxation therapy, homeopathy, aromatherapy, and therapeutic touch)[16]; and (2) menopausal outcomes (e.g., *hot flashes, night sweats, vasomotor symptoms*) in humans (**Appendix 3)**. 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 selected studies and reviews identified on the topic were searched to identify additional publications.

Study Selection and Eligibility Criteria

Intervention studies were eligible if they: (1) were randomized controlled trials (RCTs) or non-randomized trials; (2) assessed effects of any complementary or alternative therapy listed above in perimenopausal, menopausal, or postmenopausal women compared to a placebo or no treatment; and (3) collected endpoints for menopausal symptoms, including hot flashes, night sweats, the Kupperman Index [17] and Greene Climacteric Scale[18]. To maintain consistency and due to difficulty of interpreting results without a placebo or control, head-to-head trials without a placebo group that compared non-hormonal therapies with estrogen or with other medications were excluded. Study populations in the eligible trials included women experiencing menopausal symptoms recruited from health care settings or general populations. No restrictions on length of follow up were applied for RCTs.

Prospective observational studies were eligible for inclusion if they followed study participants for at least one year, and if they assessed associations between any of the exposures of interest (as defined above) and subsequent risk of perimenopausal, menopausal, or postmenopausal women experiencing menopausal symptoms.

Data Extraction

The exposures or interventions eligible for inclusion in the current review were summarized using the following broad groupings: (i) biologically-based therapies such as phytoestrogens (including dietary isoflavones and extracts of soy, red clover and other phytoestrogens), black cohosh, and *ERr731 rhubarb raponticin*; (ii) non-biological therapies, such as mind-body and behavioural therapies (including yoga and applied relaxation), manipulative, body-based, and energy therapies (including reflexology, therapeutic massage. and aromatherapy), and acupuncture; and (iii) medicinal herbs (including Chinese or other medicinal herbs). Two authors independently extracted data and a consensus was reached in case of any inconsistency with involvement of a third author. A predesigned electronic data abstraction form was used to extract relevant information. In case of multiple publications, the most up-to-date or comprehensive information was extracted.

Assessing the Risk of Bias

Two reviewers independently rated the quality of studies. The Cochrane Collaboration's tool[19] and the Newcastle-Ottawa Scale[20] were used to assess the risk of bias in RCTs and in prospective observational studies respectively. Detailed information on the assessment of study quality and risk of bias is provided in **Appendix 4**.

Statistical Analysis

For intervention studies, treatment effects were defined as the differences in outcomes between the treatment and placebo at the end of the trial. For continuous outcomes, summary measures were presented as mean differences. For data reported as medians, ranges, or 95% confidence intervals (CIs), we calculated means and standard deviations as previously described[21]. To enable a consistent approach to the meta-analysis and enhance interpretation of the findings, units of measurement were converted where appropriate. Most crossover trials in this review did not report adequate crossover analysis, and therefore, we used data from the first period only[22]. The inverse variance weighted method was used to combine summary measures using random-effects models to minimise effects of between-study heterogeneity[23]. Fixed-effect models were used in subsidiary analyses. Heterogeneity was assessed using the Cochrane χ^2 statistic and the I^2 statistic, and was distinguished as low ($I^2 \leq 25\%$), moderate ($I^2 > 25\%$ and $< 75\%$) or high ($I^2 \geq 75\%$)[24]. Publication bias was evaluated through funnel plots and Egger's regression symmetry tests[25]. Sensitivity analyses were done to assess the influence of each individual study, by omitting the studies that had the largest effect on the overall result one by one. Furthermore, we restricted the analysis to studies that did not include participants with a history of breast cancer. Study-level characteristics including geographical location, duration of treatment, number of total participants, and risk of bias were pre-specified as characteristics for assessment of heterogeneity, and were evaluated using stratified analyses and random effects meta-regression[26]. Only estimates derived from RCTs were pooled in the meta-analysis. Given the heterogeneous nature of the study characteristics, exposures evaluated, and outcomes, we did not perform a quantitative summarization of observational or non-randomized studies. A narrative synthesis and construction of descriptive summary tables were done for these studies that could not be quantitatively pooled. All tests were two-tailed and p-values of 0.05 or lower were considered significant. STATA release 13 (Stata Corp, College Station, Texas, US) software was used for all analyses.

RESULTS

Study Identification and Selection

In total, we identified 4,908 relevant citations. After screening based on titles and abstracts, 207 articles were selected for detailed evaluation of their full texts. Of those, 119 articles, based on 113 unique studies, met our inclusion criteria and were included in the review: seventy articles about biologically-based therapies, 36 on whole medical systems, nine on mind-body and behaviour-related therapies and four articles on manipulative, body-based, and energy therapies (**Figure 1; Appendix 4**).

Characteristics of Included Studies

Among the 113 included studies, 101 were RCTs and 12 were prospective non-randomized intervention or observational studies (**Table 1** and **eTables 1-3)**. In aggregate, the 113 studies reported results for 12,443 unique women. Thirty-nine studies were based in Europe; 32 in Asia; 2220 in North America; 11 in South America; eight in Australia, and one in Egypt. The baseline age of participants ranged from 18 to 75 years. For the RCTs, the duration of the interventions ranged

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

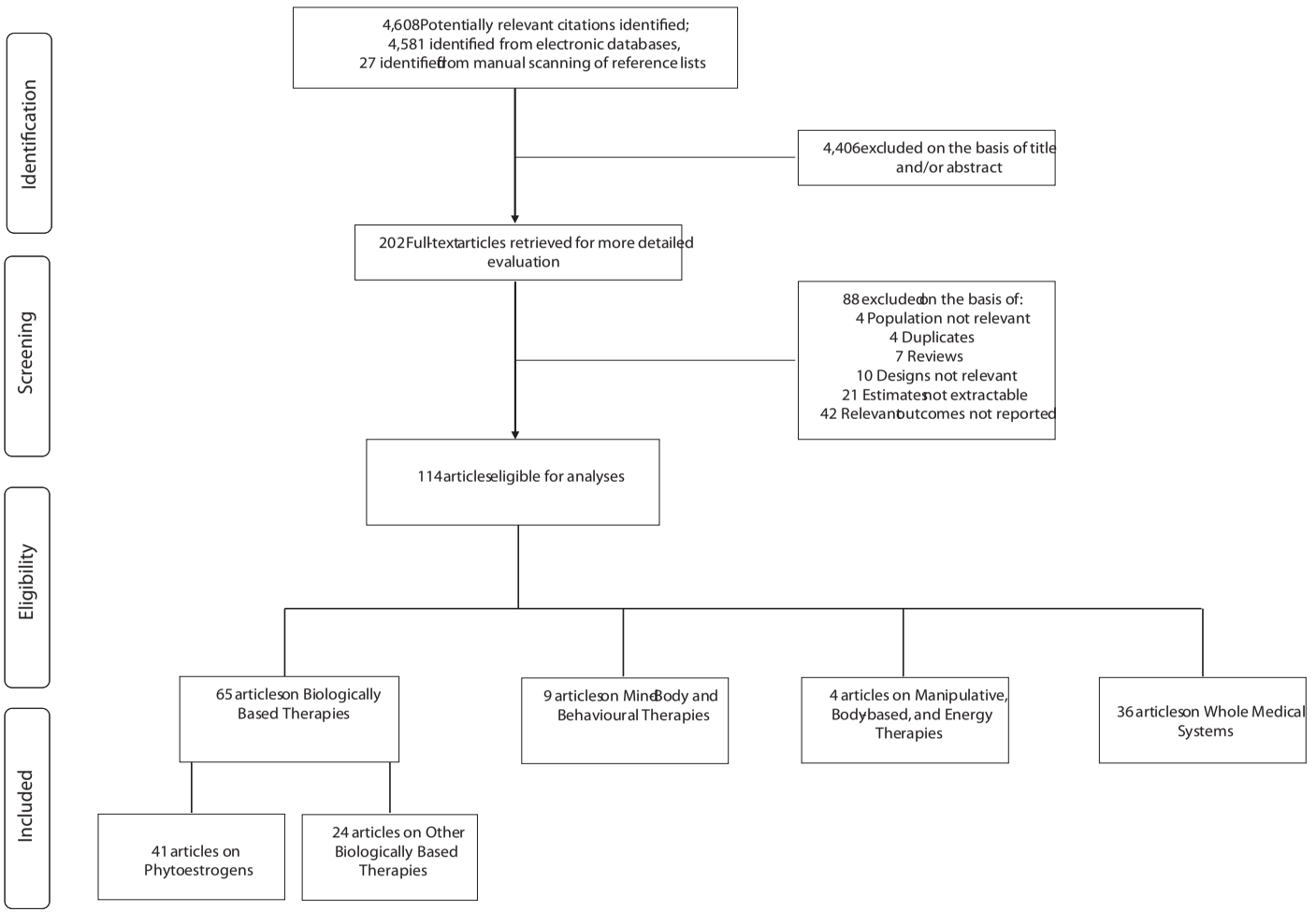


Table 1 Summary characteristics of the included studies

	Biologically-based therapies	Whole medical systems	Mind-body and behavioural therapies	Manipulative, body-based and energy therapies
Eligible studies				
No. of unique studies	69	33	7	4
Median (IQR) duration of follow-up, weeks	12.0 (12.0-24.0)	12.0 (7-13)	12.0 (12.0-13.0)	14.7 (8.0-18.2)
Study Design				
Randomised trials	64	26	7	4
Non-randomised trials	3	0	0	0
Non-randomised observational studies	2	6	0	0
Other	0	1*	0	0
Participants				
Total no. of participants	8344	2883	863	353
Median (IQR) no of participants	84 (54-145)	72 (46-102)	120 (70-200)	68 (45-132)
Median age (IQR), years	53 (52-54)	52 (50-53)	50 (49-55)	52 (51-53)
Location				
Europe	27	9	2	1
North America	12	7	2	1
Asia-Pacific	20	16	3	1
South America	10	0	0	1
Africa	0	1	0	0

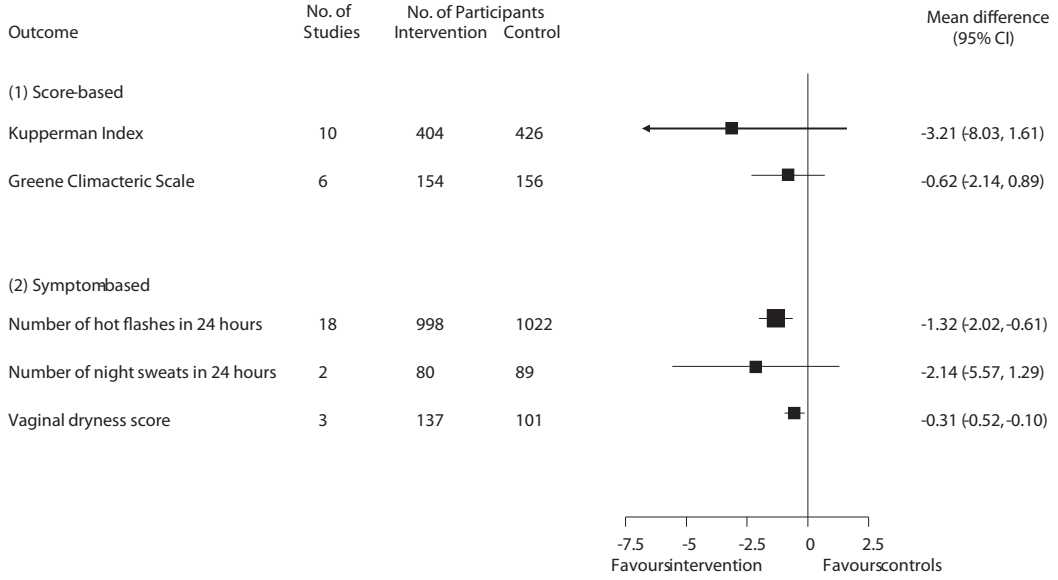
*Used both randomised trial and non-randomised observational study designs

from four weeks to two years, but the majority had a 12-week intervention period. The follow-up period or duration of treatment for prospective non-randomized intervention or observational studies ranged from four weeks to six years.

Effects of Biological Therapies on Menopausal Outcomes

Forty-six studies examined the effect of overall phytoestrogen supplementation on menopausal symptoms and scores. Of those, only data from thirty-five studies (all RCTs) contributed to the meta-analysis, which showed a decrease in the number of daily hot flashes with a pooled mean difference between the treatment groups of -1.26 (95% CI: -1.87, -0.65) (**Figure 2**). There was evidence of similar beneficial effects on vaginal dryness scores (pooled mean difference of changes between treatment groups: -0.31 (-0.52, -0.10) and 24-hour night sweat symptoms (pooled mean difference of changes: -2.14 (-5.57, 1.29), though the latter effect was not significant. There was no improvement in the score-based menopausal symptom assessments quantified by the Kupperman Index (pooled mean difference between treatment groups: -3.21 (-6.09, 1.61)) or by Greene Climacteric Scale (pooled mean difference between treatment groups: -0.62 (-2.14, 0.89)) (**Figure 2** and **eFigures 1-2)**. Study-specific estimates from studies not included in the meta-analyses generally supported improvements in individual symptoms, particularly in the number of hot flashes within 24 hours (**eTables 4-6**).

Figure 2 Effects of Phytoestrogens supplementation on menopausal outcomes in the available randomised controlled trials



Separate meta-analyses were performed for different types of interventions, including evaluation of dietary isoflavones and supplements and extracts of soy isoflavones (twenty studies), and red clover (9 studies) (**Figure 3** and **eFigures 3-6)**. The results of the analyses on the effects of supplements and extracts of soy isoflavones overall replicated the findings of the larger combined analyses (**eFigure 4**). For dietary soy isoflavones there was evidence of significant reductions in the number of night sweats in 24 hours (pooled mean difference of changes: -0.40 (-0.79, -0.01)), but the result was based on a single trial only (**eFigure 5**). There was no association between red clover and any of the menopausal symptoms or scores, except for the number of night sweats in 24 hours (**eFigure 6**).

The effect of black cohosh on menopausal symptoms was assessed in six RCTs. Overall, black cohosh did not have any beneficial effect on the number of hot flashes or night sweats within 24 hours, or the Kupperman Index. Black cohosh improved the Greene Climacteric Scale significantly (pooled mean difference of changes: -4.45 (-7.18, -1.72)), but was only assessed in one study (**Figure 3** and **eFigure 7**). Of the six studies of black cohosh that could not be included in the meta-analysis, one study found an improvement in menopausal symptoms score, another study reported an improvement in the Kupperman Index and in a variety of other menopausal symptoms, including hot flashes, sweating, and insomnia, as did two other studies that combined black cohosh with other therapies (**eTables 5** and **6**). One study did not find any effect of black cohosh on menopausal symptoms (**eTables 5** and **6**). Nine additional studies of other biological therapies were identified and have been summarized in **eTable 5**.

Figure 3 Effect of specific biological therapies on menopausal scores and symptoms.

Outcome	Studies	Intervention	Control	Mean difference (95% CI)
Dietary and Supplemental SI				
Kupperman Index	5	222	238	-2.61 (-4.78, -0.43)
Green Climacteric Scale	1	76	79	-0.92 (-3.53, 1.69)
Number of hot flashes in 24 hours	10	480	502	-0.79 (-1.35, -0.23)
Number of night sweats in 24 hours	1	30	30	-0.40 (-0.79, -0.01)
Vaginal dryness score	1	59	65	-0.26 (-0.48, -0.05)
Red clover				
Kupperman Index	2	103	112	-3.61 (-40.06, 32.85)
Green Climacteric Scale	3	53	52	-0.65 (-2.80, 1.49)
Number of hot flashes in 24 hours	7	320	329	-1.85 (-3.88, 0.19)
Number of night sweats in 24 hours	1	50	59	-3.90 (-4.51, -3.30)
Black cohosh				
Kupperman Index	2	103	63	-0.01 (-2.24, 2.23)
Green Climacteric Scale	1	15	13	-4.45 (-7.18, -1.72)
Number of hot flashes in 24 hours	4	269	232	-0.63 (-2.15, 0.89)
Number of night sweats in 24 hours	1	80	84	0.08 (-0.31, 0.47)

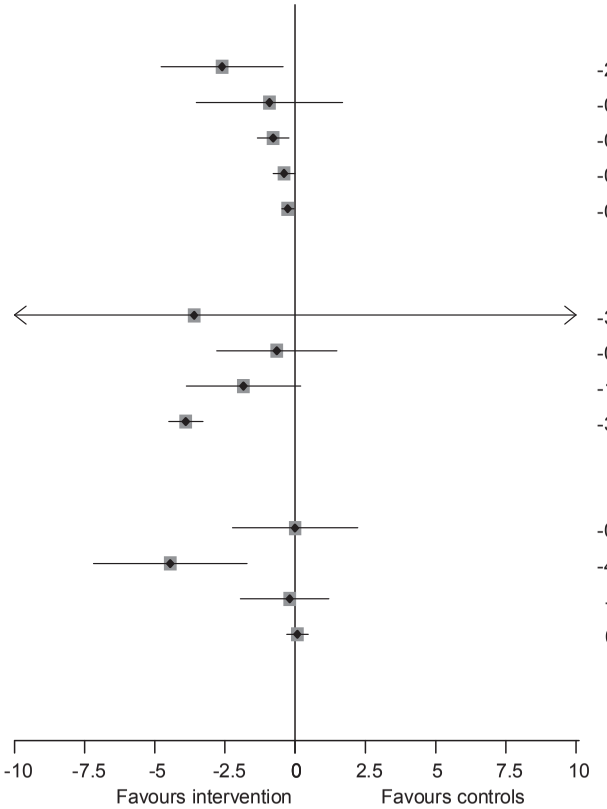
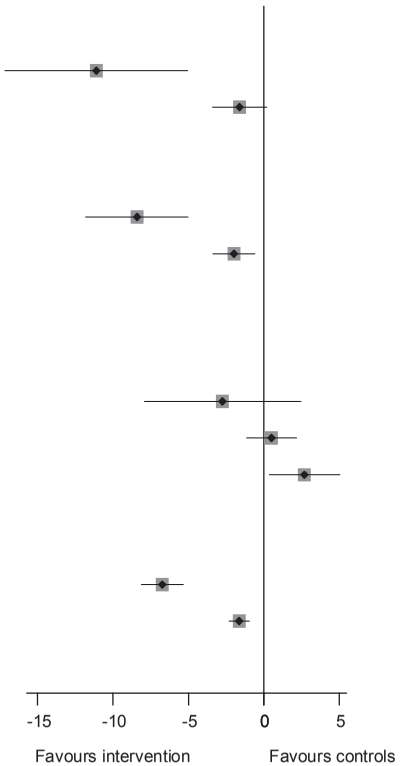


Figure 4 Effect of non-biological therapies and medical herbs on menopausal outcomes in the available randomized controlled trials

Outcome	Studies	Intervention	Control	Mean difference (95% CI)
Non-biological therapies				
(1) Mind, body, behavioral therapies				
Kupperman Index	1	13	20	-11.10 (-17.17, -5.03)
Number of hot flashes in 24 hours	3	153	189	-1.61 (-3.40, 0.19)
(2) Acupuncture				
Kupperman Index	3	93	79	-8.41 (-11.81, -5.02)
Number of hot flashes in 24 hours	6	366	292	-1.99 (-3.38, -0.60)
Medicinal herbs				
(1) Chinese medicinal herbs				
Kupperman Index	2	71	72	-2.73 (-7.92, 2.46)
Number of hot flashes in 24 hours	1	46	46	0.52 (-1.13, 2.17)
Number of night sweats in 24 hours	1	50	50	2.70 (0.35, 5.05)
(2) Other medicinal herbs*				
Kupperman Index	2	110	110	-6.72 (-8.10, -5.33)
Number of hot flashes in 24 hours	1	55	55	-1.62 (-2.29, -0.96)



*, Includes EstroG-100, Err 731, and Pycnogenol

Briefly, five studies found an improvement in symptoms with therapies including evening primrose, flaxseed, St. John's Wort, and combined therapies, while three studies of guar gum, flaxseed, and St John's Wort with Chaste tree found no difference (**eTable 5**).

Effects of Non-biological Therapies and Herbal Remedies

Findings from the meta-analyses were based solely on available data from RCTs, including three on mind, body, and behavioural therapies; eight on acupuncture; four on Chinese medicinal herbs; and three on other medicinal herbs (**Figure 4** and **eFigures 8-11)**. A single study demonstrated an improvement in overall menopausal symptoms measured by the Kupperman Index with mind, body, and behavioural therapies, specifically relaxation sessions (pooled mean difference of changes: -11.10 (-1.17, -5.03)). For the same intervention, based on three RCTs, a similar, albeit weaker, reduction in the number of hot flashes in a 24 hour period was observed (pooled mean difference of changes: -1.61 (-3.40, 0.19)). The results of additional studies that assessed other non-biological therapies are summarized in **eTable 7**. Specifically, while two studies demonstrated an improvement in symptoms through treatments including therapeutic massage and aromatherapy, studies that used yoga or passive movement as interventions found no such improvements.

The meta-analyses of six RCTs that used acupuncture as the intervention found significant reductions in the number of daily hot flashes (pooled mean difference of changes: -1.99 (-3.38, -0.60)) and an improvement in the Kupperman Index (pooled mean difference of changes: -8.41 (-11.81, -5.02)) across treatment groups (**Figure 4**). Similarly, meta-analyses of the studies that used non-Chinese medicinal herbs also demonstrated significant improvements in the number of hot flashes in 24-hours (pooled mean difference of changes: -1.62 (-2.29, -0.95)) and total Kupperman Index (pooled mean difference of changes: 6.72 (-8.10, -5.33)). No overall evidence of improvements in menopausal outcomes was observed for Chinese medicinal herbs (**Figure 4**).

Sensitivity analyses and assessments of Bias, Study Quality and Heterogeneity

For pooled analyses involving five or more studies, exclusion of any single study at one time from the meta-analysis, or exclusion of trials that included participants with a history of breast cancer had minimal effect on the pooled estimates (data available upon request). Seventy of the included RCTs demonstrated a high risk of bias within one or more areas of study quality (**eTable 8**). Three RCTs were found to have a low risk of bias in all areas. An additional 38 studies were found to be at low risk but with some lack of clarity in important aspects. All of the non-randomized studies were also considered to be at high risk of bias (**eTable 3**). Ten of 17 analyses showed high between-study heterogeneity, with an I^2 estimate exceeding 75% ($p<0.01$ for the Cochrane $\chi 2$ statistic) (**eTable 8**). This level of heterogeneity could be explained by differences between studies due to results derived from heterogeneous populations, methods, and effect estimates (**eTable 9**). Furthermore, for trials evaluating the effect of phytoestrogen supplementation on Kupperman Index and the number of hot flashes in 24 hours, the identified heterogeneity was explained to a large extent by differences in the study quality and location (**eTable 9**) (**eFigures 12-13**). For trials on the effect of soy isoflavones on the number of daily hot flashes, the identified heterogeneity was explained by location whereas for trials examining the effect of red clover supplementation on the number of

hot flashes in 24 hours, heterogeneity was not explained by any of the study-level characteristics assessed (**eTable9**) (**eFigures 14-15**). Publication bias was assessed visually using Begg's funnel plots for those meta-analyses that included five or more studies. Upon examination, four out of six of the plots were approximately symmetrical (**eFigure 16**), while the rest (n=2) were asymmetrical. The Egger's test estimates were non-significant ($p>0.05$) for all analyses that involved a minimum of five studies.

DISCUSSION

In this systematic review and meta-analysis we found that a number of plant-based and natural therapies might help improve both individual and collective menopausal symptoms in women. Composite phytoestrogen supplementation and individual phytoestrogen interventions such as dietary and supplemental soy isoflavones significantly improved menopausal symptoms in general, and vasomotor symptoms in particular. Additionally, several mind, body, and behavioural therapies including relaxation, acupuncture, and various medicinal herbs improved collective menopausal symptoms. Nonetheless, there was substantial diversity in scientific rigour and quality across the available studies.

We observed significant beneficial effects of composite phytoestrogen supplementation on menopausal symptoms in the RCTs. Our subsidiary analyses to differentiate the overall effects of phytoestrogens into whole foods, soy protein, and isoflavone extract supplementation groups yielded broadly similar results. While, supplementing with red clover, a rich source of phytoestrogens formononetin, biochanin A, daidzein, and genistein[27], was not associated with the frequency of hot flashes, it generally conferred a benefit for night sweats, but the evidence is too limited to draw a conclusion. Compared with the most recent previous quantitative reviews, our review had substantially greater power (overall and in specific associations); evaluated the impacts of a wide range of plant-based and other natural therapies on several menopausal symptoms (including hot flashes, night sweats, vaginal dryness score and Kupperman Index) in one single comprehensive investigation; and yielded similar (albeit more precise) effect estimates for various supplements such as soy isoflavone extracts and red clover[13,28].

There may be a plausible biological argument for these effects of phytoestrogens. It is well-known that estrogen levels decrease during menopause and that, menopausal symptoms are a consequence[29]. Accordingly, phytoestrogens, plant compounds with estrogen-like properties[30] can be an effective treatment. The two major subtypes of phytoestrogen, isoflavones and lignans, have a similar chemical structure to estradiol (i.e., a form of estrogen) and therefore also appear to exhibit an estrogenic effect. Taken together, this may explain the aggregate beneficial effects observed in this review in the phytoestrogen supplementation trials. However, this mechanism of action could also cause adverse effects; there is evidence that isoflavone supplement use was associated with an increased rate of endometrial hyperplasia[31].

Second, we found no association between black cohosh (Cimicifuga racemosa or Actaea racemosa) supplementation and menopausal symptoms. Although black cohosh remains a widely studied and

popular herbal remedy, there has been lack of clarity regarding the identity of its active compounds and its mechanism of action, as well as concerns over possible adverse effects[32]. Beyond these existing uncertainties, the lack of beneficial effects evident in the current meta-analysis does not support the use of black cohosh to reduce menopausal symptoms. Third, among the non-biological interventions, there was evidence that acupuncture and behavioural therapies were beneficial. It has been proposed that acupuncture, defined as the practice of inserting needles into certain points in the body for therapeutic purposes[33], may favourably affect menopausal symptoms by increasing the activity of hypothalamic β-endorphin[34], and may favourably affect the nervous system by modulating the levels of various neurotransmitters and neuropeptides[35]. Nonetheless, given the insufficient number of powerful, high-quality, and detailed trials, especially those adequately controlled for sham treatment or placebo in the comparison arm, further evidence is needed to conclude whether acupuncture effectively reduces menopausal symptoms. Similarly, while behavioral interventions appeared to be safe and potentially effective in our analysis, this early, limited-scale evidence requires further careful study. Finally, our analyses involving trials of medicinal herbal remedies showed no overall effect of Chinese medicinal herbs such as dong quai on the Kupperman index for. By contrast, combined analyses involving trials that assessed other newer herbal remedies such as EstroG-100 (a mixture of standardized extracts of Cynanchum wilfordii, Phlomis umbrosa and Angelica gigas), ERr 731 (an extract isolated from Rheum rhaponticum), and pycnogenol (extract from pine bark), appeared to improve the Kupperman index. However, more trials are needed to determine the efficacy of these products on menopausal symptoms since only a few trials have been conducted.

To the best of our knowledge, this is the first quantitative review of randomized and non-randomized studies that assesses a wide range of plant-based and natural therapies in relation to menopausal symptoms in a single comprehensive investigation. The analyses, in aggregate, included more than twelve thousand women and evaluated a broad array of menopausal symptoms, ranging from specific symptoms such as vaginal dryness and hot flashes, to collective symptom scores. There are a number of limitations to this study. Firstly, typical to any literature-based meta-analysis, it is possible that both measured and unmeasured publication bias can limit, at least in part, our overall findings. In this regard, although evaluations with the conventional funnel plots and Egger's test estimates indicate minimal impact of publication bias, these approaches are limited by a qualitative nature reliant on visual inspection and the fact that the majority of these assessments were based on between five to ten studies. Therefore, despite all efforts made to undertake a comprehensive search of the published and unpublished literature, we cannot exclude the possibility of publication bias stemming from underreporting of negative findings. Also, inclusion of data from potentially poorly conducted studies is undoubtedly a limitation of the current review. Variation in study quality has contributed to the heterogeneity of findings noted in several of the meta-analyses. Other sources of heterogeneity are likely to include population differences, including ethnicity, a factor in the presence of menopausal symptoms[36,37] and differing age ranges. Furthermore, the supplements used in the trials included in this study may vary in quality and composition (ie, how much of the active ingredient is actually in the supplement), which might have contributed in the heterogeneity in effects observed in our analyses. Also, while we assessed different outcome

measures of menopause symptoms, covering their frequency and severity, there may be problems of comparability due the varying degrees of the outcome measures[38]. Since the number of available studies in some analyses was small, it precluded our ability to quantitatively investigate the sources of the observed heterogeneity beyond the qualitative descriptions of the individual studies presented in **eTable 9**. Also, self-reported measures of vasomotor symptoms may be subject to memory and reporting bias. Therefore, future studies that assess vasomotor symptoms physiologically with, for example, an ambulatory hot flash monitor to measure skin conductance, are needed. Considering the levels of heterogeneity identified and the low degree of quality of some of the studies included, the results of this systematic review and meta-analysis should be interpreted with caution. Further randomized clinical studies utilizing an adequately designed strict protocol with sufficient sample size, adequate blinding of outcome assessment, participants and personnel, proper allocation concealment, and sufficient follow up period are needed.

There may be several implications of our review. First, our findings reinforce that a number of plant-based and natural therapies may improve both individual and collective menopausal symptoms. Second, it highlights major research and knowledge gaps both in potentially beneficial therapies and assessment of a wider range of outcomes. For instance, while the majority of the available studies focus appropriately on hot flashes, which are the most common symptom of the menopausal transition, a few studies additionally evaluated other menopausal symptoms (e.g. night sweats). There were insufficient numbers of studies on other therapies such as behavioural therapies, acupuncture, and herbal remedies. Therefore, our review should stimulate future research, concentrating on large, well-designed studies that evaluate a wider range of menopausal symptoms. Finally, it also underscores the lack of data on adverse effects that may arise from long term use of plant-based and natural therapies. Information on any detrimental health effects, typically available in long-term intervention studies, is essential given their potential relevance to post-menopausal health.

In conclusion, available evidence indicates that composite and specific phytoestrogen supplementations may confer significant benefit in reducing common menopausal symptoms in women. Additionally, several other plant-based and natural therapies may also effectively improve menopausal symptoms. However, owing to the general sub-optimal quality and heterogeneous nature of the current evidence, further rigorous studies are needed to determine the impact of plant-based and natural therapies on menopausal health.

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eTable 1 Characteristics of randomised controlled trials of biologically-based therapies

Lead Author, Publication Date	Name of study or source of participants	Location	Year of study	Age group	Allocation concealment	Blinding to subjects	Blinding to carers	Intervention form	Therapy and daily dosage	Intervention period, weeks	Control
Dietary isoflavones											
Murkies, 1995	Melbourne Bayside General Practice	Australia	NS	30-70	Unclear	Yes	Yes	Flour	45g soy flour	12	Wheat
Albertazzi, 1998	NS	Italy	NS	45-62	Yes	Yes	Yes	Powder	60 g soy, 76 mg isoflavones	12	Casein powder
Knight, 2001	University Department of Obstetrics & Gynaecology	Australia	NS	40-65	Yes	Yes	Yes	Powder	60 g soy, 134.4 mg isoflavones	12	Casein powder
Han, 2002	Federal University of Sao Paulo	Brazil	1999-2000	45-55	Unclear	Yes	Yes	Capsule	50.3 mg soy protein, 33.3 mg isoflavone	16	Placebo
Van Patten, 2002	Volunteers	Canada	1998-2000	55*	Unclear	Yes	Yes	Beverage	90 mg isoflavone	12	Rice beverage
Burke, 2003	SEA	USA	1996-1997	45-55	Unclear	Yes	Yes	Beverage	42 mg or 58 mg soy drink	96	Soy drink with no isoflavones
Lewis, 2006	Family Practice & Gynaecology Clinic	Canada	NS	45-60	Unclear	Yes	Yes	Muffins	25 g soy, 42 mg isoflavones	16	Wheat
Cheng, 2007	NS	Sweden	NS	49-69	Unclear	Yes	Yes	Fruit drink	60 mg isoflavones	12	Oatmeal
Liu, 2014	Advertisements and referrals	China	2010-2012	48-65	Yes	Yes	Yes	Flour	12.8 g soy protein, 49.3 mg isoflavones	24	Low-fat milk
Supplements and extracts of soy isoflavones											
Kotsopoulos, 2000	Clinical trial	Australia	NS	50-75	Unclear	Yes	Yes	Powder (beverage)	118 mg isoflavones	12	Casein
Faure, 2002	NS	France	NS	53*	Unclear	Yes	Yes	Capsule	70 mg genistin and daidzin	16	Micro-crystalline cellulose
Duffy, 2003	Kings College London	UK	NS	50-65	Unclear	Yes	Yes	Capsule	60 mg total isoflavone	12	Placebo
Penotti, 2003	Menopause clinic	Italy	NS	45-60	Unclear	Yes	Yes	Tablets	72 mg soy-derived isoflavones	24	Placebo

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Lead Author, Publication Date	Name of study or source of participants	Location	Year of study	Age group	Allocation concealment	Blinding to subjects	Blinding to carers	Intervention form	Therapy and daily dosage	Intervention period, weeks	Control
Colarcurci, 2004	NS	Italy	NS	52*	Unclear	Yes	Yes	Tablets or transdermal	50 mg or 75 mg ioflavones oral; 6 mg or 12 mg isoflavones transdermal	12	No treatment
Nahas, 2004	NS	Brazil	NS	53*	Unclear	No	No	Capsule	60 mg soy germ isoflavones	24	Lactose
Secreto, 2004	NS	Italy	NS	52*	Yes	Yes	Yes	Capsule	600 mg soy extract, 80 mg isoflavones	12	Placebo
Cancellieri, 2007	Menopausal centres	Italy	NS	45-65	Unclear	Yes	Yes	Tablets	72 mg isoflavones	24	Placebo
Petri, 2007	Department of Gynaecology	Brazil	NS	>=45	Unclear	Yes	Yes	Capsule	100 mg isoflavone	40	Lactose
Ferrari, 2009	Menopause units	Italy	2004-2006	40-65	Unclear	Yes	Yes	Tablets	200 mg soybean extract, 80 mg isoflavones	12	Placebo
Lee, 2010	Chonbuk National University Hospital	South Korea	NS	40-60	Unclear	Yes	Yes	Capsule	175 mg rexflavone	12	Placebo
Hachul, 2011	Advertisements	Brazil	NS	50-65	Unclear	Yes	Yes	Tablets	80 mg isoflavone	16	Placebo
Aso, 2012	Fliers, advertisements, volunteer list	Japan	2008-2009	45-60	Unclear	Yes	Yes	Tablets	10 mg S-(-) equol	12	Lactose
Liu, 2014	Advertisements and referrals	China	2010-2012	48-65	Yes	Yes	Yes	Beverage	63 mg daidzein	24	Low-fat milk
					Red clover						
Knight, 1999	University Department of Obstetrics & Gynaecology	Australia	NS	54*	Unclear	Yes	Yes	Tablets	40 mg or 160 mg red clover	12	Placebo
Baber, 1999	Volunteers	Australia	NS	45-65	Unclear	Yes	Yes	Tablets	40 mg red clover	12-14	Placebo
van de Weijer, 2002	NS	Netherlands	NS	49-65	Unclear	Yes	Yes	Tablets	80 mg red clover	12	Placebo
Jeri, 2002	NS	Peru	NS	< 60	Unclear	Yes	Yes	Tablets	40 mg red clover	16	Placebo
Tice, 2003	Isoflavone Clover Extract Study	USA	1999-2001	45-60	Yes	Yes	Yes	Tablets	57 mg or 82 mg red clover	12	Placebo

eTable 1 Characteristics of randomised controlled trials of biologically-based therapies

Lead Author, Publication Date	Name of study or source of participants	Location	Year of study	Age group	Allocation concealment	Blinding to subjects	Blinding to carers	Intervention form	Therapy and daily dosage	Intervention period, weeks	Control
Atkinson, 2004	Addenbrookes, 2004	UK	1997-1999	49-65	Unclear	Yes	Yes	Tablets	40 mg red clover	48	Placebo
Hidalgo, 2005	FUCLIM	Ecuador	2003-2004	> 40	Yes	Yes	Yes	Capsules	80 mg red clover	12	Placebo
Geller, 2009	University of Illinois at Chicago and Northwestern University	USA	2003-2007	53*	Unclear	Yes	Yes	Capsules	398 mg red clover	48	Placebo
Lipovac, 2012	Menopause Ambulance of the General Hospital & The Menox Climacteric Institute	Austria	2003-2004	>=40	No	No	No	Capsules	80 mg red clover	12	Placebo
					Other phytoestrogens						
Brzezinski, 1997	Menopause clinic	Israel	NS	45-60	Unclear	Yes	Yes	Dietary food	Phytoestrogen rich diet	12	Regular Israeli diet
Komesaroff, 2001	Media and newspaper advertisements	Australia	NS	45-60	Unclear	Yes	Yes	Topical	Wild yam cream; one teaspoonful twice daily	12	Placebo
Carranza-Lira, 2001	Climacteric clinic	Mexico	NS	51*	Unclear	Yes	Yes	Topical	Phytoestrogen cream, 4 mg	4	Placebo
Nikander, 2003	University Hospital	Finland	1999-2000	54*	Unclear	Yes	Yes	Tablets	114 mg phytoestrogen (glycitein 58%, daidzein 36%, genistein 6%)	12	Placebo
Sammartino, 2003	Menopause clinic	Italy	NS	52*	No	No	No	Tablets	36 mg genistein	48	Calcium
Albertazzi, 2005	Newspaper and radio	UK	2003	44-65	Yes	Yes	Yes	Capsules	90 mg pure genistein	6	Placebo
Danna, 2007	University of Messina	Italy	NS	50-70	Unclear	Yes	Yes	Tablets	54 mg genistein	48	Placebo
Evans, 2011	NS	Canada	NS	40-65	Yes	Yes	Yes	Capsules	30 mg genistein	12	Microcrystalline cellulose
Hsu, 2011	Medical centre	Taiwan	2004-2005	45-60	Unclear	Yes	Yes	Powder	24 mg Diascorea alata extracts	48	Placebo

eTable 1 Characteristics of randomised controlled trials of biologically-based therapies

Lead Author, Publication Date	Name of study or source of participants	Location	Year of study	Age group	Allocation concealment	Blinding to subjects	Blinding to carers	Intervention form	Therapy and daily dosage	Intervention period, weeks	Control
Other biological therapies											
Makkonen, 1993	NS	Finland	NS	53*	Unclear	Yes	Yes	Powder	15 g Guar gum	24	Wheat flour
Frei-Kleiner, 2005	Gynecological practice	Switzerland	NS	52*	Unclear	Yes	Yes	Capsule	42 mg black cohosh	12	Placebo
Shahnazi, 2013	Shahid Beheshti University of Medical Science Tehran	Iran	NS	51.6*	Unclear	Yes	Yes	Tablets	Black Cohosh	8 weeks	Placebo
Verhoeven, 2005	Advertisements	Netherlands	2002	45-65	Unclear	Yes	Yes	Capsule	125 mg soy isoflavones plus 100 mg Actaea racemosa Linnaeus	12	Placebo
Lewis, 2006	Family Practice & Gynaecological Clinic	Canada	NS	45-60	Unclear	Yes	Yes	Muffin	25 g flaxseed	16	Wheat
Newton, 2006	HALT	USA	2001-2004	45-55	Yes	Yes	Yes	Capsule	160 mg black cohosh or 200 mg multibotanical plus dietary soy counselling	48	Placebo
Pockaj, 2006	NS	USA	2003-2004	56*	Unclear	Yes	Yes	Capsule	40 mg black cohosh	8	Placebo
Sammartino, 2006	Menopause clinic	Italy	2005	50*	Yes	Yes	Yes	Tablets	Combination of isoflavones 150 mg, lignans 100 mg, and Cimicifuga racemosa 50 mg	4	Calcium
Uebelhack, 2006	Advertisements	Germany	2003-2004	45-60	Yes	Yes	Yes	Tablets	Black cohosh plus St. John's Wort	16	Placebo
Bai, 2007	NS	China	NS	40-60	Unclear	Yes	Yes	Tablets	40 mg black cohosh	12	Tibolone
Chung, 2007	NS	Korea	2005-2006	50.7*	Unclear	Yes	Yes	Tablets	264 mg black cohosh plus St. John's Wort	12	Placebo

eTable 1 Characteristics of randomised controlled trials of biologically-based therapies

Lead Author, Publication Date	Name of study or source of participants	Location	Year of study	Age group	Allocation concealment	Blinding to subjects	Blinding to carers	Intervention form	Therapy and daily dosage	Intervention period, weeks	Control
Rotem, 2007	General Health Services	Israel	NS	44-65	No	Yes	Yes	Capsule	Phyto-Female Complex containing 100 mg black cohosh, 75 mg dong quai, 75 mg herb extract, 50 mg red clover, 50 mg American ginseng extract, 50 mg chaste-berry	12	Placebo
Amsterdam, 2009	NS	USA	NS	54*	Yes	Yes	Yes	Capsule	64-128 mg black cohosh	12	Rice flour
Geller, 2009	University of Illinois at Chicago and Northwestern University	USA	2003-2007	53*	Unclear	Yes	Yes	Capsules	128 mg black cohosh	48	Placebo
van Die, 2009	NS	Australia	NS	40-60	Unclear	Yes	Yes	Tablets	900 mg St. John's Wort plus 1000 mg Chaste tree/berry	17	Placebo
Abdali, 2010	Zeinabieh Hospital in Shiraz	Iran	2008	45-55	Unclear	Yes	Yes	Drops	20 drops of St. John's Wort three times daily	8	Distilled water
Simbalista, 2010	Climacteric House, University of Sao Paulo	Brazil	2005-2006	52*	Yes	Yes	Yes	Bread	25 g flaxseed	12	Wheat bran
Colli, 2012	Gynaecology service	Brazil	2009-2010	46-68	Unclear	Yes	Yes	Capsule	1 g flaxseed extract and 90 g flaxseed meal	24	Collagen
Farzaneh, 2013	Taleghani Hospital	Iran	NS	45-59	Unclear	Yes	Yes	Capsule	1000 mg evening primrose	6	Placebo

*, mean age; FUCLIM, Foundation for Health and Well Being in the Climacteric; HALT, Herbal Alternatives for Menopause Trial; SEA, Soy Estrogen Alternative Study

eTable 2 Characteristics of randomised controlled trials of non-biological therapies

Lead Author, Publication Date	Name of study or source of participants	Location	Year of study	Age group	Allocation concealment	Blinding to subjects	Blinding to carers	Treatment and daily dosage	Intervention period, weeks	Control	Total trial participants
Mind-body and behavioural therapies											
Elavsky, 2007	NS	USA	NS	42-58	No	No	No	Yoga, 90 minute sessions twice weekly	16	No treatment	124
Chattha, 2008_a; Chattha, 2008_b	Yoga University	India	NS	40-55	No	No	Yes	1-2 hours daily sessions	8	Physical exercises	120
Joshi, 2011	Yoga Institute	India	2009-2010	40-55	No	No	No	1 hour sessions daily	12	Control	200
Yeh, 2012	Community-based	Taiwan	NS	>/= 45	No	No	No	Ping Shuai Qigong, 30 minutes	12	Control	70
Lindh, 2013	Advertisements	Sweden	2007-2010	54.9*	Yes	No	No	Applied relaxation, 10 sessions	12	No treatment	60
Newton, 2014	MsFLASH	USA	2011-2012	40-62	No	No	No	Yoga, 12 weekly sessions	12	Usual activity	249
Reed, 2014	MsFLASH	USA	2011-2012	40-62	No	No	No	Yoga, 90 minutes weekly; Aerobic exercise, three times weekly	12	Usual activity	249
Manipulative, body-based, and energy therapies											
Williamson, 2002	School of Complementary Health	NS	NS	45-60	Unclear	Yes	No	Reflexology, 9 sessions	19	Non-specific foot massage	76
Oliveira, 2012	UNIFESP	Brazil	NS	50-65	No	No	No	Therapeutic massage, 1 session twice weekly	16	Control	30
Darsareh, 2012	Menopausal clinic	Iran	2011	45-60	No	No	No	Aromatherapy and placebo massage, 30 minutes twice weekly	4	No treatment	60

eTable 2 Characteristics of randomised controlled trials of non-biological therapies

Lead Author, Publication Date	Name of study or source of participants	Location	Year of study	Age group	Allocation concealment	Blinding to subjects	Blinding to carers	Treatment and daily dosage	Intervention period, weeks	Control	Total trial participants
Elkins, 2013	Newspapers and media	USA	2008-2012	NS	Yes	Yes	Yes	Clinical hypnosis, 45 minutes five times weekly	12	Structured attention	187
Acupuncture											
Shen, 2005	Tianjin TCM College	China	NS	45-55	No	No	No	Acupuncture, 1 session daily	4	Oryzanol, vitamin B1, vitamin E	65
Nir, 2007	Advertisements at community clinics	USA	2003-2004	45-65	Unclear	Yes	No	Acupuncture, 9 sessions	7	Placebo acupuncture	29
Vincent, 2007	Mayo Clinic General Research Center	USA	2004	45-59	No	Yes	No	Acupuncture, 2 sessions weekly	5	Sham acupuncture	103
Zaborowska, 2007	University Hospital	Sweden	NS	NS	No	No	No	Acupuncture, 30 minutes twice weekly for first two weeks and once a week for 10 weeks	12	Placebo	102
Hervik, 2009	Vestfold Central Hospital	Norway	2003-2006	53*	No	Yes	Yes	Acupuncture, 30 minutes, twice weekly for the first 5 weeks, thereafter, once a week for the following 5 weeks	22 weeks	Sham acupuncture	59
Liljergen, 2009	Danderyds Hospital	Sweeden	2002-2005	58*	No	Yes	Yes	Acupuncture, 20 minutes, twice a week for 5 weeks.	5 weeks	Sham acupuncture	84
Borud, 2009	ACUFLASH	Norway	2006-2007	53.8*	No	No	No	Acupuncture, 10 sessions	12	No treatment	267
Kim, 2010	Medical Center	Korea	2008	45-60	No	No	No	Acupuncture, 12 sessions	4	Usual care	175
Venzke, 2010	Advertisements	USA	2000-2002	53.4*	No	Yes	No	Chinese medicine acupuncture,two 25-min treatments a week for 4 weeks followed by one treatment a week for 8 weeks	12	Sham acupuncture	51

eTable 2 Characteristics of randomised controlled trials of non-biological therapies

Lead Author, Publication Date	Name of study or source of participants	Location	Year of study	Age group	Allocation concealment	Blinding to subjects	Blinding to carers	Treatment and daily dosage	Intervention period, weeks	Control	Total trial participants
Painovich, 2012	Advertisements	USA	NS	> 40	Unclear	Yes	No	Acupuncture, 3 sessions weekly	12	Sham acupuncture and awaiting controls	33
Hachul, 2013	Advertisements	Brazil	NS	50-67	Unclear	Yes	Yes	Acupuncture, 2 sessions weekly	5	Sham acupuncture	18
Baccetti, 2014	Volunteers	China	2005-2008	45-56	Yes	No	No	Acupuncture, 12 sessions twice weekly; Diet; Self-massage	6	Diet and self-massage	100
Nedeljkovic, 2014	University of Bern	Switzerland	NS	53*	Yes	Yes	No	Chinese medicine acupuncture	12	Sham acupuncture	20
Chinese medicinal herbs											
Hirata, 1997	Advertisements and media	USA	NS	52.4*	Yes	Yes	Yes	4.5 g Dong quai	24	Maltodextrin	71
Kwee, 2007	Local newspapers	Netherlands	2002-2004	53.0*	No	Yes	Yes	Chinese herbal medicines, 90 drops	12	Placebo	31
Haines, 2008	Menopause clinic	Hong Kong	NS	52.0*	Yes	Yes	Yes	Dang Gui Buxue Tang, 3 g	24	Placebo	100
Qu, 2009	Womens Hospital, Zhejiang University	China	2004-2008	45-60	No	No	No	GengNianLe, one dose	12	Tibolone	47
Sluijs, 2009	Sydney Metropolitan area	Australia	2004-2005	45-65	Yes	Yes	Yes	Chinese herbal medicines plus Cimicifuga racemosa	16	Placebo	92
Sun, 2012	Yonsei University College of Medicine	South Korea	2009-2010	45-60	Yes	Yes	Yes	Red ginseng, 3 g	12	Placebo	72
Xia, 2012	Media and referrals	China	2009	45-55	Yes	Yes	Yes	Chinese herbal medicines, 3.5 g	8	Placebo	72
Nedeljkovic, 2014	University of Bern	Switzerland	NS	53*	Yes	Yes	No	Chinese herbal medicines, 3 g	12	Placebo	20
Other medicinal herbs											

eTable 2 Characteristics of randomised controlled trials of non-biological therapies

Lead Author, Publication Date	Name of study or source of participants	Location	Year of study	Age group	Allocation concealment	Blinding to subjects	Blinding to carers	Treatment and daily dosage	Intervention period, weeks	Control	Total trial participants
Winther, 2005	Advertisements	Sweden	NS	51.0*	Unclear	Yes	Yes	Femal, 80 mg pollen extract and 240 mg pistil extract	12	Placebo	64
Heger, 2006	Gynaecological clinic	Ukraine	2003-2004	45-55	Unclear	Yes	Yes	Rheum rhaponticum (ERr 731), 4mg	12	Placebo	110
Yang, 2007	NS	Taiwan	2002-2005	45-55	No	Yes	Yes	Pycnogenol, 200 mg	24	Placebo	200
Garcia, 2010	Gynaecological clinic	Singapore and Philipines	NS	45-60	Unclear	Yes	Yes	Nutrafem containing 75 mg of E. ulmoides plant extract and 150 mg of V. radiata plant extract (4 capsules daily)	13	Placebo	159
Yakoot, 2011	Outpatient clinic in Alexandria	Egypt	2007	45-60	No	Yes	No	Lady 4 (each capsule contained 250 mg evening primrose oil, 100 mg damiana, 50 mg ginseng, and 200 mg royal jelly), 2 capsules taken daily	4	Placebo	120
Chang, 2012	Newspapers and signs	South Korea	NS	NS	Yes	Yes	Yes	EstroG-100 (contained standardized extracts of Cynanchum wilfordii, Phlomis umbrosa and Angelica gigas); 257.05 mg of EstroG-100 taken twice daily powder	12	Corn starch	64
Kohama, 2013	NS	Japan	2007-2010	42-58	Yes	Yes	Yes	Pycnogenol, 60 mg	12	Placebo	170

*, mean age; ACUFLASH, Acupuncture on Hot Flushes Among Menopausal Women; NS, not stated; UNIFESP, Universidade Federal de Sao Paulo

eTable 3 Characteristics of observational cohort studies and single-arm trials contributing to review

Lead Author, Publication Date	Name of study or source of participants	Location	Year of study	Study design	Age group	Intervention form	Treatment and daily dosage	Intervention period, weeks	Total participants	Quality
Nagata, 2001	Takayama Study	Japan	1992	Prospective cohort	35-54	Intake assessed by questionnaire	Soy and isoflavone	6 years follow-up	1,106	6
Albert, 2002	Hospitals	Spain	2000	Open prospective, observational and non-randomized clinical trial	> 45	Capsule	Isoflavones, 35 mg twice daily	16	190	5
Porzio, 2002	NS	Italy	1999-2000	Single arm trial	37-56	-	Acupuncture, weekly schedule	24	15	5
Lai, 2005	Advertisement and health fairs	Taiwan	2003-2004	Prospective cohort	45-55	Granule	TMN-1, 12 g	12	63	5
Kaszkin-Bettag, 2008; Hasper, 2009	Gynaecological clinic	Germany	2005-2006	Prospective cohort	39-71	Tablets	ERr 731, 4 mg 1-2 times	96	363	6
Liu, 2009	NS	China	NS	Prospective cohort	40-65	Capsule	Menprogen, 0.4 g herbal extracts	> 12	83	5
Bommer, 2011	General Practices	Switzerland	2008	Non-randomised open clinical trial	50-65	Tablets	Fresh sage, 280 mg	8	71	6
Chedraui, 2011	University Campus	Ecuador	2009-2010	Single blinded pilot clinical trial	40-59	Capsule	Isoflavones, 100 mg	12	50	5
Otte, 2011	Clinical referrals	USA	NS	Single group, non-randomized, quasi-experimental study	53*	-	Acupuncture, 3 sessions within 2 weeks	8	10	5
Drewe, 2013	NS	Switzerland	2008-2009	Prospective cohort	52.3*	Tablets	Cimicifuga racemosa extract Ze 450, 6.5 mg or 13 mg	12	442	6

eTable 3 Characteristics of observational cohort studies and single-arm trials contributing to review

Lead Author, Publication Date	Name of study or source of participants	Location	Year of study	Study design	Age group	Intervention form	Treatment and daily dosage	Intervention period, weeks	Total participants	Quality
Hidaka, 2013	Kampo Outpatient Clinic	Japan	NS	Prospective cohort	50.6*	Extract	Japanese traditional medicine (Kami-shoyo-san), 7.5 g	4	45	5
Jeong, 2013	Daegu Catholic University Medical Center	South Korea	NS	Prospective cohort	46.6*	-	Acupuncture, 12 sessions	4	10	5

NS, not stated; USA, United States of America

eTable 4 Results of randomised controlled trials of biological therapies (phytoestrogens) that were not included in meta-analyses

Lead Author, Publication Date	Name of study or source of participants	Location	Intervention form	Therapy and daily dosage	Broad name of intervention	Control	Mean differences in symptoms comparing intervention to control/ placebo
Murkies, 1995	Melbourne Bayside General Practice	Australia	Flour	45g soy flour		Wheat	Vasomotor symptom score: -1.90 (-3.69, -0.11); Hot flash score: -1.20 (-2.20, -0.20)
Komesaroff, 2001	Media and newspaper advertisements	Australia	Topical	Wild yam cream; one teaspoonful twice daily	Other phytoestrogens	Placebo	Daytime hot flash score: 1.20 (0.36, 2.04); Night sweats score: 1.30 (0.65, 1.95)
Han, 2002	Federal University of Sao Paulo	Brazil	Capsule	50.3 mg soy protein, 33.3 mg isoflavone	Dietary soy isoflavones	Placebo	Vasomotor score of the Kupperman Index: -3.00 (-3.86, -2.14); Insomnia score of the Kupperman Index: -2.20 (-2.72, -1.68)
Burke, 2003	SEA	USA	Beverage	42 mg or 58 mg soy drink	Dietary soy isoflavones	Soy drink with no isoflavones	Number of vasomotor symptoms in 24 hours: 0.80 (0.15, 1.45)
Duffy, 2003	Kings College London	UK	Capsule	60 mg total isoflavone	Supplements/ Extracts of soy isoflavones	Placebo	Green Climacteric Scale Vasomotor Score: 0.00 (-0.68, 0.68)
Nahas, 2004	NS	Brazil	Capsule	60 mg soy germ isoflavones	Supplements/ Extracts of soy isoflavones	Lactose	Hot flash score of the Kupperman Index: -2.80 (-3.59, -2.01);
Albertazzi, 2005	Newspaper and radio	UK	Capsules	90 mg pure genistein	Other phytoestrogens	Placebo	Green Climacteric Scale Anxiety Score: -0.02 (0.21, 0.17); Green Climacteric Scale Depression Score: -0.17 (-0.51, 0.17); Green Climacteric Scale Vasomotor Score: -0.03 (-0.04, 0.38); Green Climacteric Scale Sexual Desire Score: 0.11 (-0.13, 0.34); Green Climacteric Scale Vaginal Dryness Score: 0.08 (-0.13, 0.28)
Evans, 2011	NS	Canada	Capsules	30 mg genistein	Other phytoestrogens	Micro-crystalline cellulose	Hot flash duration (min/day): 3.53 (-7.07, 14.13); Hot flash severity score: -0.06 (-0.21, 0.09)

eTable 4 Results of randomised controlled trials of biological therapies (phytoestrogens) that were not included in meta-analyses

Lead Author, Publication Date	Name of study or source of participants	Location	Intervention form	Therapy and daily dosage	Broad name of intervention	Control	Mean differences in symptoms comparing intervention to control/ placebo
Liu, 2014	Advertisements and referrals	China	Flour	12.8 g soy protein, 49.3 mg isoflavones	Dietary soy isoflavones	Low-fat milk	Vasomotor symptom score: 0.13 (-0.14, 0.39)
Liu, 2014	Advertisements and referrals	China	Beverage	63 mg daidzein	Supplements/ Extracts of soy isoflavones	Low-fat milk	Vasomotor symptom score: 0.13 (-0.15, 0.41)

SEA, Soy Estrogen Alternative Study; NS, not stated

eTable 5 Results of randomised controlled trials of other biological therapies that were not included in meta-analyses

Lead Author, Publication Date	Name of study or source of participants	Location	Intervention form	Therapy and daily dosage	Control	Mean differences in symptoms comparing intervention to control/placebo
Makkonen, 1993	NS	Finland	Powder	15 g Guar gum	Wheat flour	Kupperman Index: -1.91 (-5.15, 1.33);
Verhoeven, 2005	Advertisements	Netherlands	Capsule	125 mg soy isoflavones plus 100 mg Actaea racemosa Linnaeus	Placebo	Kupperman Index: -0.10 (-2.03, 1.83); hot flash severity score: -0.10 (-0.25, 0.05); Green Climacteric Scale Score: 0.90 (-0.83, 2.63); number of vasomotor symptoms in 24 hours: -0.70 (-1.06, -0.34); number of mild hot flashes in daytime: -0.30 (-0.74, 0.14); number of moderate hot flashes in daytime: -0.40 (-0.65, -0.15); number of severe hot flashes in daytime: 0.30 (0.11, 0.49)
Sammartino, 2006	Menopause clinic	Italy	Tablets	Combination of isoflavones 150 mg, lignans 100 mg, and Cimicifuga racemosa 50 mg	Calcium	Kupperman Index: -17.00 (-17.86, -16.14)
Uebelhack, 2006	Advertisements	Germany	Tablets	Black cohosh plus St. John's Wort	Placebo	Menopause Rating Scale Score: -0.14 (-0.17, -0.11); Hamilton Depression Scale Score: -5.50 (-6.44, -4.56)
Bai, 2007	NS	China	Tablets	40 mg black cohosh	Tibolone	Kupperman Index: 0.20 (-0.79, 1.19)
Tanmabasamut, 2015	Siriraj Menopause Clinic	Thailand	Tablets	40 mg black cohosh	Placebo	Vasomotor symptoms score: -0.3 (-1.3, 0.7)
Charandabi, 2013	NS	Iran	Tablets	6.5 mg of dried extract of Black cohosh	Placebo	Vasomotor symptom score: -12.9 (-16.2, -9.3)

eTable 5 Results of randomised controlled trials of other biological therapies that were not included in meta-analyses

Lead Author, Publication Date	Name of study or source of participants	Location	Intervention form	Therapy and daily dosage	Control	Mean differences in symptoms comparing intervention to control/placebo
Rotem, 2007	General Health Services	Israel	Capsule	Phyto-Female Complex containing 100 mg black cohosh, 75 mg dong quai, 75 mg herb extract, 50 mg red clover, 50 mg American ginseng extract, 50 mg chaste-berry	Placebo	Number of hot flashes in 24 hours: -1.85 (-2.85, -0.85); hot flash intensity score: -1.33 (-1.77, -0.89); number of night sweats in 24 hours: -1.40 (-1.90, -0.90); sleep quality score: -2.00 (-2.45, -1.55)
van Die, 2009	NS	Australia	Tablets	900 mg St. John's Wort plus 1000 mg Chaste tree/berry	Placebo	Number of hot flashes in 24 hours: -1.36 (-6.60, 3.88); Hamilton Depression Scale Score: -0.43 (-3.41, 2.55); Green Climacteric Scale Score: -2.92 (-7.15, 1.31); Green Climacteric Scale Anxiety Score: -0.92 (-2.50, 0.66); Green Climacteric Scale Depression Score: -0.59 (-2.05, 0.87); Green Climacteric Scale Vasomotor Score: -0.60 (-1.43, 0.23); Green Climacteric Scale Sexual Desire Score: -0.32 (-0.90, 0.26): Green Climacteric Scale Sleep Problem Score: 0.00 (-0.50, 0.50)
Abdali, 2010	Zeinabieh Hospital in Shiraz	Iran	Drops	20 drops of St. John's Wort three times daily	Distilled water	Number of hot flashes in 24 hours: -0.85 (-1.07, -0.63); duration of hot flashes in 24 hours (min): -6.20 (-7.46, -4.94); hot flash severity in 24 hours: -0.78 (-0.94, -0.62)
Simbalista, 2010	Climacteric House, University of Sao Paulo	Brazil	Bread	25 g flaxseed	Wheat bran	Number of hot flashes in 24 hours: 1.20 (-0.75, 3.15); Kupperman Index: -0.20 (-3.41, 3.01)
Dodin, 2005	Centre menopause Quebec	Canada	Bread	40 g flaxseed	Wheat germ	Hot flashes score: -0.12 (-2.1, 2.4) Night sweats score: -0.22 (-2.3, 2.8)

eTable 5 Results of randomised controlled trials of other biological therapies that were not included in meta-analyses

Lead Author, Publication Date	Name of study or source of participants	Location	Intervention form	Therapy and daily dosage	Control	Mean differences in symptoms comparing intervention to control/placebo
Colli, 2012	Gynaecology service	Brazil	Capsule	1 g flaxseed extract and 90 g flaxseed meal	Collagen	Kupperman Index: -1.22 (-2.86, 0.42); hot flash intensity score: -1.39 (-2.29, -0.49); hot flash intensity: -0.83 (-1.76, 0.10)
Farzaneh, 2013	Taleghani Hospital	Iran	Capsule	1000 mg evening primrose	Placebo	Number of hot flashes in 24 hours: -0.40 (-1.03, 0.23); hot flash severity score: -0.80 (-1.54, -0.06); hot flash duration per attack: 0.30 (-2.39, 2.99)

eTable 6 Results of observational cohort studies and single-arm trials contributing to review

Lead Author, Publication Date	Location	Year of study	Study design	Intervention	Estimates	Covariates adjusted for
Nagata, 2001	Japan	1992	Prospective cohort	Soy and isoflavones	Hazard ratio (95% CI) for hot flashes comparing top versus bottom thirds of soy intake 0.47 (0.28-0.79); Corresponding ratio for total isoflavone intake 0.42 (0.25-0.72)	Age and menopause
Albert, 2002	Spain	2000	Open prospective, observational and non-randomized clinical trial	Isoflavones, 35 mg twice daily	Baseline mean (SD) in percentages of menopausal symptoms of hot flashes; sleep disorders; anxiety; depression; vaginal dryness; and loss of libido were: 82.73 (17.14); 61.46 (34.61); 59.53 (27.81); 49.86 (30.67); 52.48 (33.86); and 57.77 (33.56). Corresponding final values were 36.33 (27.71); 32.66 (30.92); 33.00 (29.72); 28.83 (27.89); 31.83 (29.09); and 43.17 (34.50)	-
Porzio, 2002	Italy	1999-2000	Single arm trial	Acupuncture, weekly schedule	Baseline mean (SD) of anxiety, depression, and vasomotor scores of Green Climacteric Scale were: 8.07 (3.11); 7.93 (3.98); and 5.67 (0.69). Corresponding final values were 3.40 (1.99); 3.07 (1.81); and 2.06 (1.18)	-
Lai, 2005	Taiwan	2003-2004	Prospective cohort	TMN-1, 12 g	Baseline mean (SD) of Kupperman Index for peri- and post-menopausal women were: 18.3 (6.2) and 18.5 (5.6). Corresponding final values were 11.7 (5.8) and 14.2 (7.4).	-
Kaszkin-Bettag, 2008; Hasper, 2009	Germany	2005-2006	Prospective cohort	ERr 731, 4 mg 1-2 times	Mean (SD) changes in Menopausal Rating Scale after 48 and 96 weeks were respectively -6.80 (6.00) and -6.80 (5.90).	-
Liu, 2009	China	NS	Prospective cohort	Menprogen, 0.4 g herbal extracts	Mean (SD) change in Kupperman Index was -14.88 (10.57).	-
Bommer, 2011	Switzerland	2008	Non-randomised open clinical trial	Fresh sage, 280 mg	Mean (SD) changes in hot flash, sleep problems, depressive mood, anxiety, sexual problems, and vaginal dryness scores of the Menopause Rating Scale -1.20 (0.10); -1.00 (0.20); -0.60 (0.10); -0.60 (0.10); -0.10 (0.00; and -0.10 (0.10).	-

eTable 6 Results of observational cohort studies and single-arm trials contributing to review

Lead Author, Publication Date	Location	Year of study	Study design	Intervention	Estimates	Covariates adjusted for
Chedraui, 2011	Ecuador	2009-2010	Single blinded pilot clinical trial	Isoflavones, 100 mg	Reduction in mean (SD): number of hot flashes in 24 hours from 3.9 (2.3) to 0.4 (0.8); hot flash severity score in 24 hours from 2.6 (0.9) to 0.4 (0.8); and Hamilton Depression Rating scale score from 16.3 (5.4) to 6.9 (5.2).	-
Otte, 2011	USA	NS	Single group, non-randomized, quasi-experimental study	Acupuncture, 3 sessions within 2 weeks	Baseline mean (SD) of sleep latency (minutes); sleep efficiency (%); Pittsburgh Seep Quality Index-Global score; and objective hot flash frequency were 17.89 (6.12); 82.49 (5.11); 9.25 (4.10); and 12.58 (13.41). Corresponding final values were 24.08 (17.37); 82.27 (5.70); 6.00 (4.47); and 13.06 (11.53).	-
Drewe, 2013	Switzerland	2008-2009	Prospective cohort	Cimicifuga racemosa extract Ze 450, 6.5 mg or 13 mg	13 mg black cohosh for 3 months Mean (SD) changes in hot flash score of KI, score of KI, insomnia score of KI, and total KI were 4.81 (2.74); 2.23 (1.47); 2.11 (1.54); and 13.94 (7.20) 13 mg black cohosh for 9 months Corresponding values as above were 4.01 (2.45); 1.68 (1.38); 1.55 (1.43); and 10.56 (6.46) 6.5 mg black cohosh for 9 months Corresponding values were 3.84 (2.51); 1.90 (1.42); 1.69 (1.48); and 11.13 (6.28)	-
Hidaka, 2013	Japan	NS	Prospective cohort	Japanese traditional medicine (Kami-shoyo-san), 7.5 g	Baseline mean (SD) of total VAS score and VAS score of vasomotor symptoms were 67 (17); 60 (15); and 73 (13). Corresponding final values were 49 (17); 40 (17); and 41 (14)	-

eTable 6 Results of observational cohort studies and single-arm trials contributing to review

Lead Author, Publication Date	Location	Year of study	Study design	Intervention	Estimates	Covariates adjusted for
Jeong, 2013	South Korea	NS	Prospective cohort	Acupuncture, 12 sessions	Reduction in mean (SD): total VAS score from 71.5 (11.32) to 19.5 (13.23); number of hot flashes in 24 hours from 9.3 (9.72) to 1.5 (1.72); hot flash severity from 2.8 (0.79) to 1.1 (0.74); and total hot flash score from 31.1 (36.62) to 2.2 (2.53)	-

KI, Kupperman Index; NS, not stated; USA, United States of America; VAS, visual

eTable 7 Results of randomised controlled trials of non-biological therapies that were not included in meta-analyses

Lead Author, Publication Date	Name of study or source of participants	Location	Intervention form	Treatment and daily dosage	Broad name of intervention	Control	Mean differences in symptoms comparing intervention to control/placebo
Elavsky, 2007	NS	USA	-	Yoga, 90 minute sessions twice weekly	Mind, body, and behavioural therapies	No treatment	Green Climacteric Scale Total Score: 1.07 (-1.88, 4.02)
Chattha, 2008	Yoga University	India	-	1-2 hours daily sessions	Mind, body, and behavioural therapies	Physical exercises	Hot flash severity score: -0.44 (-0.64, -0.24); night sweats severity score: -0.20 (-0.42, 0.02); disturbed sleep severity score: -0.19 (-0.40, 0.02); Green Climacteric Scale Vasomotor Score: -0.63 (-1.03, -0.23)
Joshi, 2011	Yoga Institute	India	-	1 hour sessions daily	Mind, body, and behavioural therapies	Control	Menopause Rating Scale Score: -5.21 (-6.39, -4.03)
Cramer 2015	Malteser Hospital	Germany	-	Yoga, 90 minute, once a week	Mind, body, and behavioural therapies	Control	Menopause Rating Score: Week 12; -5.6 (-9.2, -1.9); Week 24; -4.5 (-8.3, -0.7)
Oliveira, 2012	UNIFESP	Brazil	-	Passive movement, 1 session twice weekly	Mind, body, and behavioural therapies	Control	Beck Depression Inventory Score: 0.00 (-3.65, 3.65); Beck Anxiety Inventory Score: -1.70 (-5.85, 2.45); MENQOL: -5.80 (-23.86, 12.26); Insomnia score of the Kupperman Index: -0.40 (-1.29, 0.49)
Yeh, 2012	Community-based	Taiwan	-	Ping Shuai Qigong, 30 minutes	Mind, body, and behavioural therapies	Control	Greene Climacteric Scale Vasomotor Score: -0.54 (-0.78, -0.30); Green Climacteric Scale Sexual Desire Score: -0.03 (-0.12, 0.06); Pittsburgh Sleep Quality Index Global Score: -3.20 (-3.62, -2.78)
Reed, 2014	MsFLASH	USA	-	Yoga, 90 minutes weekly; Aerobic exercise, three times weekly	Mind, body, and behavioural therapies	Usual activity	MENQOL: -0.20 (-0.42, 0.02) for aerobic exercise and -0.30 (-0.56, -0.04) for yoga; Vasomotor domain of MENQOL score: 0.00 (-0.41, 0.41) for aerobic exercise and -0.30 (-0.74, 0.14) for yoga; sexual domain of MENQOL score: -0.20 (-0.59, 0.19) for aerobic exercise and -0.50 (-0.97, -0.03) for yoga

eTable 7 Results of randomised controlled trials of non-biological therapies that were not included in meta-analyses

Lead Author, Publication Date	Name of study or source of participants	Location	Intervention form	Treatment and daily dosage	Broad name of intervention	Control	Mean differences in symptoms comparing intervention to control/placebo
Williamson, 2002	School of Complementary Health	NS	-	Reflexology, 9 sessions	Manipulative, body-based, and energy therapies	Non-specific foot massage	Women's Health Questionnaire Anxiety/Fears Score: -0.14 (-0.22, -0.06); Women's Health Questionnaire Depressive Mood Score: -0.01 (-0.08, 0.06); VAS score for hot flash severity: -2.10 (-9.13, 4.93); VAS score for night sweats severity: -2.20 (-10.00, 5.60)
Oliveira, 2012	UNIFESP	Brazil	-	Therapeutic massage, 1 session twice weekly	Manipulative, body-based, and energy therapies	Control	Beck Depression Inventory Score: -3.40 (-6.76, -0.04); Beck Anxiety Inventory Score: -1.90 (-6.00, 2.20); MENQOL: -20.60 (-36.86, -4.34); insomnia score of the Kupperman Index: -1.60 (-2.66, -0.54)
Darsareh, 2012	Menopausal clinic	Iran	-	Aromatherapy and placebo massage, 30 minutes twice weekly	Manipulative, body-based, and energy therapies	No treatment	Menopause Rating Scale Score: -8.75 (-9.79, -7.71) for aromatherapy massage and -2.65 (-3.70, -1.60) for placebo massage
Elkins, 2013	Newspapers and media	USA	-	Clinical hypnosis, 45 minutes five times weekly	Manipulative, body-based, and energy therapies	Structured attention	Weekly hot flash frequency: -42.93 (-43.48, -42.38); weekly hot flash score: -15.29 (-15.50, -15.08); Pittsburgh Sleep Quality Index: -5.04 (-5.14, -4.94)
Vincent, 2007	Mayo Clinic General Research Center	USA	-	Acupuncture, 2 sessions weekly	Whole Medical Systems (Acupuncture)	Sham acupuncture	Hot flash score: 1.00 (-2.12, 4.12); Green Climacteric Scale Vasomotor Score: 0.00 (-0.58, 0.58); Green Climacteric Sexual Desire Score: -0.10 (-0.40, 0.20); Green Climacteric Scale Total Score: 0.60 (-3.84, 5.04)
Deng, 2007	Memorial Sloan-Kettering Cancer Center	USA	-	Acupuncture, twice-weekly treatments for 4 weeks	Whole Medical Systems (Acupuncture)	Sham acupuncture	Number of hot flashes: -2 (-2.7, 0.9)
Venzke, 2010	Advertisements	USA	-	Chinese medicine acupuncture,two 25-min treatments a week for 4 weeks followed by one treatment a week for 8 weeks	Whole Medical Systems (Acupuncture)	Sham acupuncture	Beck Anxiety Inventory Score: 0.10 (-2.92, 3.12); Beck Depression Inventory Score: 3.00 (-0.38, 6.38); Hot flash score: 0.60 (-0.34, 1.54)

eTable 7 Results of randomised controlled trials of non-biological therapies that were not included in meta-analyses

Lead Author, Publication Date	Name of study or source of participants	Location	Intervention form	Treatment and daily dosage	Broad name of intervention	Control	Mean differences in symptoms comparing intervention to control/placebo
Painovich, 2012	Advertisements	USA	-	Acupuncture, 3 sessions weekly	Whole Medical Systems (Acupuncture)	Sham acupuncture and awaiting controls	Vasomotor domain of MENQOL score: 0.30 (-1.16, 1.76); sexual domain of MENQOL score: 0.00 (-1.72, 1.72); MENQOL: 0.30 (-0.70, 1.30)
Hachul, 2013	Advertisements	Brazil	-	Acupuncture, 2 sessions weekly	Whole Medical Systems (Acupuncture)	Sham acupuncture	Sleep latency (min): -2.44 (-4.72, -0.16); sleep efficiency (%): 3.57 (2.08, 5.06); Pittsburgh Sleep Quality Index Global Score: -5.23 (-5.71, -4.75); Beck Anxiety Inventory Score: 1.18 (0.34, 2.02)
Baccetti, 2014	Volunteers	China	-	Acupuncture, 12 sessions twice weekly; Diet; Self-massage	Whole Medical Systems (Acupuncture)	Diet and self-massage	Hot flash frequency score: -1.30 (-1.58, -1.02); sudden sweating frequency score: -1.30 (-1.65, -0.95); sleep disorders frequency score: -1.00 (-1.31, -0.69); vaginal dryness intensity score: -1.10 (-1.42, -0.78)
Nedeljkovic, 2014	University of Bern	Switzerland	-	Chinese medicine acupuncture	Whole Medical Systems (Acupuncture)	Sham acupuncture	Weekly hot flash severity: -45.20 (-92.31, 1.91); weekly hot flash frequency: -24.00 (-47.05, -0.95); Menopause Rating Scale Score: -12.50 (-16.83, -8.17)
Kwee, 2007	Local newspapers	Netherlands	Liquid extract	Chinese herbal medicines, 90 drops	Whole Medical Systems (Chinese Medicinal Herbs)	Placebo	Weekly hot flash frequency: 2.10 (-35.36, 39.56)
Haines, 2008	Menopause clinic	Hong Kong	Capsule	Dang Gui Buxue Tang, 3 g	Whole Medical Systems (Chinese Medicinal Herbs)	Placebo	Monthly mild hot flash frequency: 3.30 (-6.22, 12.82)
Qu, 2009	Womens Hospital, Zhejiang University	China	Liquid extract	GengNianLe, one dose	Whole Medical Systems (Chinese Medicinal Herbs)	Tibolone	Hamilton Depression Scale Score: 3.00 (-1.04, 7.04)
Xia, 2012	Media and referrals	China	Granule	Chinese herbal medicines, 3.5 g	Whole Medical Systems (Chinese Medicinal Herbs)	Placebo	Vasomotor domain of MENQOL score: -0.92 (-1.30, -0.54); sexual domain of MENQOL: -0.34 (-0.82, 0.14); hot flash score: -2.90 (-4.66, -1.14)

eTable 7 Results of randomised controlled trials of non-biological therapies that were not included in meta-analyses

Lead Author, Publication Date	Name of study or source of participants	Location	Intervention form	Treatment and daily dosage	Broad name of intervention	Control	Mean differences in symptoms comparing intervention to control/placebo
Nedeljkovic, 2014	University of Bern	Switzerland	Capsule	Chinese herbal medicines, 3 g	Whole Medical Systems (Chinese Medicinal Herbs)	Placebo	Weekly hot flash frequency: -18.60 (-49.32, 12.12); weekly hot flash severity: -34.40 (-94.18, 25.38); Menopause Rating Scale Score: -1.50 (-7.51, 4.51)
Winther, 2005	Advertisements	Sweden	Tablets	Femal, 80 mg pollen extract and 240 mg pistil extract	Whole Medical Systems (Other Medicinal Herbs)	Placebo	Menopause Rating Scale Hot Flash Score: -15.90 (-23.61, -8.19)
Yang, 2007	NS	Taiwan	Capsule	Pycnogenol, 200 mg	Whole Medical Systems (Other Medicinal Herbs)	Placebo	Women's Health Questionnaire Depressive Mood Score: 0.42 (0.24, 0.60); Women's Health Questionnaire Vasomotor Symptoms Score: 0.41 (0.22, 0.60); Women's Health Questionnaire Depressive Anxiety/Fears Score: 0.41 (0.22, 0.60); Women's Health Questionnaire Sexual Behaviour Score: 0.48 (0.30, 0.66); Women's Health Questionnaire Sleep Problems Score: 0.71 (0.53, 0.89)
Garcia, 2010	Gynaecological clinic	Singapore and Philipines	Capsule	Nutrafem containing 75 mg of E. ulmoides plant extract and 150 mg of V. radiata plant extract (4 capsules daily)	Whole Medical Systems (Other Medicinal Herbs)	Placebo	Weekly number of VMS: -6.69 (-10.07, -3.31); weekly vasomotor symptom severity score: -11.39 (-19.87, -2.91);
Yakoot, 2011	Outpatient clinic in Alexandria	Egypt	Capsule	Lady 4 (each capsule contained 250 mg evening primrose oil, 100 mg damiana, 50 mg ginseng, and 200 mg royal jelly), 2 capsules taken daily	Whole Medical Systems (Other Medicinal Herbs)	Placebo	Menopause Rating Scale Score: -2.20 (-3.50, -0.90)

NS, not stated; UNIFESP, Universidade Federal de Sao Paulo; VAS, visual analogue scale; VMS, vasomotor symptoms

eTable 8 Between-study heterogeneity statistics

Intervention	Outcome	No. of studies	I-squared (95% confidence interval)	P-value
Phytoestrogen	Kupperman index	10	99 (99-99)	< 0.001
Phytoestrogen	Green Climacteric Scale	6	0 (0-75)	0.940
Phytoestrogen	No. of hot flashes in 24 hours	18	93 (91-95)	< 0.001
Phytoestrogen	Number of night sweats in 24 hours	2	99 (98-99)	< 0.001
Phytoestrogen	Vaginal dryness score	3	48 (0-85)	0.145
Dietary and supplementary soy isoflavones	Kupperman index	5	88 (75-94)	< 0.001
Dietary and supplementary soy isoflavones	No. of hot flashes in 24 hours	10	83 (69-90)	< 0.001
Red clover	Kupperman index	2	100 (100-100)	< 0.001
Red clover	Green Climacteric Scale	3	0 (0-90)	0.747
Red clover	No. of hot flashes in 24 hours	7	97 (95-98)	< 0.001
Black cohosh	Kupperman index	2	0	0.464
Black cohosh	No. of hot flashes in 24 hours	3	60 (0-89)	0.081
Mind, body, and behavioural therapies	No. of hot flashes in 24 hours	3	85 (57-95)	0.001
Acupuncture	Kupperman index	2	0	0.925
Acupuncture	No. of hot flashes in 24 hours	4	48 (0-83)	0.121
Chinese medicinal herbs	Kupperman index	2	84 (34-96)	0.012
Other medicinal herbs	Kupperman index	2	98 (95-99)	< 0.001

eTable 9 Risk of bias assessments for the included clinical trials

Lead author, publication date	Random sequence generation	Allocation concealment	Blinding of participants & personnel	Blinding of outcome assessments	Incomplete outcome data	Selective reporting	Other bias
Makkonen, 1993	Low	Unclear	Low	Unclear	Low	Low	Unclear
Murkies, 1995	Low	Unclear	Low	Unclear	Low	Low	Low
Brzezinski, 1997	Low	Unclear	Low	Unclear	Low	Low	Low
Hirata, 1997	Low	Low	Low	Unclear	Low	Low	Low
Albertazzi, 1998	Low	Low	Low	Unclear	Low	Low	Unclear
Baber, 1999	Low	Unclear	Low	Unclear	Low	Low	Unclear
Knight, 1999	Low	Unclear	Low	Unclear	Low	Unclear	Unclear
Kotsopoulos, 2000	Low	Unclear	Low	Unclear	Low	Unclear	Low
Carranza-Lira, 2001	Low	Unclear	Low	Unclear	High	Unclear	Unclear
Knight, 2001	Low	Low	Low	Unclear	Low	Low	Unclear
Komesaroff, 2001	Low	Unclear	Low	Unclear	Low	Low	Unclear
Faure, 2002	Low	Unclear	Low	High	Low	Unclear	Unclear
Han, 2002	Low	Unclear	Low	Low	Low	Low	Unclear
Jeri, 2002	Low	Unclear	Low	Unclear	High	Low	Unclear
Van Patten, 2002	Low	Unclear	Low	High	Low	Low	Unclear
van de Weijer, 2002	Low	Unclear	Low	High	Low	Low	Unclear
Williamson, 2002	Low	Unclear	High	High	Low	Low	Unclear
Burke, 2003	Unclear	Unclear	Low	High	High	Unclear	Unclear
Duffy, 2003	Unclear	Unclear	Low	High	High	Low	Unclear
Nikander, 2003	Low	Unclear	Low	Unclear	Low	Low	Unclear
Penotti, 2003	Low	Unclear	Low	High	High	Unclear	Unclear
Sammartino, 2003	Low	High	High	High	Low	Low	Unclear
Tice, 2003	Low	Low	Low	Unclear	Low	Low	Low
Atkinson, 2004	Low	Unclear	Low	Low	Low	Low	Low
Colarcurci, 2004	Low	Unclear	Low	High	Low	Low	Unclear

eTable 9 Risk of bias assessments for the included clinical trials

Lead author, publication date	Random sequence generation	Allocation concealment	Blinding of participants & personnel	Blinding of outcome assessments	Incomplete outcome data	Selective reporting	Other bias
Nahas, 2004	Unclear	Unclear	High	High	High	Low	Unclear
Secreto, 2004	Low	Low	Low	Unclear	Low	Low	Unclear
Albertazzi, 2005	Low	Low	Low	Unclear	Low	Low	Unclear
Liljegren, 2012	Low	Low	Low	Unclear	Low	Low	Unclear
Hervik, 2008	Low	Low	Low	Unclear	Low	Low	Unclear
Deng, 2007	Low	Low	Low	Low	Low	Low	Unclear
Cramer, 2015	Low	High	Unclear	Unclear	Low	Low	Unclear
Dodin, 2005	Low	Low	Low	Low	Low	Low	Low
Shahnazi, 2013	Low	Low	Low	Unclear	Low	Low	Unclear
Tammabasamut, 2014	Low	Low	Low	Unclear	Low	Low	Unclear
Frei-Kleiner, 2005	Unclear	Unclear	Low	High	Unclear	Low	Unclear
Hidalgo, 2005	Low	Low	Low	Unclear	Low	Low	Unclear
Shen, 2005	Low	High	High	High	High	low	High
Verhoeven, 2005	Low	Unclear	Low	Unclear	Low	Low	Unclear
Winther, 2005	Low	Unclear	Low	High	Low	Low	Unclear
Heger, 2006	Low	Unclear	Low	Unclear	Low	Low	Low
Lewis, 2006	Low	Unclear	Low	Unclear	Low	Low	Unclear
Newton, 2006	Low	Low	Low	Low	Low	Low	Low
Pockaj, 2006	Unclear	Unclear	Low	High	Low	Low	Unclear
Sammartino, 2006	Low	Low	Low	Unclear	Low	Low	Unclear
Uebelhack, 2006	Low	Low	Low	Unclear	Low	Low	Unclear
Bai, 2007	Unclear	Unclear	Low	High	Low	Low	Unclear
Cheng, 2007	Unclear	Unclear	Low	High	Low	Low	Unclear
Cancellieri, 2007	Unclear	Unclear	Low	High	High	Low	Unclear
Chung, 2007	Unclear	Unclear	Low	High	Low	Low	Unclear
Danna, 2007	Low	Unclear	Low	Low	Low	Low	Unclear

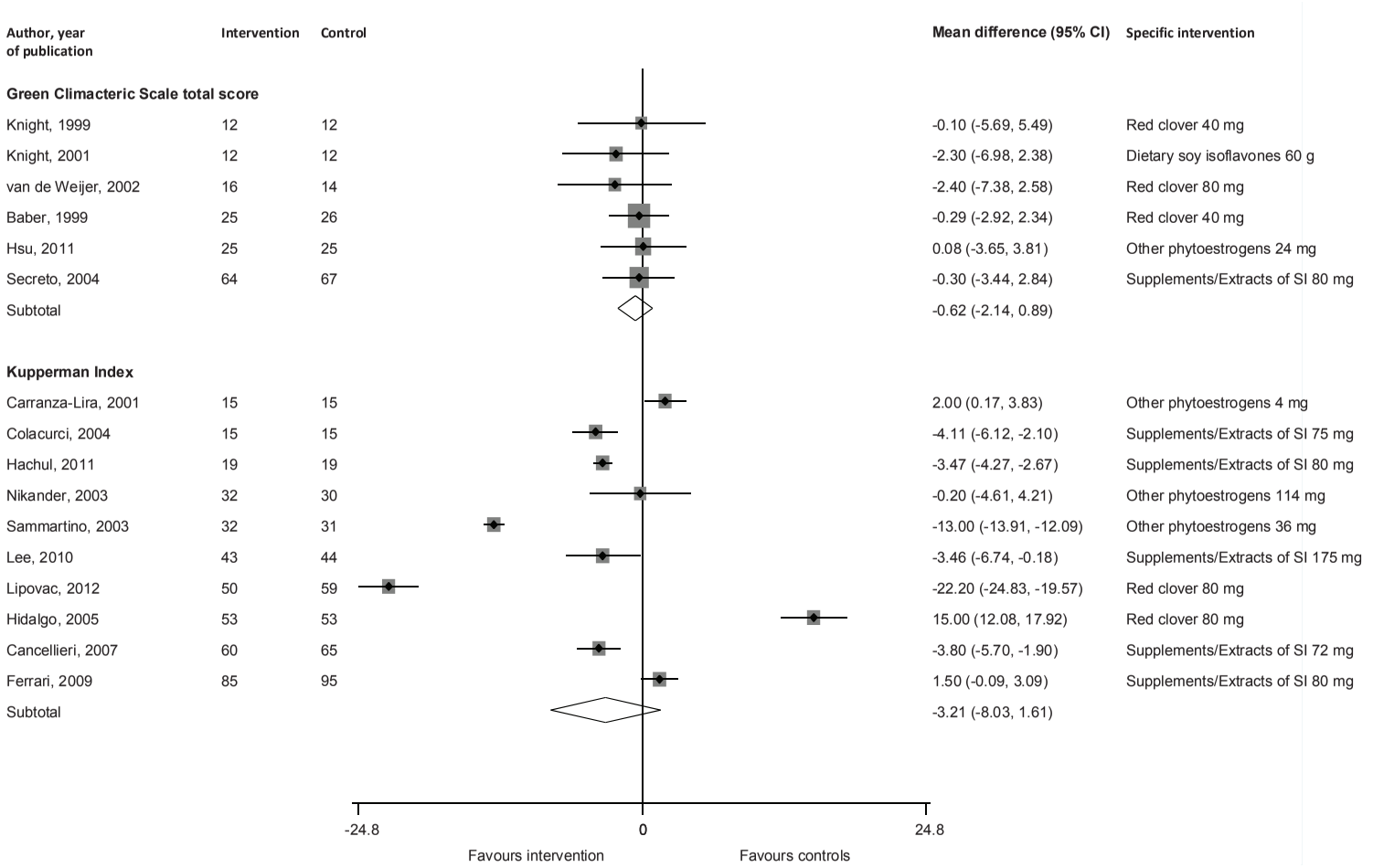
eTable 9 Risk of bias assessments for the included clinical trials

Lead author, publication date	Random sequence generation	Allocation concealment	Blinding of participants & personnel	Blinding of outcome assessments	Incomplete outcome data	Selective reporting	Other bias
Elavsky, 2007	Low	High	High	High	Low	Low	Unclear
Nir, 2007	Low	Unclear	High	Low	Low	Low	Unclear
Petri, 2007	Low	Unclear	Low	Low	Low	Low	Unclear
Kwee, 2007	High	High	Low	Unclear	Low	Low	Unclear
Rotem, 2007	Unclear	High	Low	High	Low	Low	Unclear
Vincent, 2007	Low	High	High	High	Low	Low	High
Yang, 2007	Unclear	High	Low	High	Low	Low	Unclear
Zaborowska, 2007	Low	High	High	High	High	Low	High
Chattha, 2008	Low	High	High	High	Low	Low	High
Haines, 2008	Low	Low	Low	Unclear	Low	Low	Unclear
Amsterdam, 2009	Low	Low	Low	Low	Low	Low	Unclear
Borud, 2009	Low	High	High	Low	Low	Low	Unclear
Ferrari, 2009	Low	Unclear	Low	Unclear	Low	Low	Unclear
Geller, 2009	Low	Unclear	Low	Unclear	Low	Low	Unclear
Qu, 2009	Low	High	High	High	High	Low	High
Sluijs, 2009	Low	Low	Low	Unclear	Low	Low	Unclear
van Die, 2009	Low	Unclear	Low	High	Low	Low	Unclear
Abdali, 2010	High	Unclear	Low	High	Low	Low	Unclear
Garcia, 2010	Unclear	Unclear	Low	High	Low	Low	Unclear
Kim, 2010	Low	High	High	High	Low	Low	high
Lee, 2010	Unclear	Unclear	Low	High	Unclear	Low	Unclear
Simbalista, 2010	Low	Low	Low	Unclear	Low	Low	Unclear
Venzke, 2010	Low	High	High	High	Low	Low	High
Evans, 2011	Low	Low	Low	Unclear	Low	Low	Unclear
Hachul, 2011	Unclear	Unclear	Low	High	Low	Low	Unclear
Hsu, 2011	Unclear	Unclear	Low	Unclear	High	Low	High

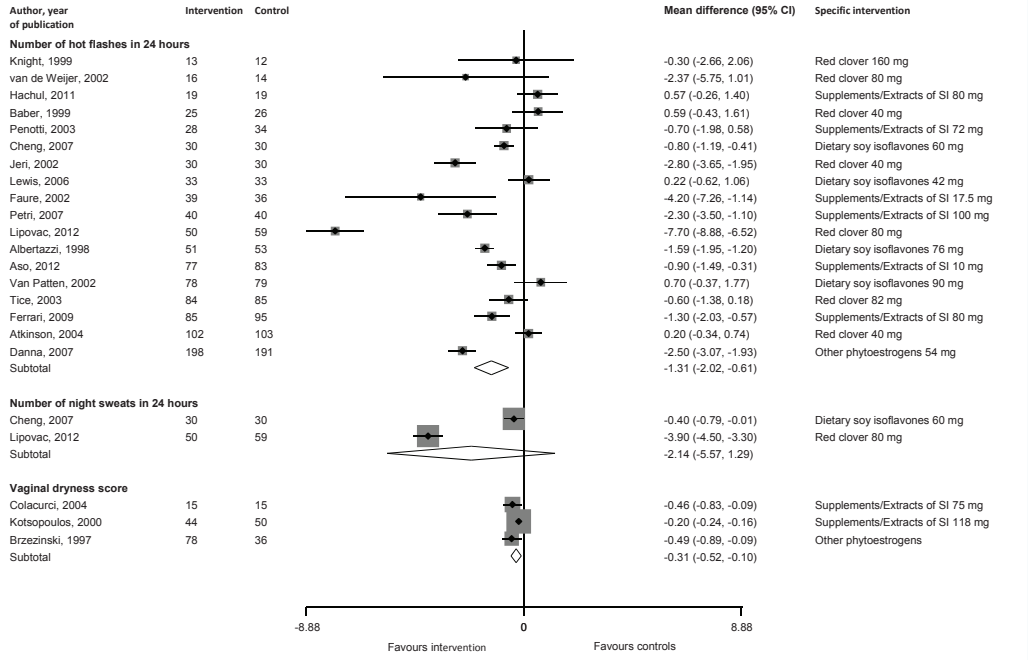
eTable 9 Risk of bias assessments for the included clinical trials

Lead author, publication date	Random sequence generation	Allocation concealment	Blinding of participants & personnel	Blinding of outcome assessments	Incomplete outcome data	Selective reporting	Other bias
Joshi, 2011	High	High	High	High	low	Low	High
Yakoot, 2011	Low	High	High	High	High	Low	High
Aso, 2012	High	Unclear	Low	High	High	Low	High
Chang, 2012	Low	Low	Low	Unclear	Low	Low	Unclear
Colli, 2012	Unclear	Unclear	Low	Unclear	High	Low	Unclear
Darsareh, 2012	Low	High	High	High	Low	Low	High
Lipovac, 2012	Unclear	High	High	High	Low	Low	High
Oliveira, 2012	Low	High	High	High	Low	Low	Unclear
Painovich, 2012	Low	Unclear	High	High	Low	Low	High
Sun, 2012	Low	Low	Low	Low	Low	Low	Low
Xia, 2012	Low	Low	Low	Unclear	High	Low	Unclear
Yeh, 2012	High	High	High	High	Low	Low	High
Elkins, 2013	Low	High	High	High	Low	Low	High
Farzaneh, 2013	Low	Unclear	Low	Unclear	high	low	Unclear
Hachul, 2013	Unclear	Unclear	Low	High	High	Low	High
Kohama, 2013	Low	Low	Low	Unclear	Low	Low	Unclear
Lindh, 2013	Low	Low	High	Unclear	Low	Low	Unclear
Baccetti, 2014	Low	Low	High	Unclear	Low	Low	Unclear
Liu, 2014	Low	Low	Low	Unclear	Low	Low	Low
Newton, 2014	Low	High	High	Unclear	Low	Low	Unclear
Nedeljkovik, 2014	Low	Low	High	Unclear	Low	Low	Unclear
Reed, 2014	Low	High	High	High	High	Low	High

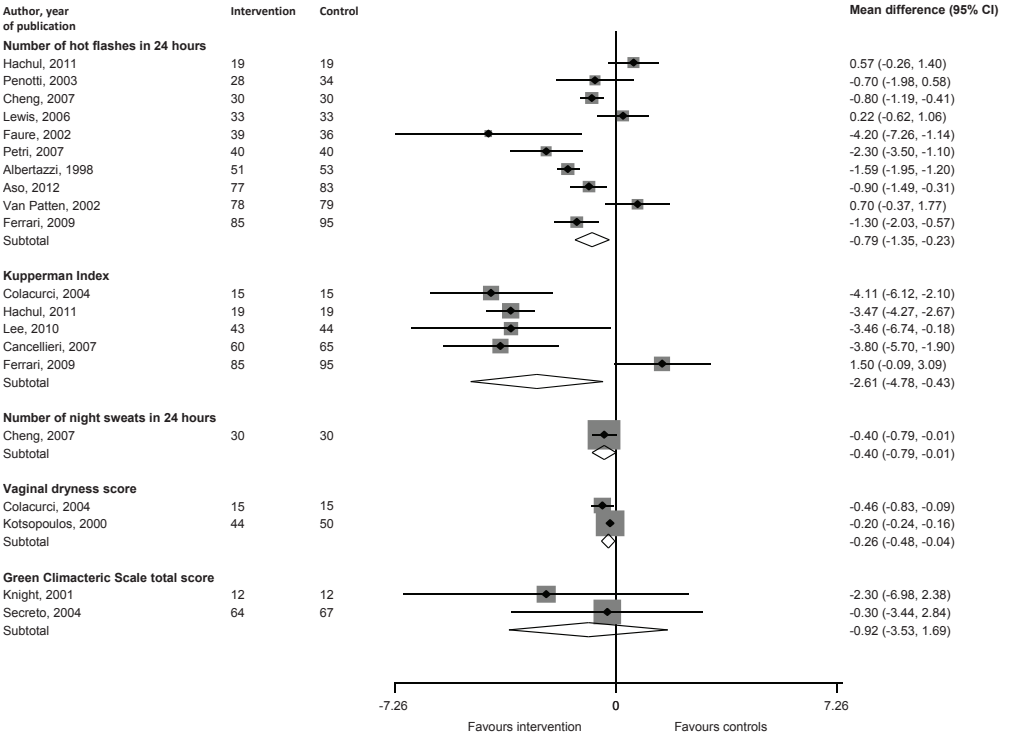
eFigure 1 Effect of Phytoestrogens supplementation on menopausal scores



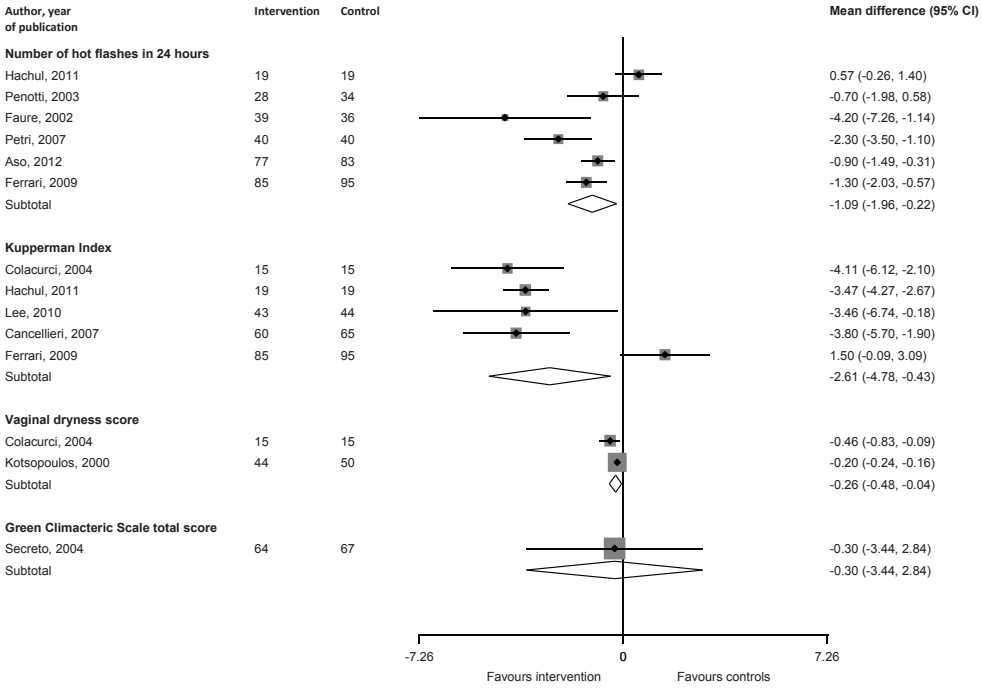
eFigure 2 Effect of Phytoestrogens supplementation on vasomotor symptoms



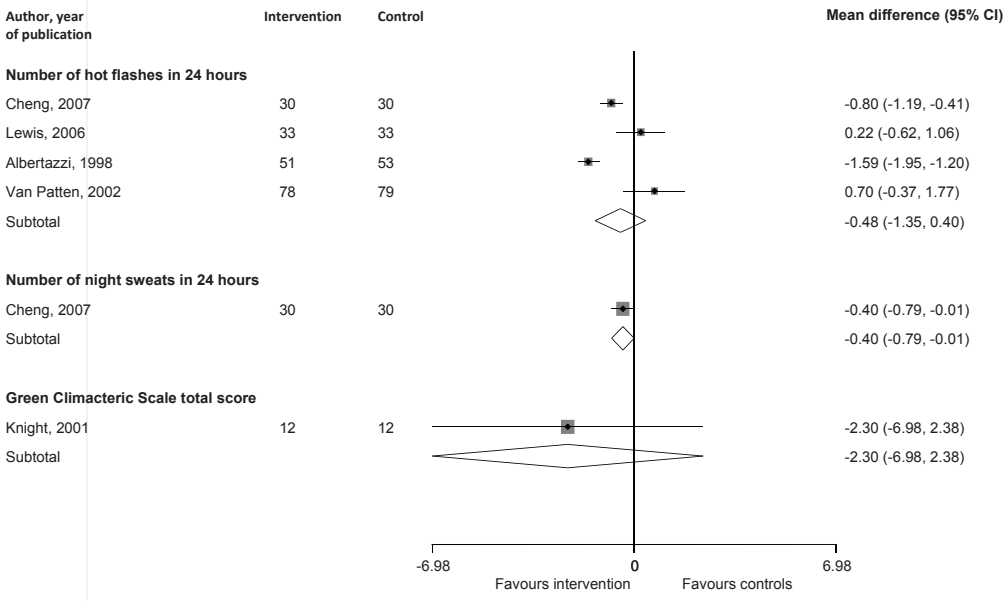
eFigure 3 Effects of dietary and supplemental soy isoflavones on menopausal scores and symptoms



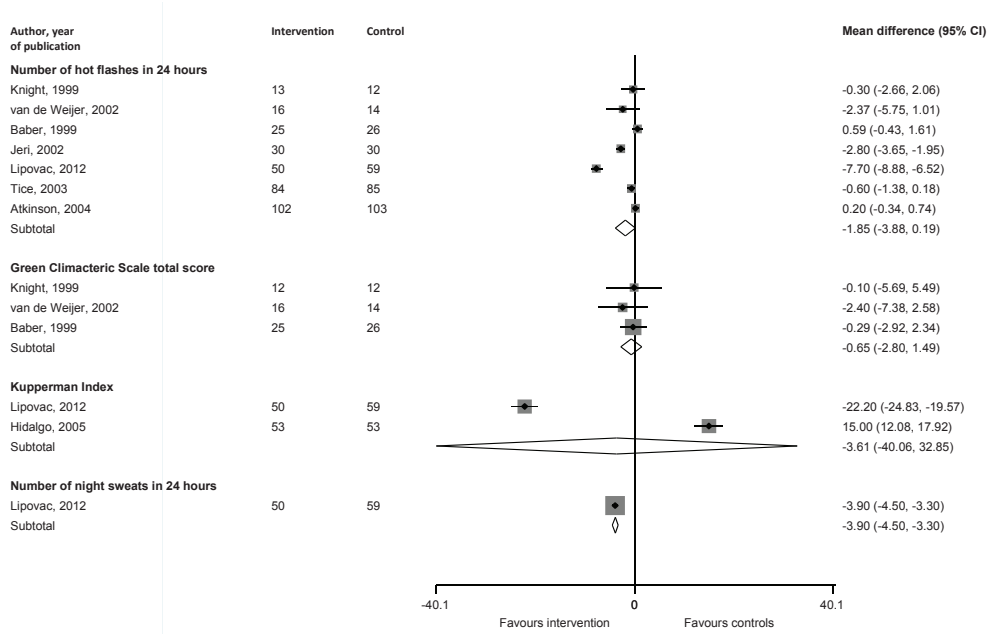
eFigure 4 Effects of supplements and extracts of soy isoflavones on menopausal scores and symptoms



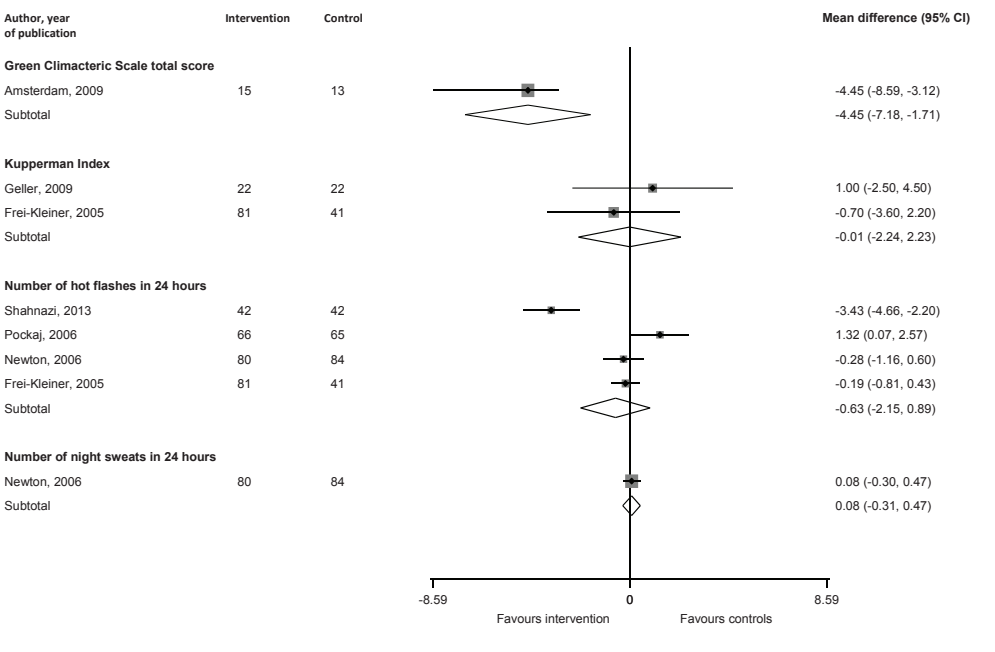
eFigure 5 Effects of dietary soy isoflavones on menopausal scores and symptoms



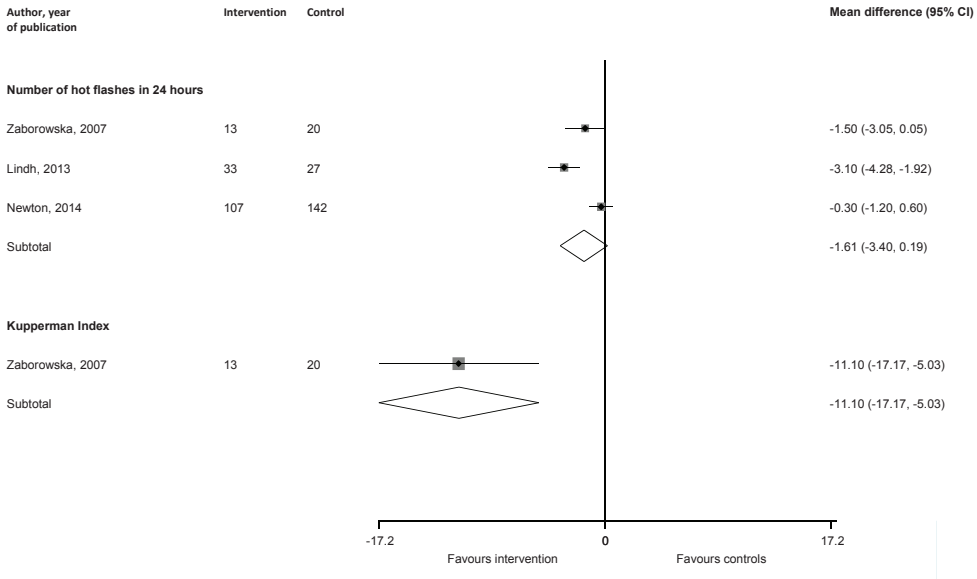
eFigure 6 Effects of red clover on menopausal scores and symptoms



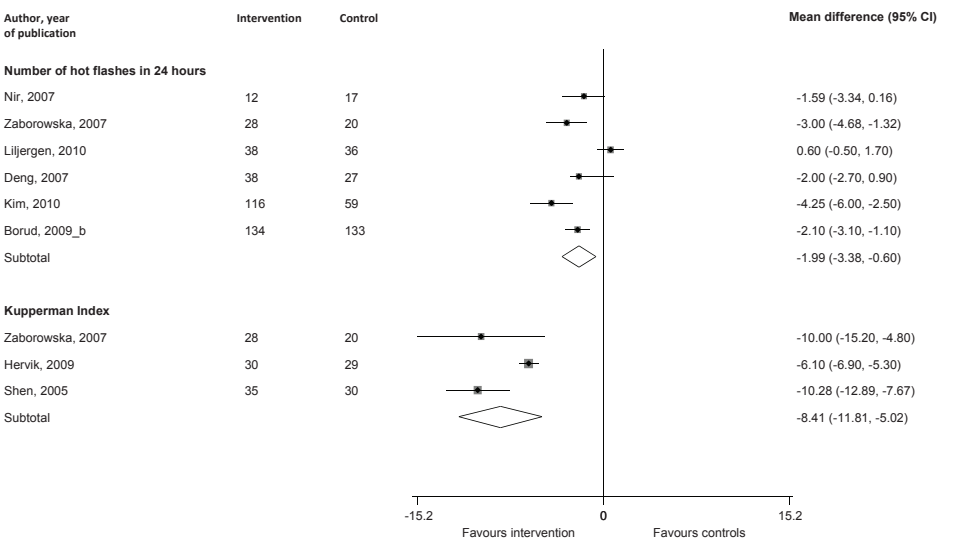
eFigure 7: Effects of black cohosh on menopausal scores and symptoms



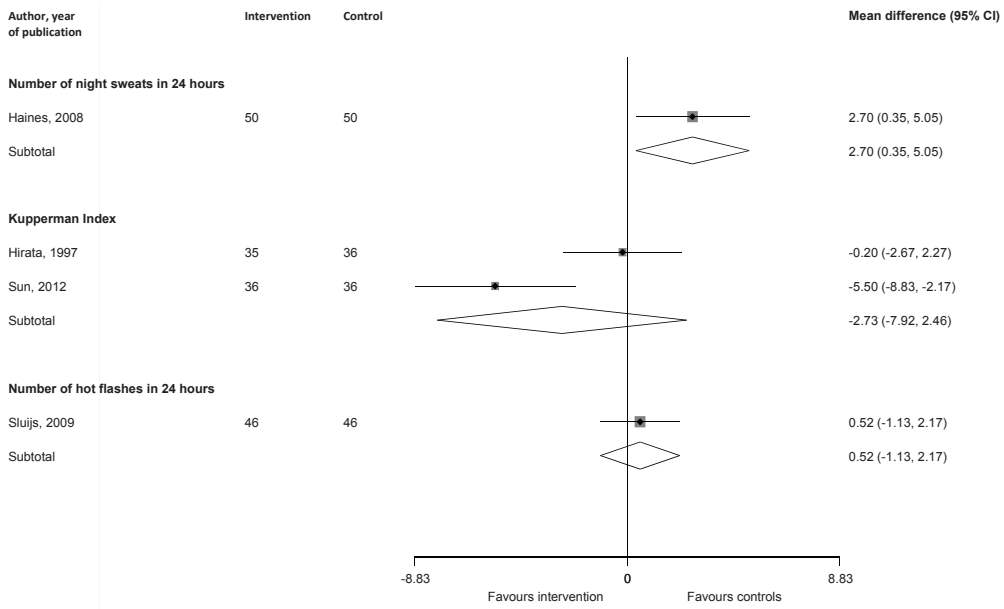
eFigure 8 Effects of mind, body, and behavioural therapies on menopausal scores and symptoms



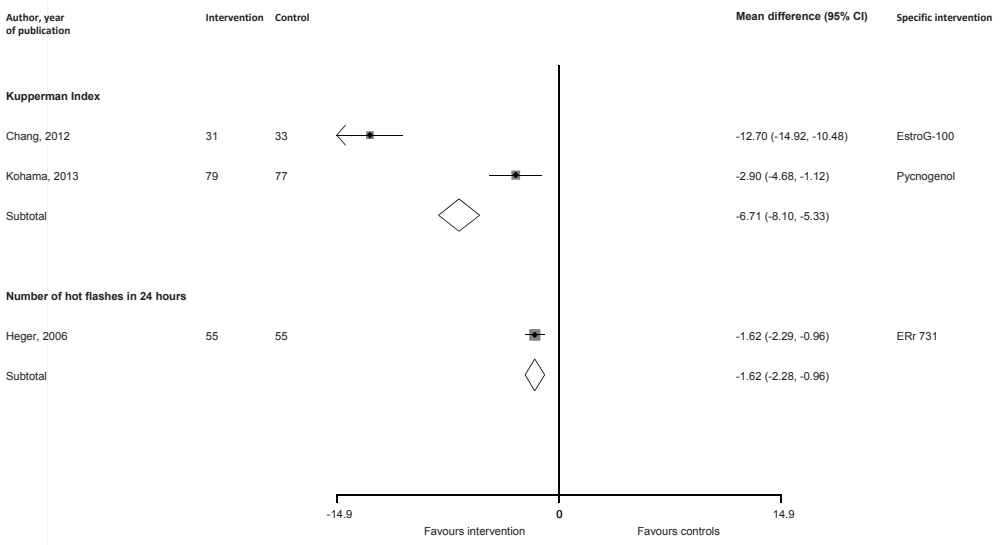
eFigure 9: Effects of acupuncture on menopausal scores and symptoms



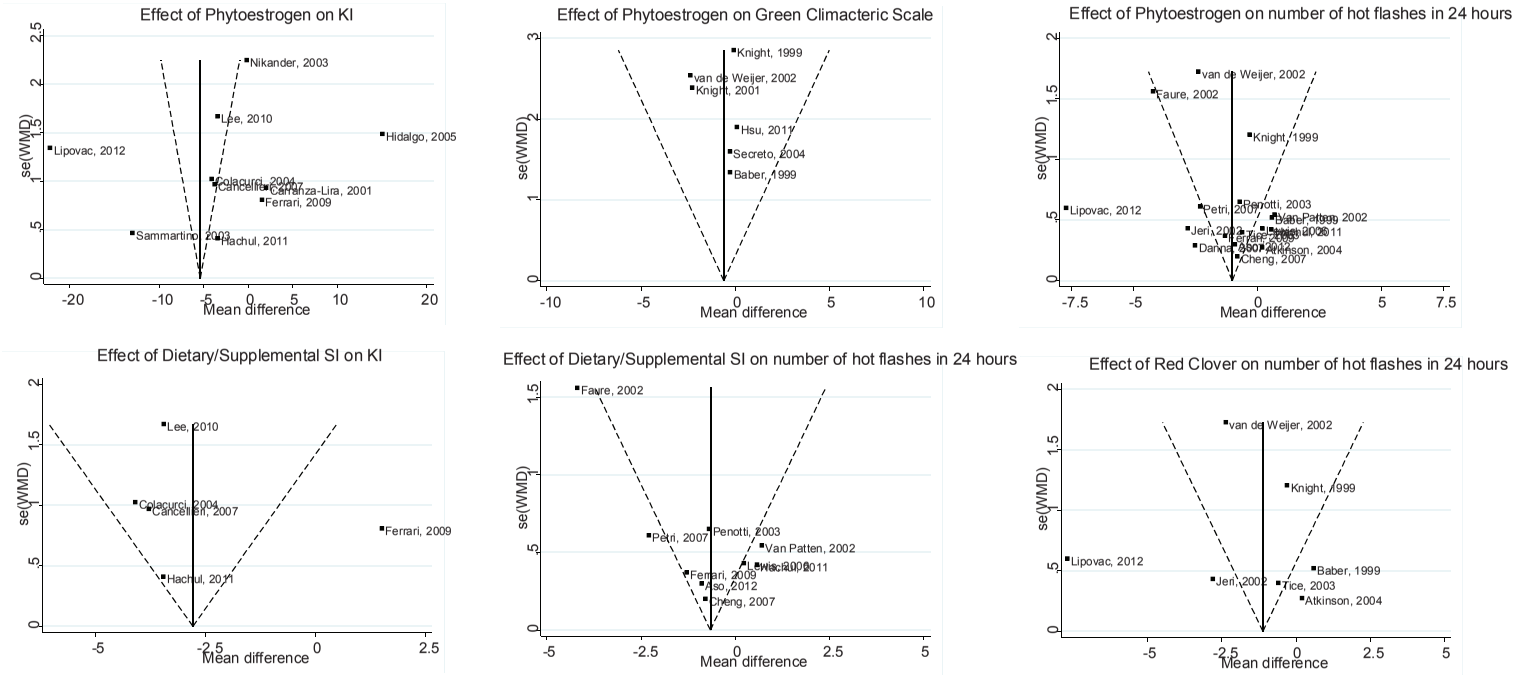
eFigure 10: Effects of Chinese medicinal herbs on menopausal scores and symptoms



eFigure 11: Effects of other medicinal herbs on menopausal scores and symptoms



eFigure 12: Assessment of small study effects by funnel plots and Egger's test in randomised controlled trials of natural remedies and menopausal symptoms



The dotted lines show 95% confidence intervals around the overall summary estimate calculated using a fixed effect model;; P-values for bias calculated using Egger's test was 0.415; 0.255; 0.450; 0.792; 0.841; and 0.435 for effect of phytoestrogen on Kupperman Index; effect of phytoestrogen on Green Climacteric Scale; effect of phytoestrogen in number of hot flashes in 24 hours; effect of dietary and supplemental soy isoflavones on Kupperman Index; effect of dietary and supplemental soy isoflavones on number of hot flashes in 24 hours; and effect of red clover on number of hot flashes in 24 hours respectively; KI, Kupperman Index; SI, soy isoflavones

eAppendix 3 Literature search strategy for studies of natural and plant-based therapies in menopausal health

Relevant studies, published before May 01, 2015 (date last searched), were identified through electronic searches not limited to the English language using MEDLINE, EMBASE, and the Cochrane databases. Electronic searches were supplemented by scanning reference lists of articles identified for all relevant studies (including review articles), by hand searching of relevant journals and by correspondence with study investigators. The computer-based searches combined search terms related to markers related to vasomotor symptoms, related to vascular outcomes and related to study design without language restriction.

(i) MEDLINE strategy to identify relevant exposures:
("Panax"[MeSH Terms] OR "Panax*"[All Fields] OR "Renshen*"[All Fields] OR "Shinseng*"[All Fields] OR "Ninjin*" [All Fields] OR "Schinseng*" [All Fields] OR "Jen Shen*" [All Fields] OR "Ginseng*" [All Fields] OR "Soy Foods"[MeSH Terms] OR "Soy*" [All Fields] OR "Natto"[All Fields] OR "Tempeh"[All Fields] OR "Texturized Soy Protein*"[All Fields] OR "Texturized Vegetable Protein*"[All Fields] OR "Tofu"[All Fields] OR "Miso"[All Fields] OR "vitex"[MeSH Terms] OR "Vitex"[All Fields] OR "Vitices"[All Fields] OR "Chasteberr*"[All Fields] OR "Chasteberry Tree*"[All Fields] OR "Chaste Tree*" [All Fields] OR "Chastetree*" [All Fields] OR "Passiflora"[MeSH Terms] OR "Passiflora*"[All Fields] OR "Passionfruit*"[All Fields] OR "Passion Flower*" [All Fields] OR "Granadilla*"[All Fields] OR "Hypericum"[MeSH Terms] OR "Hypericum*"[All Fields] OR "Hypericum perforatum*"[All Fields] OR "St. Johns Wort"[All Fields] OR "St. Johnswort"[All Fields] OR "Saint Johns Wort"[All Fields] OR "Saint Johnswort"[All Fields] OR "St. John's Wort"[All Fields] OR "St. John Wort"[All Fields] OR "Saint John's Wort"[All Fields] OR "Saint John Wort"[All Fields] OR "Cimicifuga"[MeSH Terms] OR "Cimicifuga*"[All Fields] OR "Cimicifuga racemosa*"[All Fields] OR "Black Bugbane*"[All Fields] OR "Actaea racemosa*"[All Fields] OR "Black Cohosh*"[All Fields] OR "Kava"[MeSH Terms] OR "Kava*"[All Fields] "Kawa*"[All Fields] OR "Piper methysticum*"[All Fields] OR "Medicine, Traditional"[Mesh] OR "Traditional Chinese Medicine"[All Fields] OR "Folk Remed*"[All Fields] OR "Folk Remed*"[All Fields] OR "Home Remed*"[All Fields] OR "Primitive Medicine"[All Fields] OR "Folk Medicine"[All Fields] OR "Indigenous Medicine"[All Fields] OR "Ethnomedicine"[All Fields] OR "Efamol"[Supplementary Concept] OR "efamol"[All Fields] OR "Efamol G"[All Fields] OR "Preglandin"[All Fields] OR "evening primrose oil"[All Fields] OR "naudicelle"[All Fields] OR "Efamast"[All Fields] OR "Epogam"[All Fields] OR "Trifolium"[MeSH Terms] OR "Trifolium*"[All Fields] OR "red clover"[All Fields] OR "Dietary fiber"[MeSH Terms] OR "fibre"[All Fields] OR "fiber"[All Fields] OR "Roughage*"[All Fields] OR "Wheat Bran*"[All Fields] OR "Isoflavones"[MeSH Terms] OR "*Isoflavone*"[All Fields] OR "3-Benzylchroman-4-*"[All Fields] OR "3 Benzylchroman 4 Ones"[All Fields] OR "Benzylidene 4 Chromanones" [All Fields] OR "rheum"[MeSH Terms] OR "rheum"[All Fields] OR "Da Huang"[All Fields] OR "rhubarb"[All Fields] OR "Complementary Therapies"[MeSH Terms] OR "Complementary Therap*"[All Fields] OR "Complementary Medicine*"[All Fields] OR "alternative medicine*"[All Fields] OR "aromatherapy"[MeSH Terms] OR "aromatherap*"[All Fields] OR "Aroma Therap*"[All Fields] OR "Withania"[MeSH Terms] OR "Withania*"[All Fields] OR "therapeutic touch"[MeSH Terms] OR "therapeutic touch"[All Fields]

OR “Reiki”[All Fields] OR “Laying-on-of-Hands”[All Fields] OR “Phytoestrogens”[MeSH Terms] OR “Phytoestrogens”[Pharmacological Action] OR “Phyto-Estrogen*”[All Fields] OR “Phyto Estrogen*”[All Fields] OR “Phytoestrogen*”[All Fields] OR “Angelica sinensis”[MeSH Terms] OR “dong quai”[Supplementary Concept] OR “Angelica sinens*”[All Fields] OR “herbal medicine”[MeSH Terms] OR “herbal medicine*”[All Fields] OR “Ayurveda”[All Fields] OR “Herbalism”[All Fields] OR “naturopathy”[MeSH Terms] OR “naturopath*”[All Fields] OR “relaxation therapy”[MeSH Terms] OR “relaxation therap*”[All Fields] OR “relaxation technique*”[All Fields] OR “chiropractic”[MeSH Terms] OR “chiropractic*”[All Fields] OR “ashwagandha”[MeSH Terms] OR “ashwagandha*”[All Fields] OR “wild yam*”[All Fields] OR “coumestrol”[MeSH Terms] OR “coumestrol*”[All Fields] OR “homeopathy”[MeSH Terms] OR “homeopath*”[All Fields] OR “Massage”[MeSH Terms] OR “Massage*”[All Fields] OR “osteopath*”[All Fields] OR “yoga”[MeSH Terms] OR “yoga”[All Fields] OR “acupuncture”[MeSH Terms] OR “acupuncture*”[All Fields] OR “mindfulness”[MeSH Terms] OR “mindfulness”[All Fields])

(ii) MEDLINE strategy to identify relevant outcomes:
(“hot flash*”[All Fields] OR “night sweat*”[All Fields] OR “menopause”[MeSH Terms] OR “menopaus*”[All Fields] OR “post menopaus*”[All Fields] OR “post-menopaus*”[All Fields] OR “postmenopaus*”[All Fields] OR “perimenopaus*”[All Fields] OR “peri-menopaus*”[All Fields] OR “peri menopaus*”[All Fields] OR “climacteric”[MeSH Terms] OR “climacteric”[All Fields] OR “Vaginal dryness”[All Fields] OR “mood swing*”[All Fields] OR “unstable mood*”[All Fields] OR “Postmenopausal Osteoporosis”[MeSH Terms] OR “Postmenopausal Osteoporos*”[All Fields] OR “Postmenopausal Bone Loss*”[All Fields] OR “Perimenopausal Bone Loss*”[All Fields] OR “Post Menopausal Osteoporos*”[All Fields] OR “Post-Menopausal Osteoporos*”[All Fields] OR “headache”[MeSH Terms] OR “headache*”[All Fields] OR “libido”[MeSH Terms] OR “Decreased Libido”[All Fields] OR “loss of libido”[All Fields] OR “palpitations”[All Fields] OR (“depression”[MeSH Terms] AND “menopause” [MeSH Terms]) OR (“depression”[All Fields] AND “menopause”[MeSH Terms]) OR (“anxiety”[MeSH Terms] AND “menopause”[MeSH Terms]) OR (“anxiety”[All Fields] AND “menopause”[MeSH Terms]) OR “sleep* problem*”[All Fields] OR “sleep* difficult*”[All Fields] OR “difficult* sleep*”[All Fields] OR “problem* sleep*”[All Fields])

(iii) MEDLINE strategy to identify relevant population:
(“humans”[MeSH Terms])

(iv) MEDLINE strategy to identify relevant study designs:
(“longitudinal studies”[MeSH Terms] OR “prospective”[All Fields] OR “cohort”[All Fields] OR “follow up”[All Fields] OR (“Clinical Trials as Topic”[Mesh]) OR “Randomized Controlled Trial” [Publication Type])

Parts i, ii, iii, and iv were combined using ‘AND’ to search MEDLINE. Each part was specifically translated for searching alternative databases.

Appendix 4 Reference list of included studies

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Chapter 5

General Discussion

INTRODUCTION

Despite improvements in prevention and treatment, cardiovascular disease (CVD) remains the leading cause of death worldwide and is a major public health problem[1]. Type 2 diabetes (T2D) is an important risk factor for CVD, and most subjects with T2D die from CVD[2]. Investigating the economic impact of non-communicable diseases (NCDs), including CVD and T2D, can help shape future healthcare plans and strategies to reduce the economic burden of these diseases. The need for prevention strategies to maintain optimal cardiometabolic health is a priority in middle- and high income countries, given the increasing prevalence of cardiometabolic disorders in the population and the increasing economic burden of these disorders to society. Maintaining optimal cardiometabolic health is a multifactorial endeavor. Several dietary and metabolic factors determine the overall risk of poor cardiometabolic health[3-6]. Identifying new risk factors and pathways for these cardiometabolic diseases could thus shed more light into the complex pathophysiology of cardiometabolic diseases and in potential novel preventing strategies to reduce the burden of these diseases. In addition, the etiology of CVD might be different in women, which can result from unique biologic characteristics in the coronary systems of women[7]. In particular, the onset of menopause is associated with a sharply increased risk of CVD risk in women[8], highlighting the need to evaluate menopause and its related physiological changes and symptoms as cardiometabolic risk factors.

In this chapter, we give an overview of the main findings of the studies in this thesis, what they add to the existing body of evidence and possible implications. Furthermore, we consider methodological issues and give suggestions for future research.

MAIN FINDINGS

Economic Impact of Non-Communicable Diseases

In chapter 2 we found that NCDs (CVD, T2D, cancer, chronic obstructive pulmonary disease and chronic kidney disease) pose a significant financial burden on health care budgets, macroeconomic productivity and nation's welfare by summarizing the findings from more than 200 papers. Among all NCDs, healthcare expenditures for CVD were the highest. The estimated DALY (a measure of productivity loss) for CVD ranged from 4.2 in Spain to 68 in Kenya. Additionally, in our review, we found that costs increased as did severity of the disease and years lived with the disease. We identified a gap in literature concerning the economic impact of NCDs in low and middle income countries. Limited research capacity, inadequate financial investment, healthcare system development, a lack of electronic health records, and language restrictions may contribute to this shortage of data and analysis[9]. Also, we identified several methodological concerns of the studies reviewed, related to sample selection, case definition and the nature of costs included.

Fatty acids

In recent decades, the quality of nutrition worldwide has changed [10]. The level of certain fatty acids intake, including polyunsaturated fatty acids (PUFAs), has increased, currently representing 28-42% of total energy consumed by European populations[10,11]. Among subtypes of fatty acids, the current guidelines for CVD encourage high consumption of PUFAs as a replacement for other types of fatty acids such as saturated fatty acids[12,13]. Therefore, due also to the increased consumption of linoleic acid-rich vegetable oils in the Western diet, consumption of n-6 PUFA has progressively increased much more than has consumption of n-3 PUFA[14]. Concerns have been raised that high dietary consumption of n-6 PUFAs along with low intake of n-3 PUFA may shift the physiological state to one that is pro-inflammatory and that, therefore, may contribute to increased risk of cardiometabolic diseases, including an unfavorable body fat distribution[15,16]. These concerns come from the fact that n-6 PUFAs intake, contrary to n-3 PUFA, has been generally associated with production of eicosanoids which have pro-inflammatory properties[17]. Furthermore, different fatty acids have different oxidation rates, and the last is associated with the development of obesity[18-20]. Therefore, the shift in n-3 to n-6 PUFA ratio may have an impact in body fat distribution. Moreover, in the Western diet, the consumption of plant derived n-3 PUFAs has almost doubled during the last decades (from 1 to 1.9 g/day)[21]. Recent evidence show that marine derived n-3 PUFA may have a different effect on health outcomes like cancer than do non-marine derived n-3 PUFA[22, 23]. In our study we examined the association between total PUFAs and individual PUFAs intake (n-3, n-6 and n-3/n-6 PUFAs ratio) and serum C-reactive protein, a marker of chronic inflammation. We found that high intake of PUFA were associated with diminished chronic inflammation, which was mainly driven by n-6 PUFA.

We did not find an association between total n-3 PUFA and C-reactive protein levels, However, when we stratified the analysis by gender, we found that high n-3 PUFA intake was associated with increased levels of inflammation only in women. Although, the underlying mechanism for the observed gender differences in the association between n-3 PUFAs and CRP is not known, previous evidence showed that women have more ALA availability than men because of lower partitioning of women toward beta oxidation[24,25]. Furthermore, gender differences have been reported in the activity of desaturation-elongation pathway for the conversion of EPA to DHA[26]. In our study, the main sources of n-3 PUFAs were butter and margarine (the main source of short chain n-3 PUFA), and 39% of the study participants did not consume fish (the main food source of long chain n-3 PUFA). Thus far, studies have been consistent in showing the beneficial role of marine-derived n-3 PUFAs supplementation on chronic systemic inflammation[27], but little is known about the role of plant derived n-3 PUFAs on inflammation and cardiometabolic health[22]. Our findings suggest that it is important to consider the dietary source of n-3 PUFAs when evaluating their role on inflammation and other health outcomes. Our results may provide additional support for the maintenance of the present recommended level of n-6 PUFAs intake. At the same time, these results suggest that substitution of plant-derived n-3 PUFAs with marine derived n-3 PUFAs should

be considered and further evaluated. Furthermore, in chapter 3.2 we examined the hypothesis that dietary fat composition (i.e. dietary ratios of n-3/n-6 PUFAs and SFAs/PUFAs) is associated with total body fat and regional body fat distribution, as measured by dual-energy X-ray absorptiometry (DXA). To test this hypothesis, we used data from two population-based cohorts in the Netherlands and Australia, including middle-aged and elderly women. Overall, we found no consistent association of dietary fat composition with total fat and regional body fat distribution. Although not statistically significant, effects in opposite directions were observed in the two studies for the association between dietary fat composition and total body fat. This may be due to differences in the food sources of PUFAs in the two populations used for this analysis.

Vitamin D

Main sources of vitamin D include exposure to sunlight, diet and dietary supplements[28]. An inappropriate diet or exposure to ultraviolet B rays may lead to vitamin D deficiency, which is estimated to be highly prevalent in elderly people with prevalence estimates usually reported to be above 40% [29,30]. Although the best-characterized effects of vitamin D deficiency involve the musculoskeletal system, recent evidence suggests that vitamin D has effects on human health beyond bone health[29]. Low serum 25(OH)D concentrations have been associated with many obesity-related disorders including CVD and T2D[29,31]. Receptors for vitamin D are present in adipocytes[32-34], suggesting a role of vitamin D in obesity. Also, low serum 25 hydroxy vitamin D (25(OH)D) concentrations lead to an increase in parathyroid hormone levels as a normal physiological response, which, in turn, favors the lipid storage metabolism [35,36]. Furthermore, a study using the bidirectional Mendelian randomization approach found that higher body mass index leads to lower 25(OH)D, but lower 25(OH)D did not lead to higher body mass index[37]. However, results from long-term studies linking vitamin D to body fat, and in particular body fat distribution, remain scarce. Furthermore, most of the research on this topic has been performed in younger populations and has generally used less precise anthropometric measures such as waist circumference or body mass index instead of more accurate measures of body composition by DXA.

We studied the relationship between vitamin D status and body composition including measures of fat and lean mass and fat distribution in the elderly. In our study we found an inverse association between vitamin D status and total body fat mass and body mass index. Furthermore, we found that adequate vitamin D status was positively associated with lean mass percentage in the elderly. Nevertheless, these results should be interpreted with caution, since adjustment for baseline body mass index (which highly correlates with total fat mass, Pearson Correlation=0.87) abolished the association between vitamin D, total body fat and lean mass (data not shown), and therefore, providing some evidence that these associations might not be causal. Our results do not support the hypothesis that vitamin D plays a role in the regional distribution of body fat. Moreover, we found an interaction between 25(OHD), total body fat and the presence of cardio-metabolic diseases. The association between vitamin D and total fat percentage was present only in subjects free of cardio-metabolic disease. Although the reasons for this effect modification are not clear, this observation may be due to changes in lifestyle after diagnosis among people with cardiometabolic diseases.

Alternatively, it may be due to increased clearance of vitamin D associated with the development of cardiometabolic diseases, which may dilute the association in individuals with CVD and T2D[38].

Biomarkers

A growing body of evidence shows gamma-glutamyltransferase (GGT) to be associated with adverse cardiometabolic health, including obesity, T2D, and CVD [39-44]. GGT has been associated with oxidative stress [45,46], which has been implicated in both the pathogenesis of obesity and T2D[47]. Furthermore, GGT is capable of inducing lipid oxidation[46], a process involved in the etiology of insulin resistance[48] and that is closely linked to abdominal obesity and T2D[49]. Nevertheless, little attention has been given to normal range GGT and its role on obesity and/or body fat distribution. Even though the studies are consistent in showing a strong association between high circulating levels of GGT and T2D risk, it is yet unclear whether this association is causal[50]. Measurement of serum GGT is accurate, reliable, easy and inexpensive [51]. Therefore, if serum GGT is a marker that may play a role in the etiology of T2D, it could have implications for prevention and clinical care of T2D. In our study, we found no association between circulating GGT levels and total body fat. However, higher circulating levels of GGT within normal range were associated with higher android fat and higher android/gynoid fat ratio, independent of the presence of nonalcoholic fatty liver disease or insulin resistance. These findings may suggest that an increase in GGT concentrations within its physiological normal range is a sensitive and early biomarker of unfavorable body fat distribution. We also found a strong association between GGT plasmatic levels, glycemic traits (glucose and insulin) and T2D. However, using GGT-related genetic variants combined in a genetic risk score, we found no evidence for a causal relationship between GGT levels and these outcomes. The lack of evidence of a causal relationship may suggest that the corresponding observational association is due to confounding and/or reverse causation.

Cardiometabolic Health in Women

Endogenous sex hormones

The shift in hormonal balance during the menopause transition, which is characterized by a decline in endogenous estradiol levels and a relative androgen excess[52], has been associated with impairment of glucose regulation and visceral adiposity[53]. Therefore, it has been suggested that endogenous sex hormones, and in particular estradiol, may play an important role in the pathophysiology of T2D in women[53,54]. This hypothesis is in line with data showing that pregnancy, characterized by high endogenous estrogen levels and polycystic ovary syndrome, a condition of anovulation and hyperandrogenism, have been both associated with insulin resistance and increased risk of T2D [55,56]. This thesis provides further insights in the association of endogenous sex hormones (sex hormone-binding globulin (SHBG), free testosterone (FT), total testosterone (TT), free estradiol (FE) and total estradiol (TE)) with the risk of T2D. By using data from the Rotterdam Study and a systematic review approach, we showed that lower levels of SHBG and higher levels of TE are associated with higher risk of T2D in postmenopausal women, independent of established risk factors such as glucose and inulin. Our study showed no association between TT and the risk of T2D though a suggestive positive association was observed between FT and T2D. Our findings support the notion that endogenous sex hormones may play a role in the pathophysiology of T2D.

Estrogen receptor beta

Estrogen, including estradiol, has many physiological effects in the female cardiovascular system, all of which are mediated by estrogen receptors[57,58]. A newly discovered estrogen receptor beta (ERβ) is widely distributed throughout the vascular system[58,59]. Consequently, high expression of ERβ in the vascular bed has raised interest in the question of whether this receptor could offer a new possibility for pharmacological interventions to prevent and treat CVD. However, the role of ERβ and its mechanism of action in the cardiovascular system remain unclear. Furthermore, the vascular response to estrogens appears to change with increasing age and to depend on years after menopause[60,61]. It is therefore unknown whether age- and menopause-related changes of vascular ERβ may explain the influence of these factors on estrogen's actions. By summarizing the evidence from 88 studies, we found that ERβ signaling possess vasodilator and antiangiogenic properties by regulating the activity of nitric oxide, altering membrane ionic permeability in vascular smooth muscle cells, inhibiting vascular smooth muscle cell migration and proliferation, and by regulating adrenergic control of the arteries. Also, there was a possible protective effect of ERβ signaling against left ventricular hypertrophy and ischemia/reperfusion injury. Furthermore, we found that the vascular effects of ERβ may be vessel-specific and may differ by age and menopause status. These findings contribute to the generation of hypotheses and the identification of potential therapeutic targets for the prevention and treatment of CVD. However, the majority of the studies were conducted in animals and further studies in humans are needed to evaluate whether isoform-selective ERβ-ligands might contribute in CVD prevention.

Menopause

Menopause transition, a physiological state of a women's life, is associated with the emergence of a preferential increase in intra-abdominal fat, a shift towards a more atherogenic lipid profile and increased glucose and insulin levels, particularly aggressive for the female cardiovascular system[62-65]. These adverse changes in cardiovascular risk factors during the menopause transition have been mainly attributed to the loss of the protective effect of estrogens, but also to the activation of the renin-angiotensin-aldosterone system[66]. Thus, early menopause has been hypothesized to be especially detrimental to cardiovascular health. About 5 to 10% of women experience early natural menopause by the age of 45[67-69]. Hence, it is important to examine the impact that early menopause might have on CVD. Furthermore, during the menopausal transition and afterwards, up to 80% of women experience vasomotor symptoms (hot flushes and night sweats), depression, fatigue, insomnia and panic attacks [70]. Previous studies have linked menopausal symptoms with adverse cardiometabolic health, including hypertension and dyslipidemia, but the results are contradictory[71-79]. Furthermore, many women chose to use HT as medical treatment for these symptoms. However, there are concerns that HT may have a negative impact on cardiovascular health[61], and therefore, identifying other medical treatments for managing these symptoms avoiding the adverse effects on cardiometabolic health is of public health importance. In our systematic review, we found an increased risk of coronary heart disease, cardiovascular mortality and overall mortality in women who experienced the menopause transition before the age of 45 years. Also, vasomotor symptoms and other menopausal symptoms were associated with an

unfavorable cardiovascular risk profile and increased risk of CVD. However, the association between menopausal symptoms and CVD was mainly explained by cardiovascular risk factors such as blood lipids and body mass index. Further studies are needed to reliably establish which menopausal symptoms are independently associated with CVD outcomes and further clarify the potential mechanisms behind these associations. Moreover, in our systematic review and meta-analysis, by summarizing the evidence from 113 randomized clinical trials, we found that composite and certain specific phytoestrogen supplementations (i.e. soy isoflavones, soy protein) may confer a benefit of reducing menopausal symptoms in women, including hot flashes and night sweats. We found no association between black cohosh (Cimicifuga racemosa or Actaea racemosa) supplementation and menopausal symptoms. Behavioral therapies, acupuncture, and several herbal remedies in aggregate improved overall menopausal symptoms. However, in our systematic review, there was substantial diversity in quality across the available studies, limiting the validity and the generalizability of our findings.

METHODOLOGICAL CONSIDERATIONS

Bias in Dietary Intake and Biomarkers

The dietary intake in the studies presented in Chapter 3 was assessed through a food frequency questionnaire (FFQ). FFQs are relatively inexpensive and easy to administer, and therefore they are frequently used in epidemiological studies to evaluate an individual's usual intake of the type and amount of foods over a defined period of time. However, FFQs and other self-report measures of diet are prone to information bias due to errors in reporting and by incomplete assessment of all sources of micro- and macronutrient intake. This may introduce misclassification. In the study presented in Chapter 3.1, because the outcome was assessed prospectively, the subjective measure of dietary intake would likely lead to non-differential misclassification with respect to the outcome, and therefore would likely bias the estimates toward the null. On the other hand, in the studies presented in Chapter 3.2 and 3.3, since the exposure and outcome were both assessed in the same time point, it can be possible the misclassification to be differential. However, the data obtained by an FFQ are suitable for ranking people and the FFQ we used was validated and showed a relatively high correlation with the intake of fatty acids. Furthermore, dietary fat intake was assessed at baseline, and there may have been changes in fat consumption over time. Nevertheless, it has been shown that dietary habits change little over time in middle-aged adults[80]. Similarly, to examine the association between vitamin D and body fat, we assessed vitamin D in fasting samples only in one time point. The vitamin D levels fluctuate over time and therefore a single measurement of vitamin D in the blood may not be sufficient to assess its relationship with different health outcomes. However, it has been shown that the correlation of vitamin D blood levels between baseline, 12-months and 14 years were 0.8 and 0.52 respectively, providing some support for the use of a single measurement of vitamin D in predicting future health outcomes[81]. Furthermore, the current immunoassays used in assessing vitamin D levels are not able to accurately detect 25OHD2, which can lead to underestimation of 25OHD levels, and therefore may result in a biased estimate[82]. Developments of more accurate measures of vitamin D are needed.

Confounding

A confounding factor is an extraneous variable in a statistical model that is not an intermediate in the causal pathway between the exposure and the outcome but correlates with both the independent variable (fatty acids) and the dependent variable (total body fat)[83]. Biased estimates may be obtained if a confounding factor is not taken into account in analyses. However, in all our analysis, we adjusted for multiple potential confounders, which were selected based on the literature and/or when the potential confounder factor provided a change in effect estimate of more than 10%[84]. However, as in any observational study, residual confounding may be present. Some of the unmeasured confounders might include physical activity at the time when exposure was evaluated and the non-fasting blood samples collected in the baseline measurement, and therefore the results in Chapter 3 may be explained by residual confounding. We did not have ultrasound measures of fatty liver from the same time the GGT was assessed. Ultrasound is a more accurate measure for the diagnosis of fatty liver, and therefore we cannot exclude the presence of residual confounding of the presence of nonalcoholic fatty liver disease in the results presented in Chapter 3.4 and 3.5. Also, our results presented in Chapter 3.5 show a non-causal effect of GGT levels on T2D risk and that the observed association between GGT levels and the risk for prediabetes and T2D may be due to residual confounding, such as mediators related to oxidative stress and inflammation pathways. These unmeasured confounders may also explain the observed association between GGT levels and body fat distribution and will need to be further elucidated in future studies. However, the results presented in Chapter 3.5 should be interpreted with caution since several issues may have compromised our approach in assessing causality, e.g., use of a weak instrument, the pleiotropic effect of the genetic variants, and lack of sufficient power. Moreover, some confounding factors we considered for the analysis in Chapter 3 were self-reported (smoking status, socio-economic status and alcohol consumption), and therefore measurement error of the confounding variable might have occurred. Residual confounding can lead to either overestimation or underestimation of the observed effect estimate, depending on how the confounding factor is related to both outcome and exposure[85].

Heterogeneity and Publication Bias

A systematic review of studies addressing a research question will inevitably bring together studies with different population characteristics, design, interventions, and exposure and outcome definitions. Such diversity is defined as heterogeneity and is a key factor to consider, evaluate, and report in studies summarizing the evidence (e.g. systematic reviews and meta-analyses)[86]. Depending on the level of heterogeneity, effect estimates from individual studies may not be able to be combined. In some of the systematic reviews presented in this thesis, a meaningful quantitative pooling of the existing data was unfeasible due to heterogeneity in the input parameters (e.g., exposure and outcome assessment), assumptions, and the study design. Furthermore, in the studies in which we performed a meta-analysis, there was some evidence of heterogeneity between studies (I^2 estimates ranged from 0% to 96% in these meta-analyses). Since the number of available studies in each analysis was generally small, it precluded our ability to extensively explore the sources of the observed heterogeneity by subgroup analyses involving various study-level characteristics,

including age, ethnicity and socio-economic stats. In this respect, literature-based meta-analyses are influenced by the level of diversity across the studies and do not provide detailed information necessary for the reliable assessment of associations independent of potential confounding factors. In the systematic reviews presented in this thesis, we included data from potentially poorly conducted studies and therefore, variation in study quality has contributed to the heterogeneity of findings noted in several of the meta-analyses. Therefore the results should be interpreted with caution. Another threat to a meta-analysis is the issue of publication bias due to underreporting of negative findings. The results of a meta-analysis are threatened if the sample of studies retrieved for review is biased. In the meta-analyses presented in this thesis, although evaluations with the conventional funnel plots and Egger's test estimates indicate minimal impact of publication bias in most of our analyses, these approaches are limited by a qualitative nature reliant on visual inspection and the fact that the majority of these assessments were based on between five to ten studies. Therefore, despite all efforts made to undertake a comprehensive search of the published and unpublished literature, we cannot exclude the possibility of publication bias stemming from underreporting of negative findings.

Missing Data, Validity and The Generalizability of The Results

In the studies presented in Chapter 3 and Chapter 4.1, a general methodological concern is that there were missing data on exposure (e.g., dietary intake or GGT levels) and/or outcome data (e.g. body composition measurements). This may have happened in our study because participants were lost to follow-up, not invited to participate in the assessment, or they did not return or complete the food frequency questionnaire. Selection bias might be present and, therefore, the missing data in exposure and outcome could have influenced the validity of our results. However, we did not observe significant differences between the examined characteristics of the participants included in the analyses and those excluded (e.g., in the study in Chapter 3.4, there was no differences in GGT levels among participants included in our study and those excluded due to no data on DXA measurements). Therefore, it is unlikely that selection bias happened. A selection towards a healthier population might have occurred in the studies focused on the role of diet. Nevertheless, it has been shown that using a restricted source population for a cohort study may not compromise validity of exposure-outcome association[87]. Also, in the studies examining the role of diet and biomarkers in relation to body fat in Chapter 3, the assessment of the exposure (diet, vitamin D and GGT levels) and the outcome of body fat distribution occurred at different times, and changes could have occurred in the interim. We were not able to study the change of diet over time, because we assumed that diet is constant over time or that the rank order of dietary variables is the same across time if changes occur. It has been shown that the potential of a single FFQ measurement to rank subjects according to nutrient intake is relatively stable over time in middle-aged adults[80]. Also, use of a single baseline dietary assessment usually leads to attenuation of the effect estimates, compared to the use of repeated dietary measurements [88]. For some of biomarkers used in the studies of Chapter 3 we were able to assess the changes over time in a subgroup of participants, supporting internal consistency and validity (e.g., we measured serum GGT levels at the baseline visit of The Rotterdam Study (1990-1993) in a subgroup of study participants (n=2753 participants)

and at the fifth visit (2009-2011) (n=1395 participants). In the subgroup analysis, we observed high correlations with GGT measure at the third visit (1997-1999) (interclass correlation coefficients between the first and the third visit and the third and the fifth visit of the Rotterdam Study were 0.84 and 0.81 respectively)). Another concern is the generalizability of the results presented in Chapter 3 and 4.1. The studies included in this chapter are performed in Caucasian populations. Ethnic differences in exposures (vitamin D) or outcomes (body composition) examined have been previously reported[89,90]. Thus, our findings may not be extended to non-Caucasian groups. Also, in Chapter 4.1 we included only postmenopausal women in the analysis, and therefore our findings cannot be generalized to pre-menopausal women or men.

FUTURE RESEARCH

Diet and Cardiometabolic Health

Polyunsaturated fatty acids

Our study showed that higher levels of both classes of PUFA were associated with diminished chronic inflammation, which was mainly driven by n-6 PUFA. Our findings are in line with previous studies showing a non-detrimental effect of PUFA on CVD and provide additional support for the maintenance of the present level of n-6 PUFA intake cardiovascular outcomes[91,92]. However, in our study we used CRP as a marker of inflammation, which has not yet been established to have a causal effect on cardiometabolic diseases[93]. Therefore, clinical trials are needed to assess whether PUFA supplementation can reduce the cardiometabolic risk by reducing inflammation. In this respect, examination of other inflammatory markers including interleukin-6 and tumor necrosis alpha would further clarify the association between fatty acids intake and inflammation. Also, investigation of other inflammatory markers, such as interleukin-6 or tumor necrosis-alpha can help to give insight into the role of fatty acids in the inflammation process. Furthermore, our results suggested that dietary sources of n-3 PUFA may be important to take into account when evaluating their role on inflammation and other health outcomes. Further studies are needed in order to assess the role of dietary sources of n-3 PUFA in cardiometabolic health. Also, we found that the presence of chronic diseases may influence the association between dietary fat intake and body composition, which needs to be clarified in future studies. Also, randomized control studies should examine whether supplementation of different fatty acids may impact total body fat and its distribution.

Vitamin D

We observed that vitamin D sufficiency was associated with body composition in elderly, and was particularly closely associated with total body fat mass and lean mass. Our study used accurate measures of body composition, but it was limited from the the use of one single measurement of body composition. Furthermore, adjustment for baseline BMI in these analyses (data not shown), abolished the association between vitamin D, total body fat and lean mass, providing some evidence that these associations are not causal. However, this should be interpreted with coution since BMI and total body fat are highly correlated, and it ramins a challenge in analyzing data

that are highly correlated withing subject. Failure to incorporate properly these correlations on depdentent variables, can lead to incorrect estimation of regression model parameters, particularly when such correlations are large. The literature lacks studies assessing whether vitamin D levels can have a causal effect on body composition, and further studies are needed to address this aspect. Since obesity is a major public health problem, examining whether vitamin D can have a causal effect, would present a novel preventive measurement in combating obesity. This can also be investigated with longitudinal studies using repeteaed meassurements of body composition and proper statistical methots to account for the correlation of responses on the dependent variables (such as generelaized estimating equations) or well-organized clinical trials examining whether vitamin D supplementation can be of additional value in weight loss treatment. Also, it may be valuable to assess whether screening for vitamin D deficiency in obese individuals and providing vitamin D supplementation could be cost-effective intervention. Furthermore, experimental studies are needed to investigate the underlying mechanisms linking vitamin D levels with body composition. These future studies should also examine further the role of cardometabolic diseases in the association between vitamin D and body fat. Also, as many epidemiological studies, we used total circulating 25OHD which is the sum of 25OHD2 and 25OHD3. 25OH3 is synthesized in skin after ultraviolet B exposure and obtained from animal food sources, whereas 25OHD2 is synthesized from vitamin D2 obtained from plant sources. Previous studies have shown that these subtypes of vitamin D may have different effect on health, including the risk for CVD[31,94], and therefore, studies looking at the role of these metabolites individually in body composition are needed.

Biomarkers

In Chapter 3.4 we found that an increase in GGT concentrations within its physiological normal range is a sensitive and early biomarker of unfavorable body fat distribution. As measurement of serum GGT is reliable, easy and inexpensive, its assessment may have clinical utility in identifying individuals who are at high risk of metabolic disturbances later in life and who could benefit from effective preventive interventions. However, our study was of cross-sectional design and therefore the causality could not be addressed. Future studies using repeated measurements of both GGT and body fat measures are necessary to determine whether levels of GGT can affect body fat distribution. Furthermore, clinical trials examining whether medication or lifestyle factors that alter GGT metabolism can be used to prevent obesity and its complications may help to clarify the role of GGT in obesity. Also, future studies should examine whether GGT levels can be used as an indicator of visceral fat levels, which is associated with more adverse outcomes. It has been shown that visceral adipose tissue is associated with more adverse cardiometabolic risk factor profiles than is abdominal subcutaneous adipose tissue. [95] Furthermore, in Chapter 3.5 we found no evidence that genetically influenced elevation of GGT levels affect glycemic traits and increase risk of prediabetes and T2D. Therefore, we do not support the hypothesis that there is a causal relationship between GGT levels and these outcomes. However, large-scale studies (observational studies using a mendelian randomization approach or randomized trials) are needed to establish the causality and explore the role of metabolic pathways, including mediators of oxidative stress and inflammation in the association between GGT and T2D. Furthermore, it has been reported that

GGT is made up of four fractions namely big-, medium-, small- and free- GGT, each one having its own molecular weight and distinct physiochemical property[96]. There might be differences in types and levels of GGT fractions in serum and plasma samples[97]. Future studies investigating the role of individual fractions of GGT in obesity and T2D would be of interest.

Cardiovascular Health in Women

Estrogen receptor beta and estradiol

In many of the studies in this thesis, we evaluated cardiometabolic health in women. We showed that estrogen receptor has multiple functions in the female cardiovascular system that may contribute to protect the cardiovascular system. However, the majority of studies were conducted in animals, and therefore it remains unclear if these findings apply to humans. Future prospective population-based studies and randomized controlled trials in humans should examine whether targeting ERβ ligands can confer the cardioprotective effects of estradiol while avoiding its specific adverse effects on other tissues such as those of uterus and breast and in cardiometabolic health, including diabetes as shown in this thesis. The findings of an association between estradiol levels and the risk of T2D raise a concern on the safety of hormone replacement therapy (HRT) use in postmenopausal women. HRT has been shown to affect the risk of CVD as well[61,98]. Thus, studies further evaluating the role of HRT in cardiometabolic health are needed. Furthermore, the clinical usefulness of plasma levels of sex hormones in patient stratification and intervention based on the risk of developing T2D in women warrants further investigation.

Menopause

Menopause is a natural phase of a women's life cycle which is associated with changes in CVD risk factors, such body fat distribution, reduce glucose tolerance, increased blood pressure that contribute to increased risk of CVD[63]. In this thesis, early menopause, defined as a menopausal age of younger than 45 years, was shown to be associated with increased CVD risk, independent of traditional CVD risk factors. Furthermore, in our study, early menopause was associated with increased risk of all-cause mortality. The frequency of ovarian failure before age 40 is ≤ 1% but about 5-10% of women experience natural menopause by age of 45[67-69]. Therefore, the increased risk of CVD and all-cause mortality associated with early menopause, represents an important factor affecting risk of disease and mortality among older women. Larger prospective population-based studies focusing on the association between early menopause and intermediate cardiovascular traits, including obesity, hypertension, and dyslipidemia are required, since they may provide new insights into the underlying mechanisms of the observed excess CVD risk. Also, to date, there is little information on the changes that occur in cardiovascular risk factors during the perimenopause and different stages of the menopause transition and the impact of these changes on the risk of CVD. Identifying the changes that might occur in cardiovascular risk factors during the menopause transition (i.e. early vs. late menopause) might help to identify which women should be target for intervention while they cross through different stages of menopause. Also, future studies exploring the hormonal changes in perimenopause and early menopause and how they correlate with cardiovascular risk factors and the risk of cardiovascular disease, might help to shape future

preventive strategies in reducing the burden of CVD in postmenopausal years. Furthermore, vasomotor symptoms during the menopause transition, may present a new factor affecting CVD risk in women. However, the current evidence is too limited to draw any conclusions regarding the potential usefulness of menopausal symptoms in assessing CVD risk among women. Thus far, findings have largely been derived from post hoc analyses of studies using brief self-report measures of vasomotor symptoms, which are subject to faulty memory and reporting bias. Therefore, prospective studies looking at physiologically-assessed vasomotor symptoms and the risk of CVD are needed in order to reliably establish whether menopausal symptoms are independently associated with CVD outcomes and in order to further clarify the potential mechanisms behind this association. In this respect, future studies should adjust for sex hormones levels to investigate whether the observed association is due to changes in hormone levels associated with menopause. Our findings showed that a number of plant-based and natural therapies (i.e. phytoestrogens, yoga) may be used as a complementary therapy in managing menopausal symptoms. Nevertheless, the majority of the available studies focus appropriately on hot flashes, and few studies additionally evaluated other menopausal symptoms (i.e. night sweats). Therefore, future randomized control trials should look at the impact of plant-based and natural therapies on a wide range of menopausal symptoms. Also, there is lack of data on adverse effects that may arise from long term use of plant-based and natural therapies, and therefore, future studies providing information on any detrimental health effects, typically available in long-term intervention studies, is essential. Moreover, in our systematic review, we found low degree of quality of randomized control trials evaluating the impact of plant-based and natural therapies on menopausal symptoms. Further randomized clinical studies utilizing an adequately designed strict protocol with sufficient sample size, adequate blinding of outcome assessment, participants and personnel, proper allocation concealment, and sufficient follow up period are needed.

CLINICAL AND PUBLIC HEALTH IMPLICATIONS

Our results on the financial burden of NCDs on healthcare expenditure and national income may be important for policy makers to help shape future healthcare plans and strategies to reduce the impact of NCDs. These strategies may include various activities, ranging from disease surveillance, drug research and development, health staff training, preparedness and response planning and execution, to public education, behavior change and disease prevention campaigns. Furthermore, our results highlight the need for future studies, assessing the economic burden of NCDs in low and middle-income country settings. We identified several nutritional factors and biomarkers to be associated with the risk of adverse cardiometabolic health. Our findings can have important clinical implications since they may contribute to identify high-risk individuals for the development of preventive strategies. For example, since measurement of serum GGT is reliable, easy, and inexpensive, its assessment may have clinical utility in identifying individuals at high risk of metabolic disturbances later in life who could benefit from effective preventive interventions. Our findings provide further support for the maintenance of the present level of n-6 PUFAs intake. At the same time, our results suggest that substitution of plant-derived n-3 PUFAs with marine

derived n-3 PUFAs may be considered when shaping future guidelines and recommendations for CVD and T2D prevention. The results presented on estrogen receptor beta may present new frontiers in the development of novel therapies for cardiometabolic diseases. For instance, specific ERβ agonists might be a therapeutic option in occlusive artery disease, whereas ERβ antagonist may be useful in vascular disease characterized by arterial wall distention and aneurysm. The combination of ERβ ligands with specific targeting or drug delivery techniques such as drug-eluting stents and perivascular gel might be effective in modulating the activity of ERβ in a specific blood vessel without altering other vessels in the systemic circulation. Also, our results presented within the framework of women's health indicate that menopause is a critical period to evaluate women's risk for CVD and that it may be an appropriate time to introduce interventions to reduce the risk of adverse cardiometabolic health. For instance, screening for medical conditions such as hypertension, dyslipidemia, insulin resistance and other cardiometabolic risk factors may be considered for women going into menopause at or before age 45, since it may help in identifying women at high risk for developing cardiovascular disease, who could profit from lifestyle or pharmacological interventions. Furthermore, our findings on the impact of plant-based and natural therapies on menopausal symptoms, stimulate the use of phytoestrogens, behavioral therapies and acupuncture in managing menopausal symptoms while avoiding the adverse cardiovascular health effects of HT. Nevertheless, the available evidence remains scarce and of poor quality, well designed studies with sufficient sample size and follow up are key for the establishment of effective strategies in the prevention of cardiovascular disease and diabetes.

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Chapter 6

Short Summary

Short Summary

In **chapter 1**, we describe the background of the research presented in this thesis. NCDs, particularly cardiometabolic diseases, are the world's main killer and pose a heavy financial burden on governments and society. Obesity is among the major risk factors of cardiometabolic diseases including hyperglycemia, hypertension and dyslipidemia. Nutrition is suggested to play a crucial role in maintaining cardiometabolic health. Biomarkers on the other hand, provide a dynamic and powerful approach for homogenous classification of a disease and risk factors that can extend our knowledge about the underlying pathogenesis of cardiometabolic disease and may help to identify subgroups in the population with greater susceptibility to these diseases. Furthermore, women face a higher CVD risk after reaching menopause, which has been attributed, in part, to the physiological and hormonal changes that occur during the menopause transition. Therefore, we aimed to study the role of nutrition, biomarkers, menopause and its related changes on cardiometabolic health.

In **chapter 2**, we performed systematic reviews of the literature on the economic impact of NCDs. In **chapter 2.1** we present a systematic review of the literature on the global economic impact of NCDs on health care spending and national income. The results showed that NCDs pose a significant financial burden on healthcare budgets and nations' welfare, which is likely to increase over time. Our study also shows that among the NCDs, CVD and cancer present the highest costs. In **chapter 2.2** we present a systematic review of the literature on macro-economic productivity. Overall NCDs generate a large impact on macro-economic productivity in most World Health Organization regions irrespective of continent and income. However, the absolute global impact in terms of dollars and disability adjusted life years remains an elusive challenge due to the wide heterogeneity in the included studies as well as limited information from low and middle-income countries.

In **chapter 3**, we examined the associations of dietary fatty acids intake, serum vitamin D and GGT levels with cardiometabolic risk factors, including inflammation, body composition and glycemic traits. In **chapter 3.1**, we describe the association of total and individual PUFA intake with C-reactive protein levels. We observed that total PUFA intake and n-6 PUFA intake, but not n-3 PUFA intake, were associated with diminished chronic inflammation. Stratification by sex revealed that these associations were more prominent in women. Possible sex differences in the association of PUFA intake and cardiometabolic health should be further explored. In **chapter 3.2**, we assessed whether dietary fat composition was associated with total body fat and regional body fat composition in two Caucasian populations. Our results showed no consistent association between dietary fat composition and total body fat or regional body fat distribution. However, we

found evidence for potential effect modification by chronic disease on the association between dietary fat intake and body fat distribution. Future studies should clarify to what extent these findings may be influenced by the presence of chronic disease. In **chapter 3.3**, we aimed to assess the association between serum vitamin D levels, body fat, lean mass and regional body fat distribution. We observed that lower vitamin D concentrations were associated with a lower lean mass and a higher fat mass. Furthermore, we found an interaction between serum vitamin D levels, total body fat and cardio-metabolic diseases. The stratification analysis showed that vitamin D was inversely associated with fat mass only in subjects free of cardiometabolic diseases. Our results did not support a role for vitamin D status in the regional distribution of body fat. In **chapter 3.4**, we examined whether GGT levels within normal range are prospectively associated with total body fat and regional body fat distribution. We found no association between GGT concentrations and total body fat mass. However, increased GGT was associated with higher android fat, lower gynoid fat and higher android fat/gynoid fat ratio. Our results suggest that an increase in GGT concentrations within its physiological normal range is a sensitive and early biomarker of unfavorable body fat distribution. In **chapter 3.5**, we investigated whether GGT related genetic variants, combined in a genetic risk score, and GGT1 genetic variant, rs2017869, were associated with GGT levels and, in turn, with an increased risk prediabetes and T2D or influencing glycemic traits in the Rotterdam Study. In this study we found that GGT levels predict risk of prediabetes and T2D. However, we did not find any significant association between formerly studied GGT1 genetic variant or GGT genetic risk score with glycemic traits, prediabetes and T2D. Our results provide no evidence that genetically elevated GGT levels affect the risk prediabetes and of T2D, and thus do not support a causal relationship role for GGT levels.

In **chapter 4**, we present original data analysis, systematic reviews and meta-analysis of the literature on the association of unique cardiometabolic risk factors in women. In **chapter 4.1** we present an original data analysis and comprehensive review on the association of endogenous sex hormones levels with the risk of T2D in women. We showed that lower levels of sex hormone-binding globulin and higher levels of total estradiol were associated with the risk of T2D. These associations were independent of established risk factors, including body mass index, glucose and insulin. In our study, however, we did not find any association between testosterone and the risk of T2D. Further studies are needed to establish hormones thresholds at which diabetes risk is increased, because this may aid in identifying high-risk postmenopausal women in the clinical setting. In **chapter 4.2**, we present a comprehensive review of the literature on the role of estrogen receptor beta in the female cardiovascular system. By summarizing findings from 88 studies, we found that estrogen receptor beta possess abundant functions that might contribute to protecting the cardiovascular system in women. Our findings suggest that estrogen receptor beta signaling may cause vasodilation, and therefore prevents the pathological effect of excessive vasoconstriction by regulating the bio-availability of nitric oxide, altering membrane ionic permeability in vascular smooth muscle cells and by regulating adrenergic control of the arteries. Also, estrogen receptor beta may have angiogenic properties and may protect against vascular injury by inhibiting vascular smooth muscle cell migration and proliferation. Additionally, the results of the current study indicate a possible protective effect of estrogen receptor beta signaling

in left ventricular hypertrophy and ischemia/reperfusion injury via genomic and non-genomic pathways. Furthermore, our systematic review suggests that the vascular effects of estrogen receptor beta may be vessel specific and may differ by age and menopause status. In **chapter 4.3**, we undertook a systematic review and meta-analysis of studies assessing the association of vasomotor symptoms with various cardiovascular risk factors such as systolic and diastolic blood pressure, hypertension, blood lipids, body mass index and measures of subclinical atherosclerosis. By quantifying data of nearly 20,000 individuals, we found that women with vasomotor symptoms including flushing and/or night sweats have significantly higher systolic and diastolic blood pressure, higher circulating total cholesterol and low-density lipoprotein levels, higher body mass index and higher odds of having hypertension than their counterparts with no symptoms. However, our study found no consistent associations between vasomotor symptoms and measures of subclinical atherosclerosis. In **chapter 4.4**, we present a systematic review and meta-analysis of the literature on the association of either vasomotor symptoms or other menopausal symptoms with the risk of coronary heart disease, stroke and CVD. By quantifying data of nearly 214,000 individuals, we found that women with vasomotor symptoms or other menopausal symptoms had up to 48% increased risk of coronary heart disease, stroke and CVD. However, these associations were mainly explained by the conventional cardiovascular risk factors. Further prospective large-scale studies are needed to extend our understating on the possible role of menopausal symptoms in cardiovascular disease. In **chapter 4.5**, we performed a systematic review and meta-analysis of all available observational evidence to quantify the associations of age at menopause and duration since onset of menopause with (i) primarily clinical CVD outcomes and intermediate vascular traits; and (ii) all-cause mortality. We found that women who experienced an early menopause (i.e. younger than 45 years) have an excess risk of coronary heart disease, CVD-mortality and all-cause mortality. Furthermore, being 45-49 years at menopause compared to ≥50 years was associated with increased risk of carotid atherosclerosis. Duration since menopause in relation to risk of developing intermediate cardiovascular traits or CVD outcomes was reported in 4 observational studies, reporting no consistent results. Our review also highlights important gaps in the existing literature, calling for further research to reliably establish whether cardiovascular risk may vary in relation to the duration since menopause and the mechanisms leading early menopause to cardiovascular outcomes and mortality. In **chapter 4.6**, we undertook a systematic review and meta-analysis of randomized controlled trials and prospective studies assessing the impact of plant-based and natural therapies on menopausal symptoms. By summarizing the evidence from 101 randomized controlled trials and 12 prospective non-randomized intervention or observational studies, including 12,443 individual women, we found that composite phytoestrogens and individual phytoestrogen interventions such as dietary and supplemental soy isoflavones were beneficial for menopausal outcomes in general, and number of night sweats in 24 hours in particular. Additionally, we found that behavioral therapies, acupuncture, and several herbal remedies in aggregate improved overall menopausal symptoms.

In **chapter 5**, we provide a general discussion in which the studies described in this thesis are described in broader context. Furthermore, this chapter describes major methodological considerations, as well as implications for clinical practice and suggestions for future research.

Korte samenvatting

In **hoofdstuk 1** wordt de achtergrond van de onderzoeken die zijn uitgevoerd in dit proefschrift beschreven. Niet overdraagbare chronische ziekten (in het Engels "Non Communicable Diseases"; NCDs), waaronder cardiometabole ziekten, zijn wereldwijd de belangrijkste oorzaak van sterfte en dragen bij aan verhoogde ziektekosten van de maatschappij. Overgewicht is een belangrijke risicofactor voor het krijgen van cardiometabole ziekten zoals een verhoogde bloedsuikerspiegel, of verhoogde bloeddruk. Voeding kan een belangrijke rol spelen bij het behouden van een goede gezondheid van het hart en bloedvaten. Ook het gebruik van specifieke biomarkers is een aanpak die zou kunnen bijdragen aan het identificeren van risicogroepen voor het ontstaan van cardiometabole ziekten. Vrouwen hebben een hoger risico om hart- en vaatziekten te krijgen na de overgang. Dit verhoogde risico wordt deels veroorzaakt door fysiologische en hormonale veranderingen die opreden tijdens de overgang. Samengenomen, wasdaarom het doel van dit proefschrift om te bestuderen in hoeverre voeding, biomarkers en de overgang geassocieerd zijn met cardiometabole ziektes.

In **hoofdstuk 2** is de beschikbare literatuur over de economische impact van NCDs op een systematische manier samengevat. In **hoofdstuk 2.1.** hebben we de literatuur over de wereldwijde impact van NDS op zorgkosten en het nationaal inkomen bestudeert. De resultaten laten zien dat NCDs een belangrijke bijdrage leveren aan de nationale zorgkosten en nationaal inkomen en dat deze bijdrage waarschijnlijk zal groeien over de tijd. Dit geldt met name voor hart- en vaatziekten en kanker. In **hoofdstuk 2.2** is de literatuur over de impact van NCDs op macro-economische productiviteit samengevat. NCDs genereren een grote impact op macro-economische productiviteit in de meeste regio's van de Wereld Gezondheidsraad ("World Healh Organisation (WHO)") ongeacht het land of het inkomen. Echter, het vaststellen van de absolute wereldwijde impact uitgedrukt in valuta per beperking-gecorrigeerde levensjaren ("Disability adjusted life years (DALYs)") blijft een uitdaging door de grote heterogeniteit van de beschikbare studies en door het gebrek aan studies uit landen met lagere inkomens.

In **hoofdstuk 3** hebben we de relatie tussen vetzuren uit de voeding, vitamine D, gamma GT waardes en cardiometabole risicofactoren bestudeerd. In **hoofdstuk 3.1.** beschrijven we de relatie tussen totale en individuele meervoudig onverzadigde vetzuren (Polyunsaturated fatty acid (PUFAs)) met C-reactive protein levels. We hebben gevonden dat de totale inname van PUFAs en de inname van n-6 PUFAs maar niet van n-3 PUFA's gerelateerd was aan verminderde chronische ontstekingen. Stratificatie op basis van geslacht liet zien dat deze relatie sterker was in vrouwen dan in mannen. Deze mogelijke verschillen tussen mannen en vrouwen in de relatie tussen PUFAs uit de voeding en cardiometabole gezondheid dienen in de toekomst verder te worden onderzocht. In **hoofdstuk 3.2**. hebben we bestudeerd of vetten uit de voeding gerelateerd waren aan totaal lichaamsvet en de

verdeling van lichaamsvet in twee Kaukasische populaties. Onze resultaten lieten een consistente relatie zien tussen vetinname uit de voeding en vetmassa. Echter, onze resultaten lieten zien dat deze effecten mogelijk anders zijn voor mensen met chronische ziekten. Deze mogelijke interactie met chronische ziekten dient verder te worden bestudeerd in toekomstige studies. In **hoofdstuk 3.3**. hebben we de relaties tussen vitamine D in het bloed en verschillende componenten van lichaamssamentelling bestudeerd (vetmassa, ververdeling en vetvrije massa) . We hebben gevonden dat lage vitamine D concentraties gerelateerd waren aan een lagere vetvrije massa en een hogere vetmassa. Daarnaast hebben we gevonden dat de relatie tussen lage vitamine D spiegel en hoge vetmassa alleen aanwezig was in gezonde mensen zonder cardiometabole ziekten terwijl er geen relatie met vetverdeling werd gevonden. In **hoofdstuk 3.4.** hebben we bestudeerd of GGT spiegels binnen de normale range gerelateerd zijn aan vetmassa en de verdeling van lichaamsvet. We vonden geen relatie tussen GGT en vetmassa. Echter, hogere GGT spiegels waren wel gerelateerd aan hogere hoeveelheid buikvet, lagere hoeveelheid heupvet en een hogere middel-heup ratio. Daarmee suggereren onze resultaten dat verhoogde GGT spiegels binnen de normale range mogelijk een biomarker van ongunstige vetverdeling zou kunnen zijn. In **hoofdstuk 3.5** hebben we bestudeerd of een gecombineerde genetische risicoscore en een specifieke genetische variant (rs2017869) gerelateerd waren aan GGT spiegels en (pre-)diabetes in de Rotterdam Studie. We hebben gevonden dat GGT spiegels het risico op (pre-)diabetes voorspelde. We vonden echter dat de genetische score en de losse genetische variant gelinked aan GG niet significant gerelateerd was aan (pre-)diabetes. Onze resultaten vormen geen bewijs voor hypothese dat genetisch verhoogde GGT spiegels het risico op (pre-)diabetes verhogen en onderbouwen daarom geen causale relatie tussen GGT en (pre)diabetes.

In **hoofdstuk 4** beschrijven we originele data analyses, een systematische review en een meta-analyse van de literatuur over cardiometabole risicofactoren in vrouwen. In **hoofdstuk 4.1** beschrijven we de resultaten van een originele data analyse en een samenvatting van de relatie tussen endogene geslachtshormonen en het risico op diabetes type 2. We lieten zien dat lage spiegels van het sexhormoonbindend globuline en hogere estradiol spiegels gerelateerd waren aan het risico op diabetes. De relatie van onafhankelijk van bestaande risicofactoren zoals body mass index (BMI), glucose en insuline concentraties. In onze studie vonden we geen relatie tussen testosteron spigels en het risico op diabetes. Vervolgstudies zijn nodig om de minimum waardes van deze hormoonspiegels te bepalen waarbij het risico op diabetes begint te stijgen. Het vaststellen van een dergelijk minimum kan zinvol zijn om diabetes risicogroepen van postmenopauzale vrouwen te identificeren in de klinische setting. In **hoofdstuk 4.2**. hebben we de literatuur over de rol van de estrogen receptor beta (ER-β) in het vrouwelijk hart- en vaatsysteem samengevat. Op basis van 88 studies hebben we geconcludeerd dat de receptor een breed scala aan functies vervult die zouden kunnen bijdragen aan de bescherming van het vrouwelijk hart- en vaatsysteem. Onze bevindingen suggereren dat de ER-β vaatverwijding veroorzaakt en daardoor de pathologische effecten van vaatvernauwing mogelijk helpt te voorkomen. Vaatverwijding wordt veroorzaakt doordat de ER-β de biobeschikbaarheid van productie van stikstofoxide reguleert, de idoorlaatbaarheid van ioenn en membranen van gladde spiercellen van de vaten verandert en de adrenerge controle van de arteriën reguleert. Daarnaast, heeft ER-β angiogene eigenschappen en kan mogen beschermen tegen vaatschade door het tegengaan van migratie en proliferatie van gladde spiercellen in de bloedvaten.

Daarnaast laten de resultaten van de huidige studie zien dat ER-β signalering mogelijk beschermt tegen hypertrofie van de linker ventrikels en vaatschade vermindert. Ook suggereren de resultaten van onze systematische review dat de effecten van de ER-β vaat-specifiek zijn en mogelijk anders zijn voor vrouwen van verschillende leeftijden en menopausale status. In **hoofdstuk 4.3** beschrijven we een systematische review en meta-analyse van studies over de relatie tussen vasomotorische symptomen (zoals opvliegers) en verschillende risicofactoren voor hart- en vaatziekten. Daarbij valt te denken aan bloeddruk, cholesterol, overgewicht en maten van subklinische aderverkalking. Op basis van data van bijna 20.000 individuen hebben we geconcludeerd dat vrouwen met vasomotor symptomen zoals opvliegers en nachtzweten een signficant hogere bloeddruk, totaal en LDL-cholesterol waarden en BMI hadden dan vrouwen zonder deze symptomen. Onze studie vond echter geen consistente relatie tussen vasomotorische symptomen en subklinische aderverkalking.

In **hoofdstuk 4.4** worden de resultaten van een systematische review en meta-analyse van de literatuur over de relatie tussen vasomotorische- of andere overgangssymptomen en het risico op cardiovasculaire ziekten beschreven. Gebaseerd op gegevens van bijna 214,000 personen hebben we gevonden dat vrouwen met vasomotorische- of andere overgangssymptomen een 48% hoger risico hadden op cardiovasculaire ziekten dan vrouwen zonder deze symptomen. Deze relatie werd echter voornamelijk verklaard door verschillen in bestaande risicofactoren voor hart- en vaatziekten. Grote prospectieve studies zullen in de toekomst nodig zijn om onze huidige kennis over de mogelijke rol van overgangssymptomen in het ontstaan van hart- en vaatziekten uit te breiden. In **hoofdstuk 4.5** hebben we een systematische review en meta-analyse uitgevoerd op basis van alle beschikbare observationele studies over de leeftijd waarop de menopauze is begonnen en de duur van de menopauze in relatie tot (i) primaire klinische cardiovasculaire uitkomsten en intermediaire vaatmetingen en (ii) sterfte door alle oorzaken. We hebben gevonden dat vrouwen die op jonge leeftijd (<45 jaar) in de overgang raken een verhoogd risico hebben op coronaire hartziekte, sterfte door hart- en vaatziekten en totale sterfte (door alle oorzaken). Daarnaast was het risico op aderverkalking hoger voor vrouwen die tussen de 45 en 49 in de overgang raakten groter dan voor vrouwen die ouder dan 50 waren. Duur van de overgang in relatie tot hart- en vaat uitkomsten werd onderzocht in 4 studies, maar deze lieten geen consistente resultaten zien. Onze review laat ook de gaten in de huidige kennis zien. Toekomstige studies zullen moeten uitwijzen of de duur van de overgang gerelateerd is aan hart- en vaat uitkomsten en welke mechanismes de relatie tussen overgang op vroege leeftijd en hart- en vaatziekten en sterfte zouden kunnen verklaren. In **hoofdstuk 4.6** hebben we een systematische review en meta-analyse uitgevoerd op basis van gerandomiseerde interventie studies (RCTs) en prospectieve studies over plantaardige en natuurlijke behandelingen voor het verminderen van overgangsklachten. We hebben 101 RCTs en prospectieve (niet- gerandomiseerde of observationele) studies geïncludeerd waarin 12,443 vrouwen zijn bekeken. We vonden dat phytoestrogeen interventies bestaande uit soja isoflavonen uit de voeding of uit supplementen mogelijk gunstig werken tegen algemene overgangsklachten , en in eht bijzonder tegen de mate van nachtzweten. Ook vonden we dat gedragstherapie, acupunctuur en verschillende kruidentherapieën mogelijk overgangsklachten kunnen verminderen.

In **hoofdstuk 5** hebben we in een algemene discussie de studies beschreven in dit proefschrift in een bredere context geplaatst. Ook komen belangrijke methodologische overwegingen, klinische implicaties en aanbevelingen voor toekomstig onderzoek in dit hoofdstuk aan bod.

Chapter 7

Appendices

PhD PORTFOLIO

Name of PhD Student	Tualant Muka
Erasmus MC Department	Epidemiology
PhD Period	October 2013-May 2016
Promotor	Prof. dr. Oscar H. Franco Prof. dr. Albert Hofman
Co-promotor	Dr. Jessica C. Kiefte-de Jong

Training

Courses and Workshops	Year	ECTS
Topics in Meta-analysis	2014	0.7
Genome Wide Association Analysis	2014	1.4
Principle of Genetic Epidemiology	2014	0.7
Causal Inference	2014	0.7
History of Epidemiological Ideas	2014	0.7
Advances in Epidemiological Analaysis	2014	0.4
Bayseian Statistics	2014	1.4
Women's Health	2014	0.9
Principles of Epidemiological Data-analysis	2014	0.7
Mendelian Randomization	2014	0.9
Courses for the Quantitative Researcher	2014	0.9
Attended Conferences		
Charge investigator meeting, Washington DC, USA	2014	0.5
European Society of Cardiology Congress, London, UK	2015	1
Attended Seminars		
Seminars of the department of epidemiology	2013-2016	2
2020 meetings	2013-2016	2
Cardiovascular group meetings	2013-2016	2
ErasmusAge group meetings	2013-2016	2
Teaching		
Biostatistical Methods I: basic principles (teaching assistant)	2015	2

MSc thesis of Kris Vargas (Supervisor)	2014-2015	2
MSc thesis of Najada Stringa (Supervisor)	2015-2016	2
MSc thesis of Eralda Asllanaj (Supervisor)	2015-2016	2
Supervision of visting researchers (Ejona Nela, Tedi Minarolli, Tammy Pulido. Eylul Taneri, Asija Zaciragic, Bledar Kraja)	2015-2016	6
Other		
Per review of articles for scientific journals	2014-2016	1
Research visit in Harvard T. H. Chan School of Public Health	September 2015-December 2015	3
Research visit in MRC Epidemiology Unit, Cambridge University	January 2016-March 2016	3

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**denotes equal contribution*

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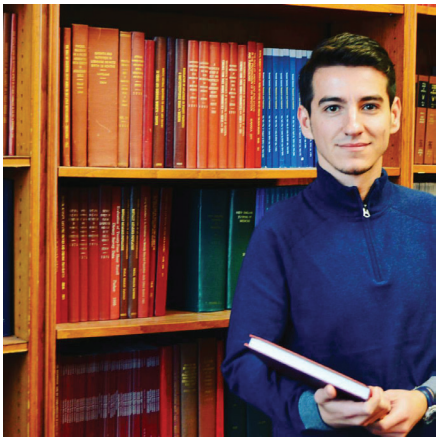

Taulant Muka was born in Çorovodë-Skrapar, Albania, on June 29th, 1988. In 2012, he completed the high school at "Qemal Stafa" in Tirana, Albania. In 2012, Taulant Muka obtained his MD degree at the University of Tirana in Albania. During medical school, in 2011, he won the JoinEU-SEE scholarship and went to the University of Bologna in Italy for 6 months. In 2012, Taulant came to Erasmus Medical Center on an ERAWEB scholarship, and in 2013 he obtained a Master of Science degree in Public Health. His master thesis supervised by Prof. Albert Hofman and Prof. Roy Thurik, was on whether entreprenuers live longer than non-entreprenuers. In October 2013, Taulant continued his studies with a Doctor of Science degree in Clinical Epidemiology and a PhD in ErasmusAGE and Cardiovascular Epidemiology group, Department of Epidemiology, Rotterdam, The Netherlands. Within ErasmusAGE and Cardiovascular Epidemiology group, he worked on cardiometabolic health, cancer and bone health, looking at nutrition, lifestyle and clinical determinants of health in these areas. During this period, he worked closely under the supervision of Dr. Jessica C. Kiefte-de Jong, Dr. Fernando Rivadeneira and Prof. Oscar H. Franco. Later, he was involved in the Women's health project, focusing on the role of menopause and sex-hormones in cardiometabolic risk. Before defending his PhD, Taulant spent seven months as a visting researcher in Harvard T. H. Chan School of Public Health and in the MRC Epidemiology Unit, University of Cambridge School of Clinical Medicine. He will keep working in the Department of Epidemiology as a postodoctoral researcher, where we will work with Prof. Oscar H. Franco on menopause and its impact on women's health.

Final Words

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