Sex Disparities in Cardiometabolic Health

Eralda Asllanaj

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Sex disparities in cardiometabolic health

Sekseverschillen in cardiometabolische gezondheid

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Asllanaj E*, Muka T*, Avazverdi N, Jaspers L, Stringa N, Milic J, Ligthart S, Ikram MA, Laven JSE, Kavousi M, Dehghan A, Franco OH. Age at natural menopause and risk of type 2 diabetes: a prospective cohort study. Diabetologia. 2017 Oct;60(10):1951-1960. doi: 10.1007/s00125-017-4346-8

Asllanaj E, Bano A, Glisic M, Jaspers L, Ikram MA, Laven JSE, Vőlzke H, Muka T, Franco OH. Age at natural menopause and life expectancy with and without diabetes. Menopause. 2018 Oct 8. doi: 10.1097/GME.0000000000001246

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

Asllanaj E*, Rojas L.R*, Rueda-Ochoa O.L*, FernandezE.P, Ochoa-Rosales C, Day F, Trajanoska K, Nano J, Ikram M.A, Burgess S, Franco O.H, Glisic M*, Muka T*. Mendelian randomization provides evidence for a causal role of dehydroepiandrosterone sulfate in decreasing NT-proBNP levels in a Caucasian population. *Revision at Circulation Research*

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E Asllanaj*, C Ochoa-Rosales*, M Glisic, J Nano, T Muka, OH. Franco. Chromatin landscape and epigenetic biomarkers for clinical diagnosis and prognosis of type 2 diabetes mellitus. Prognostic EpigeneticsVolume 15 in Translational Epigenetics 2019, Pages 289-324. doi: 10.1016/B978-0-12-814259-2.00012-1

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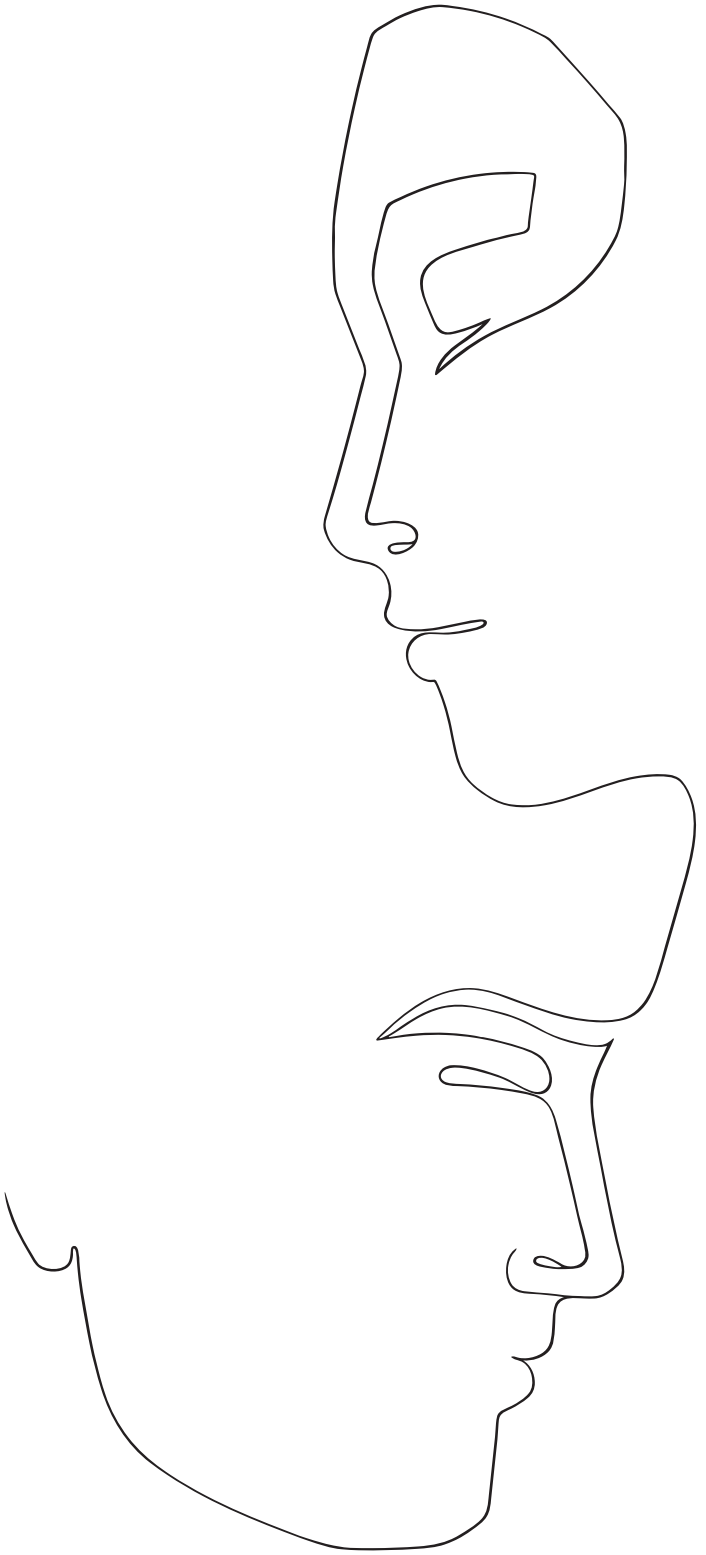


1

General Introduction

Epidemiology of cardiometabolic diseases: the importance of sex

Cardiometabolic health encompasses cardiovascular and metabolic diseases, including type 2 diabetes (T2D), cardiovascular disease (CVD) and their associated risk factors such as obesity, hypertension and unhealthy lifestyle [1]. Over 17.7 million CVD-related deaths were reported in 2015, amounting for 31% of all the deaths that year and making CVDs still the leading cause of mortality in both men and women globally [2]. Also for T2D, as a major risk factor for CVD, the estimated number of deaths caused directly by diabetes were 1.6 million in 2016 and these numbers are expected to increase since both the prevalence and incidence of T2D continuous to rise[3]. In addition to the burden on patients and their families, cardiometabolic diseases also affect the healthcare system worldwide. As a result, research and clinical practice are moving towards precision medicine for which sex differences are a critical component in prevention, diagnosis and treatment of cardiometabolic diseases. Differences due to sex in cardiometabolic outcomes have been described in several stages of diseases such as in prevalence, severity, prognosis and also in risk factors. Women and men seem to respond differently to cardiometabolic conditions such as T2D, dyslipidaemia, and smoking may play a bigger role in the origination and development of CVD in women compared to men[4-8]. Similarly, the risk of development of CVD due to obesity is greater among women than men also because the prevalence of obesity is higher in women than in men[9 10]. Moreover, studies on physical activity report increasing levels of exercise to be associated with a better cardiovascular profile in men than in women[11]. In addition to lifestyle factors, sex disparities exist also in manifestation and severity of cardiometabolic diseases with middle-aged men having higher mortality rates and higher CVD prevalence than same age women[12]. However, this female advantage in CVD rates compared with same aged men, gradually disappears with increased age and the menopause transition and particularly changes in sex hormones during this transition seem to play a crucial role[13 14]. Indeed, the postmenopausal state in women is associated with a worsening of the cardiometabolic risk profile including adverse changes in body composition, blood pressure and blood lipids, suggesting that sex hormones, and the relative balance between oestrogens and androgens, play some role in modulating sex-based differences in cardiometabolic diseases[15 16]. Although the obvious changes during the menopause transition, evidence from interventional studies comparing cardiometabolic risk profiles and the underlying biological factors in pre-vs. postmenopausal women is very limited.

Reproductive factors and cardiometabolic diseases

The menopause transition is a major life event in women's life which results in the loss of ovarian follicle development that leads to a permanent cessation of the menstrual period[17]. Although menopause is a universal phenomenon in women, timing of the final menstrual period differs greatly between women[17 18] and it is considered a marker of

ageing and cardiovascular health [18]. Women with early onset of menopause (<45 years) have an increased CVD risk and overall mortality, whereas late onset of menopause is linked to a reduced risk of CVD and mortality[19]. While the relationship between age at menopause and CVD risk is well established, its association with T2D, one of the major CVD risk factors, remains unclear. Some evidence from women who have undergone oophorectomy report less favourable glucose and insulin levels in these women[20 21], which is suggestive of a link between early menopause and diabetes risk. However, the few epidemiological studies that have investigated the association of menopause and diabetes have been scarce in numbers, of low quality, with small numbers of participants and have even reported conflicting results. Some studies have suggested no association between age at menopause and T2D onset[22 23]. In contrast, some others found early age at menopause to be associated with an increased risk of T2D[24 25], whereas other studies even found that late age at menopause was a risk factor for T2D[26 27]. Besides the conflicting results, seems that all studies agree that sex hormones and the subsequent decline in endogenous oestrogens during the menopause transition are the main determinants in cardiometabolic disease risk [21 25 28 29]. Findings from observational studies have shown that higher androgen and lower oestrogen levels are associated with CVD risk factors in post-menopausal women, including blood pressure, C-reactive protein, glucose tolerance and insulin resistance[30-34]. In addition, emerging evidence indicates an association between low dehydroepiandrosterone (DHEA) and heart failure and CVD[35-37] by influencing DHEA and its sulphate conjugate (DHEAs) are the most abundant sex hormones with serum concentrations up to 20-fold higher than the other sex steroids[38] and it is suggested that they could play a role in cardiometabolic health by modulating the natriuretic peptides levels in blood[37]. Nevertheless, these changes in hormonal balance explain partly the differences in several cardiometabolic risk factors[39], and the role of other factors such as lifestyle factors may be of great importance in understanding these disparities in cardiometabolic diseases.

Lifestyle, cardiometabolic risk and longevity

Another factor that may explain sex differences in cardiometabolic diseases are differences in lifestyle risk factors. For years it was thought that the excess male mortality was explained by unhealthy behaviours that were more socially acceptable for men than women. These behaviours include cigarette smoking, heavy alcohol use, eating more red meat and fewer fruits and vegetables, obesity, and exposure to physical hazards[40]. The differences in behaviour were thought to play a more important role explaining the higher male mortality than inherent sex differences in physiology[41]. The search to discover and establish risk factors for CVD has started in the early 1940s in the community-based Framingham Heart Study and since then other global efforts are daily made[42]. These efforts have established that modifiable risk factors such as smoking, hy-

pertension, diabetes, abdominal obesity, poor diet, psychosocial factors, lack of physical activity and alcohol use, play an important role in the development and progression of CVD in both men and women[42 43].

Smoking is the leading avoidable cause of death in the world by killing around 6 million people a year and is a key cardiac risk factor for development of CVD in both women and men[44]. Although the difference has been getting smaller in the past decades , the prevalence of smoking remains much higher in men than in women. Based on statistics from 2014, prevalence of smoking in men versus women was respectively 21.4% vs. 14.8% in Canada and 16.7% vs. 13.6% in the U.S. Besides the well-documented risk for cancer and cardiovascular disease from smoking, a recent meta-analysis reported a 37% increased risk of developing T2D for current smokers compared to never smokers[45]. The beneficial effects of smoking cessation in reducing the risk of disease and in preventing T2D is supported by a large amount of evidence[46 47]. However, smoking cessation is often accompanied by weight gain, with various studies reporting average increases of 4–8 kg, but with 10% to 13% of quitters gaining at least 11 kg [48 49]. Consequently, post smoking cessation weight gain is reported among smokers who have tried to quit as the main cause for their relapse, and among women, as the main reason for not trying to quit[50 51]. As obesity is a main risk factor for T2D, this increase in adiposity could blur the benefits of smoking cessation and paradoxically increase the risk of having T2D[52]. Besides smoking and obesity for cardiometabolic diseases, other factors such as low to moderate levels of alcohol[53], moderate to high physical activity[54] and adherence to dietary guidelines[55 56] have also been associated with better cardiometabolic risk profiles and longer life expectancy in both men and women. Therefore, better overall healthy lifestyle seems to be crucial in reducing cardiovascular and mortality risk[56]. People that engage in multiple unfavourable lifestyle behaviours have a higher risk for mortality and incidence of chronic diseases than people who have no unfavourable lifestyle behaviours or only one and the sum of these single components might be more important than the single components itself. Although its importance, seems that only a few studies have investigated the combined impact of the lifestyle-related factors and mortality outcomes and total life expectancy. Research to quantify the overall impact of lifestyle-related factors on mortality outcomes will provide important information valuable for disease prevention. Moreover, for highly lifestyle dependent conditions such as obesity and diabetes mellitus identifying patterns and markers in lifestyle could help not only in prevention but also in management and progression to reverse such condition.

Epigenetic evidence and cardiometabolic outcomes

Recent evidence shows that several lifestyle and environmental risk factors may partly affect health via epigenetic changes. The epigenome includes a series of chemical modifications that occur on the DNA or its associated proteins and are very important in gene

function[57]. The field of epigenetics is rapidly growing, with increasingly more focus and highlight on the link between our epigenetic makeup and CVD aetiology and predisposition. Several GWAS have identified loci that explain a fraction of the variance in T2D and CVD or their related risk factors[58 59]. Beyond this, the role of epigenetic determinants is increasingly recognized as a potential important link between environmental exposure and disease risk. Thus, epigenetic determinants may be a benchmark to capture the influences of environmental exposures and disease risk in cardiometabolic health[60]. Epigenetic mechanisms may subsequently influence gene expression, independently of the genetic code[61]. DNA methylation, histone modification, and noncoding RNA are three major types of epigenetic marks[62]. The best understood and most studied epigenetic mechanism is DNA methylation, the attachment of a methyl group to a CpG site. While the amount of research linking epigenetics and cardiometabolic outcomes is climbing, it is of critical importance that these studies should be stratified according to sex[63]. Epigenetic mechanisms ensure the inactivation of the second X-chromosome in women, securing dosage compensation of the X-chromosome between men and women[64], they are thought to control sex-specific gene expression during development [65] and they might play a role in the sex-specific disease profiles later in life[65]. In addition, the sex chromosomes contain multiple epigenetic modifiers that are differentially expressed between the sexes, which might influence the autosome in a sex-specific manner[66]. Furthermore, steroid sex hormones such as oestrogen and testosterone have been shown to affect epigenetic modifications[63 67 68].

AIM AND OUTLINE OF THIS THESIS

The overall aim of this thesis was to study traditional and novel sex specific risk factors of cardiometabolic diseases and longevity. Therefore, the objectives to achieve this aim were as following in the respective chapters and are presented in **Table 1.1**.

*In **chapter 2** of this thesis, we investigated the association of reproductive factors such as age at menopause and sex hormones with cardiometabolic risk*. We investigated the causality and direction of the relation between age at natural menopause and blood pressure using genetic variants as instrumental variables in a bi-directional Mendelian Randomization analysis (*Chapter 2.1*). Further, we examined the association between age at natural menopause and risk of T2D and calculate total life expectancy and the number of years lived with and without T2D (*Chapter 2.2 and 2.3*). Moreover, we assessed the associations between sex hormones and natriuretic peptide levels and examined whether the observed association with dehydroepiandrosterone sulphate is causal (*Chapter 2.4 and 2.5*).

Table 1.1. General overview of the studies included in the thesis.

Chapter	Exposure	Outcome	Epidemiological method used	Data source	Article type
2.1	Genetic risk score of age at natural menopause/ blood pressure	Age at natural menopause/ blood pressure	Mendelian randomization	The Rotterdam Study	Original data analysis
2.2	Age at natural menopause	Type 2 Diabetes	Cox Regression	The Rotterdam Study/Publicly available GWAS results	Original data analysis
2.3	Age at natural menopause	Type 2 Diabetes/ Mortality	Life tables	The Rotterdam Study	Original data analysis
2.4	Sex steroids, sex hormone-binding globulin	N-terminal pro-brain natriuretic peptide		The Rotterdam Study	Original data analysis
2.5	Genetic risk score of dehydroepiandrosterone sulphate	N-terminal pro-brain natriuretic peptide	Mendelian randomization	The Rotterdam Study/ Publicly available GWAS results	Original data analysis
3.1	Smoking status/ Body Mass Index	Type 2 Diabetes/ Mortality	Life tables	The Rotterdam Study	Original data analysis
3.2	Lifestyle score	Type 2 Diabetes/ Mortality	Life tables	The Rotterdam Study	Original data analysis
3.3	Body Mass Index	Type 2 Diabetes	Latent class trajectory analysis	The Rotterdam Study	Original data analysis
4.1	Type 2 Diabetes	Cardiovascular disease/Mortality	Life tables	THIN study	Original data analysis
5.1	DNA-methylation	Cardiometabolic outcomes	NA	Literature search from electronic databases	Systematic Review
5.2	DNA-methylation/ Histone modifications	Type 2 Diabetes	NA	Literature search from electronic databases	Review (Book chapter)

*In **chapter 3** we assessed the associations between lifestyle factors and cardiometabolic risk and life expectancy for men and women separately.* We calculated total life expectancy and life expectancy with and without T2D for smokers, overweight and obese ex-smokers, by comparing them to non-smokers and normal weight current smokers (*Chapter 3.1*). We further identified change of body mass index trajectories prior to diabetes development. Within these patterns, additional exploration of trajectories of other cardiometabolic risk factors including glycaemic indices (such as glucose, insulin, insulin resistance, beta cell dysfunction), blood pressure and lipid profile are examined (*Chapter 3.2*). By combining the most relevant lifestyle factors in a score and categorizing it in healthier, moderate and unhealthier score, in *chapter 3.3*, we aimed to calculate total

life expectancy and the number of years lived with and without diabetes for individuals with an overall healthier or unhealthier lifestyle.

*In **chapter 4** we studied several CVD risk factors and their associations with longevity.* The association between T2D and incident CVD and total life expectancy is investigated in chapter 4.1 comprising more than 17 million participants from UK using data from the THIN database.

*In **chapter 5** we quantitatively summarize current evidence on the relation between DNA methylation and cardiometabolic health. Chapter 5.1* describes the potential role of DNA methylation and histone modifications in explaining the sex differences in cardiometabolic diseases. In *chapter 5.2* we summarize some of the most up to date findings in the field of epigenetics for diabetes and its risk factors. Thus, the role of chromatin landscape and epigenetic biomarkers for clinical diagnosis and prognosis of type 2 diabetes mellitus was reviewed

Finally, in **Chapter 6**, we discuss the main findings of this thesis and we further address the methodological considerations, potential clinical implications and directions for future research.

METHODS

Study design

Rotterdam Study

The studies described in this thesis are performed within a large population based cohort study, the Rotterdam Study (RS), also known in Dutch as "Erasmus Rotterdam Gezondheid Onderzoek (ERGO)[69] (**Figure 1.1**). The study started in Ommoord, a well-defined suburb of Rotterdam, the Netherlands. In 1989, all residents aged 55 years or older were invited to participate in the study (RSI). 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 (RSII). The third cohort was formed in 2006 and included 3,932 participants 45 years and older (RSIII). There were no eligibility criteria to enter the Rotterdam Study cohorts except the minimum age and residential area based on postal codes. In total, the Rotterdam Study comprises 14,926 individuals. All participants were examined in detail at baseline. In summary, a home interview was conducted (approximately 2 hours) and the subjects had an extensive set of examinations (~ 5hours) in a specially built research facility in the centre of their district. Participants have been re-examined every 35 years, and have been followed up for a variety of diseases. Genotyping was conducted, in self-reported white participants in all three cohorts using the Illumina Infinium HumanHap550K Beadchip in RSI and RSII and the Illumina Infinitum HumanHap 610 Quad chip in RSIII at the

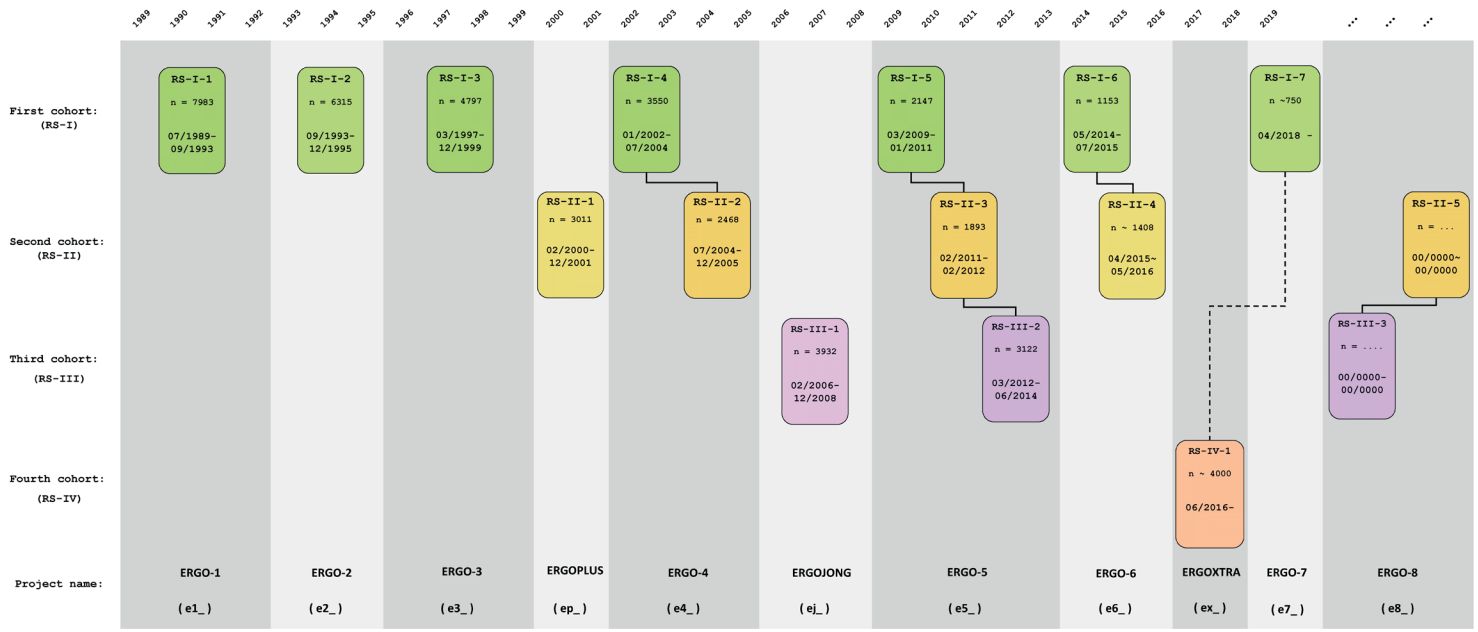


Figure 1.1. Diagram of examination cycles of the Rotterdam Study.

Genetic Laboratory of the Erasmus MC, Department of Internal Medicine, Rotterdam, the Netherlands. SNPs were imputed based on the 1000 Genomes cosmopolitan phase 1 version 3 reference. 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 provided written informed consent to participate and to obtain information from their treating physicians.

The Health Improvement Network (THIN) database

The study presented in **chapter 4.1** was conducted within the Health Improvement Network Database (THIN). THIN is a national database of electronic primary care records generalizable to the UK population[70]. It contains coded information for more than 15 million patients from 787 primary care general practices, including patient demographics, symptoms, diagnoses, drug prescriptions, consultations, and laboratory test results[70] (**Figure 1.2**). Cardiovascular diseases, diabetes, and hypertension are included in the Quality and Outcomes Framework, a scheme that incentivizes appropriate identification and management of patients with these diagnoses.

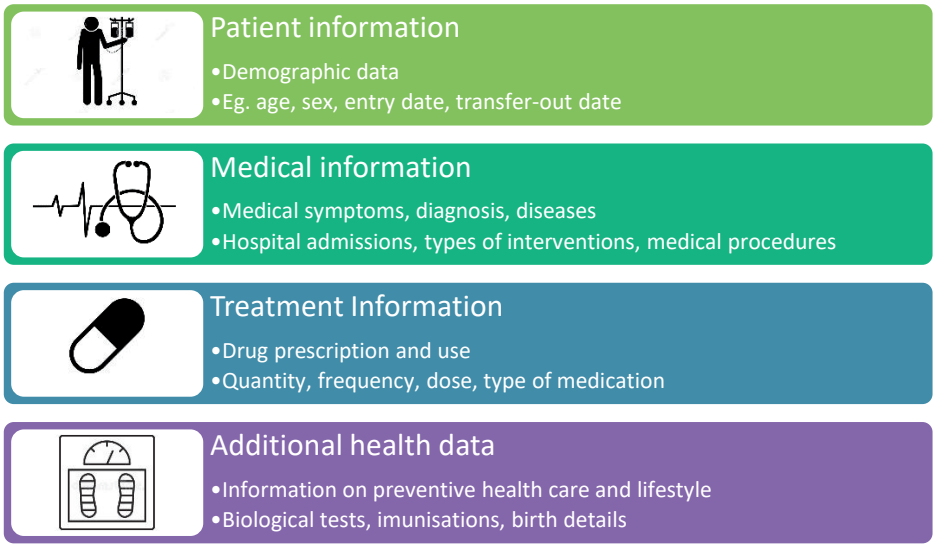


Figure 1.2. Structure of the health improvement network (THIN) database and information of the data collected

Systematic Reviews

Some projects included in **chapter 5** are systematic reviews and reviews of the literature. Relevant research articles were identified using different electronic medical databases.

The studies were conducted using a predefined protocol and in accordance with PRISMA guidelines[71]. Two independent reviewers screened the retrieved titles and abstracts and selected eligible studies. Discrepancies between the two reviewers were resolved through discussion and consensus with a third independent reviewer. We retrieved full texts for studies that satisfied all selection criteria. Further, reference lists of the included studies were screened to identify additional relevant studies. Study quality was judged on the selection criteria of participants, comparability of cases and controls, exposure and outcome assessment. Details on the methods can be found in **chapter 5**.

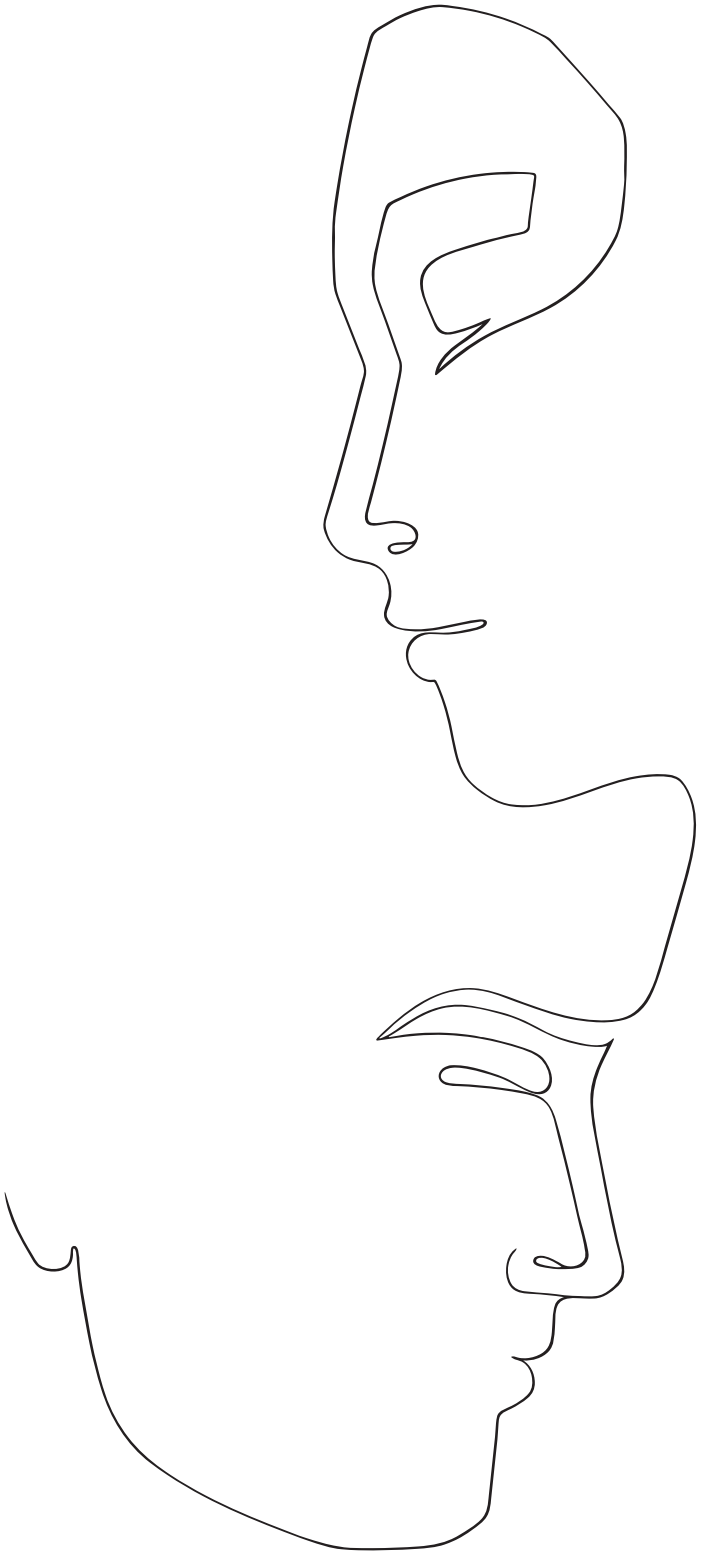
REFERENCES

1. Sasson C, Eckel R, Alger H, et al. American Heart Association Diabetes and Cardiometabolic Health Summit: Summary and Recommendations. J Am Heart Assoc 2018;**7**(15):e009271.
2. WHO. World Health Organization. Cardiovascular diseases (CVDs). 2017.
3. WHO. Global Action Plan for the Prevention and Control of NCDs 2013-2020.
4. Lansky AJ, Ng VG, Maehara A, et al. Gender and the extent of coronary atherosclerosis, plaque composition, and clinical outcomes in acute coronary syndromes. JACC Cardiovasc Imaging 2012;**5**(3 Suppl):S62-72.
5. Lerner DJ, Kannel WB. Patterns of coronary heart disease morbidity and mortality in the sexes: a 26-year follow-up of the Framingham population. Am Heart J 1986;**111**(2):383-90.
6. Berger JS, Elliott L, Gallup D, et al. Sex differences in mortality following acute coronary syndromes. JAMA 2009;**302**(8):874-82.
7. Huxley R, Barzi F, Woodward M. Excess risk of fatal coronary heart disease associated with diabetes in men and women: meta-analysis of 37 prospective cohort studies. BMJ 2006;**332**(7533):73-8.
8. Huxley RR, Woodward M. Cigarette smoking as a risk factor for coronary heart disease in women compared with men: a systematic review and meta-analysis of prospective cohort studies. Lancet 2011;**378**(9799):1297-305.
9. Barstad LH, Juliusson PB, Johnson LK, et al. Gender-related differences in cardiometabolic risk factors and lifestyle behaviors in treatment-seeking adolescents with severe obesity. BMC Pediatr 2018;**18**(1):61.
10. Collaboration NCDRF. Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19.2 million participants. Lancet 2016;**387**(10026):1377-96.
11. Blair SN, Kohl HW, 3rd, Paffenbarger RS, Jr., et al. Physical fitness and all-cause mortality. A prospective study of healthy men and women. JAMA 1989;**262**(17):2395-401.
12. Bots SH, Peters SAE, Woodward M. Sex differences in coronary heart disease and stroke mortality: a global assessment of the effect of ageing between 1980 and 2010. Bmj Global Health 2017;**2**(2).
13. Rossouw JE. Hormones, genetic factors, and gender differences in cardiovascular disease. Cardiovascular Research 2002;**53**(3):550-57.
14. Grady D, Herrington D, Bittner V, et al. Cardiovascular disease outcomes during 6.8 years of hormone therapy - Heart and Estrogen/progestin Replacement Study follow-up (HERS II). Jama-J Am Med Assoc 2002;**288**(1):49-57.
15. Mehta LS, Beckie TM, DeVon HA, et al. Acute Myocardial Infarction in Women: A Scientific Statement From the American Heart Association. Circulation 2016;**133**(9):916-47.
16. Lew J, Sanghavi M, Ayers CR, et al. Sex-Based Differences in Cardiometabolic Biomarkers. Circulation 2017;**135**(6):544-55.
17. Jaspers L, Daan NMP, van Dijk GM, et al. Health in middle-aged and elderly women: A conceptual framework for healthy menopause. Maturitas 2015;**81**(1):93-98.
18. Gold EB. The Timing of the Age at Which Natural Menopause Occurs. Obstet Gyn Clin N Am 2011;**38**(3):425-+.
19. Canto JG, Iskandrian AE. Major risk factors for cardiovascular disease - Debunking the "only 50%" myth. Jama-J Am Med Assoc 2003;**290**(7):947-49.
20. KritzSilverstein D, BarrettConnor E, Wingard DL. Hysterectomy, oophorectomy, and heart disease risk factors in older women. Am J Public Health 1997;**87**(4):676-80.

21. Pirimoglu ZM, Arslan C, Buyukbayrak EE, et al. Glucose tolerance of premenopausal women after menopause due to surgical removal of ovaries. Climacteric 2011;**14**(4):453-57.
22. Parazzini F. Risk factors for type 2 diabetes in women attending menopause clinics in Italy: a cross-sectional study. Climacteric 2005;**8**(3):287-93.
23. Qiu CS, Chen HJ, Wen JP, et al. Associations Between Age at Menarche and Menopause With Cardiovascular Disease, Diabetes, and Osteoporosis in Chinese Women. J Clin Endocr Metab 2013;**98**(4):1612-21.
24. Shen L, Song L, Li H, et al. Association between earlier age at natural menopause and risk of diabetes in middle-aged and older Chinese women: The Dongfeng-Tongji cohort study. Diabetes Metab 2017;**43**(4):345-50.
25. Brand JS, van der Schouw YT, Onland-Moret NC, et al. Age at Menopause, Reproductive Life Span, and Type 2 Diabetes Risk. Diabetes Care 2013;**36**(4):1012-19.
26. Yang AM, Liu SM, Cheng N, et al. Reproductive factors and risk of type 2 diabetes in an occupational cohort of Chinese women. J Diabetes Complicat 2016;**30**(7):1217-22.
27. Fu YL, Yu YQ, Wang SB, et al. Menopausal Age and Chronic Diseases in Elderly Women: A Cross-Sectional Study in Northeast China. Int J Env Res Pub He 2016;**13**(10).
28. Stefanick ML. New perspectives on menopausal hormones & cardiovascular disease: Role of age & years since menopause - The Women's Health Initiative (WHI) randomized trials. Menopause 2007;**14**(6):1076-76.
29. Tehrani FR, Behboudi-Gandevani S, Ghanbarian A, et al. Effect of menopause on cardiovascular disease and its risk factors: a 9-year follow-up study. Climacteric 2014;**17**(2):164-72.
30. Crandall CJ, Barrett-Connor E. Endogenous Sex Steroid Levels and Cardiovascular Disease in Relation to the Menopause A Systematic Review. Endocrin Metab Clin 2013;**42**(2):227-+.
31. Sutton-Tyrrell K, Wildman RP, Matthews KA, et al. Sex hormone-binding globulin and the free androgen index are related to cardiovascular risk factors in multiethnic premenopausal and perimenopausal women enrolled in the study of women across the nation (SWAN). Circulation 2005;**111**(10):1242-49.
32. Golden SH, Dobs AS, Vaidya D, et al. Endogenous sex hormones and glucose tolerance status in postmenopausal women. J Clin Endocr Metab 2007;**92**(4):1289-95.
33. Wang L, Szklo M, Folsom AR, et al. Endogenous sex hormones, blood pressure change, and risk of hypertension in postmenopausal women: The Multi-Ethnic Study of Atherosclerosis. Atherosclerosis 2012;**224**(1):228-34.
34. Zhao D, Guallar E, Ouyang P, et al. Endogenous Sex Hormones and Incident Cardiovascular Disease in Post-Menopausal Women. Journal of the American College of Cardiology 2018;**71**(22):2555-66.
35. Wu TT, Chen Y, Zhou Y, et al. Prognostic Value of Dehydroepiandrosterone Sulfate for Patients With Cardiovascular Disease: A Systematic Review and Meta-Analysis. J Am Heart Assoc 2017;**6**(5).
36. Glisic M, Rojas LZ, Asllanaj E, et al. Sex steroids, sex hormone-binding globulin and levels of N-terminal pro-brain natriuretic peptide in postmenopausal women. Int J Cardiol 2018.
37. Lam CS, Cheng S, Choong K, et al. Influence of sex and hormone status on circulating natriuretic peptides. J Am Coll Cardiol 2011;**58**(6):618-26.
38. Moriyama Y, Yasue H, Yoshimura M, et al. The plasma levels of dehydroepiandrosterone sulfate are decreased in patients with chronic heart failure in proportion to the severity. J Clin Endocrinol Metab 2000;**85**(5):1834-40.
39. Gambacciani M, Ciaponi M, Cappagli B, et al. Prospective evaluation of body weight and body fat distribution in early postmenopausal women with and without hormonal replacement therapy. Maturitas 2001;**39**(2):125-32.

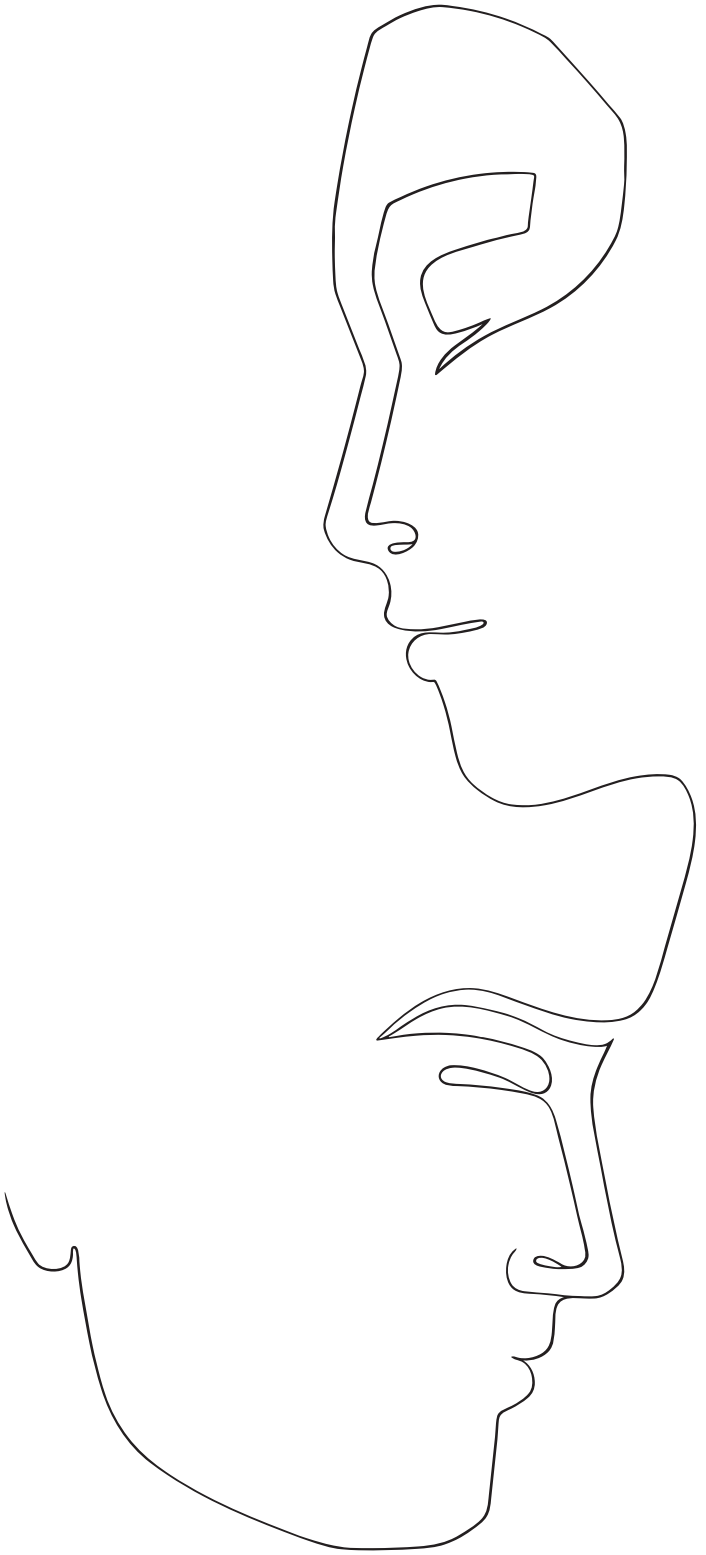
40. Barrett-Connor E. Sex differences in coronary heart disease. Why are women so superior? The 1995 Ancel Keys Lecture. Circulation 1997;**95**(1):252-64.
41. Waldron I. Why Do Women Live Longer Than Men. Social Science & Medicine 1976;**10**(7-8):349-62.
42. Anand SS, Islam S, Rosengren A, et al. Risk factors for myocardial infarction in women and men: insights from the INTERHEART study. European Heart Journal 2008;**29**(7):932-40.
43. Appelman Y, van Rijn BB, ten Haaf ME, et al. Sex differences in cardiovascular risk factors and disease prevention. Atherosclerosis 2015;**241**(1):211-18.
44. WHO report on the global tobacco epidemic, 2008: The MPOWER package. Popul Dev Rev 2008;**34**(3):581-81.
45. Pan A, Wang Y, Talaei M, et al. Relation of active, passive, and quitting smoking with incident type 2 diabetes: a systematic review and meta-analysis. Lancet Diabetes Endocrinol 2015;**3**(12):958-67.
46. Pan A, Wang YL, Talaei M, et al. Relation of active, passive, and quitting smoking with incident type 2 diabetes: a systematic review and meta-analysis. Lancet Diabetes Endo 2015;**3**(12):958-67.
47. Nagrebetsky A, Brettell R, Roberts N, et al. Smoking cessation in adults with diabetes: a systematic review and meta-analysis of data from randomised controlled trials. Bmj Open 2014;**4**(3).
48. Aubin HJ, Farley A, Lycett D, et al. Weight gain in smokers after quitting cigarettes: meta-analysis. Brit Med J 2012;**345**.
49. Lycett D, Munafo M, Johnstone E, et al. Associations between weight change over 8 years and baseline body mass index in a cohort of continuing and quitting smokers. Addiction 2011;**106**(1):188-96.
50. Pisinger C, Jorgensen T. Weight concerns and smoking in a general population: the Inter99 study. Prev Med 2007;**44**(4):283-9.
51. Pomerleau CS, Zucker AN, Stewart AJ. Characterizing concerns about post-cessation weight gain: results from a national survey of women smokers. Nicotine Tob Res 2001;**3**(1):51-60.
52. Dhana K, Nano J, Ligthart S, et al. Obesity and Life Expectancy with and without Diabetes in Adults Aged 55 Years and Older in the Netherlands: A Prospective Cohort Study. Plos Medicine 2016;**13**(7).
53. Djousse L, Lee IM, Buring JE, et al. Alcohol consumption and risk of cardiovascular disease and death in women: potential mediating mechanisms. Circulation 2009;**120**(3):237-44.
54. Dhana K, Koolhaas CM, Berghout MA, et al. Physical activity types and life expectancy with and without cardiovascular disease: the Rotterdam Study. J Public Health-Uk 2017;**39**(4):E209-E18.
55. Anand SS, Hawkes C, de Souza RJ, et al. Food Consumption and its Impact on Cardiovascular Disease: Importance of Solutions Focused on the Globalized Food System: A Report From the Workshop Convened by the World Heart Federation (vol 66, pg 1590, 2015). Journal of the American College of Cardiology 2015;**66**(17):1948-48.
56. Li Y, Pan A, Wang DD, et al. Impact of Healthy Lifestyle Factors on Life Expectancies in the US Population. Circulation 2018;**138**(4):345-55.
57. Kalish JM, Jiang C, Bartolomei MS. Epigenetics and imprinting in human disease. Int J Dev Biol 2014;**58**(2-4):291-98.
58. Deloukas P, Kanoni S, Willenborg C, et al. Large-scale association analysis identifies new risk loci for coronary artery disease. Nature Genetics 2013;**45**(1):25-U52.
59. Ehret GB, Munroe PB, Rice KM, et al. Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. Nature 2011;**478**(7367):103-09.
60. Muka T, Koromani F, Portilla E, et al. The role of epigenetic modifications in cardiovascular disease: A systematic review. Int J Cardiol 2016;**212**:174-83.

61. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet 2012;**13**(7):484-92.
62. Dunham I, Kundaje A, Aldred SF, et al. An integrated encyclopedia of DNA elements in the human genome. Nature 2012;**489**(7414):57-74.
63. Hartman RJG, Huisman SE, den Ruijter HM. Sex differences in cardiovascular epigenetics-a systematic review. Biol Sex Differ 2018;**9**(1):19.
64. Augui S, Nora EP, Heard E. Regulation of X-chromosome inactivation by the X-inactivation centre. Nat Rev Genet 2011;**12**(6):429-42.
65. Hochberg Z, Feil R, Constancia M, et al. Child health, developmental plasticity, and epigenetic programming. Endocr Rev 2011;**32**(2):159-224.
66. Wijchers PJ, Festenstein RJ. Epigenetic regulation of autosomal gene expression by sex chromosomes. Trends Genet 2011;**27**(4):132-40.
67. Mourad R, Hsu PY, Juan L, et al. Estrogen induces global reorganization of chromatin structure in human breast cancer cells. PLoS One 2014;**9**(12):e113354.
68. Dkhil MA, Al-Quraishy S, Abdel-Baki AA, et al. Epigenetic modifications of gene promoter DNA in the liver of adult female mice masculinized by testosterone. J Steroid Biochem Mol Biol 2015;**145**:121-30.
69. Hofman A, Brusselle GG, Darwish Murad S, et al. The Rotterdam Study: 2016 objectives and design update. Eur J Epidemiol 2015;**30**(8):661-708.
70. Blak BT, Thompson M, Dattani H, et al. Generalisability of The Health Improvement Network (THIN) database: demographics, chronic disease prevalence and mortality rates. Inform Prim Care 2011;**19**(4):251-5.
71. Moher D, Liberati A, Tetzlaff J, et al. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. Journal of Clinical Epidemiology 2009;**62**(10):1006-12.



2

Reproductive factors and cardiometabolic risk



2.1

Age at natural menopause and blood pressure: A bi-directional Mendelian randomization analysis

ABSTRACT

Objective

Age at natural menopause (ANM) varies considerably and has been associated with hypertension and cardiovascular health. We aimed to study the causality and direction of the relation between ANM and blood pressure using genetic markers as instrumental variables in a bi-directional Mendelian randomization analysis.

Methods

We studied 3,994 postmenopausal women participants of the Rotterdam Study. Multivariable linear and logistic regression models were used to assess the association between ANM (continuous), systolic blood pressure (SBP), diastolic blood pressure (DBP) and presence of hypertension (HTA). We also compared levels of blood pressure and prevalence of HTA by categories of ANM (early; 40-44 years, intermediate; 45-49 years, normal: 50 to 54 years (reference) and late menopause ≥ 55 years). We used genetic variants related to SBP, DBP and ANM to compute genetic risk scores and performed bi-directional Mendelian randomization analysis.

Results

There was a non-linear association between ANM and SBP (*p* for non-linearity = 0.026). Early menopause, compared to menopause age 50-54 years, was associated with lower SBP (β = -3.39, 95%CI: -5.8; -1.01) after adjustment for age, cardiovascular risk factors and medication, presence of comorbidity, hormone replacement therapy and estradiol levels. No association was found between ANM and DBP. Early menopause was associated with lower prevalence of HTA (early menopause vs. menopause age 50-54: odds ratio (OR) = 0.72, 95%CI: 0.54-0.95). Also, younger age at menopause was associated with lower prevalence of HTA (per 1 year younger age at menopause, OR= 0.97, 95%CI: 0.95-0.99). Bi-directional Mendelian randomization analysis showed no association between ANM genetic risk score, SBP, DBP or HTA. Higher genetic risk score of SBP (β=0.025; 95%CI: 0.002-0.04) and DBP (β=0.028; 95%CI: 0.01-0.05). Higher genetic risk score of SBP (*p*<0.0001) and DBP (*p*<0.0001) were also associated with a higher probability of taking antihypertensive medication.

Conclusion

These results suggest that higher blood pressure, or some environmental exposure related with higher blood pressure, such as use of antihypertensive medications, are causally associated with a later onset of natural menopause.

INTRODUCTION

Menopause, or the permanent cessation of menstruation, is a marker of the end of a woman's reproductive life. Approximately 40 million women will experience menopause over the next decade[1]. The age at menopause varies considerably, with some women experiencing menopause around the age of 40 and other later in their 50s. Women with early onset of menopause (<45 years) have increased risk of cardiovascular disease, and late menopause onset (>50) is linked to reduced risk of CVD[2]. The increase in CVD risk associated with early onset of menopause is believed to be due to its adverse effects on CVD risk factors, but the independent influence of age at natural menopause on levels of cardiovascular risk factors remains unclear.

Hypertension is a major risk factor for CVD and by far, the most important risk factor that affects women in the early postmenopausal years. About 30 to 50% of women develop hypertension (RR >140/90 mmHg) before the age of 60[3 4]. Early menopause has been suggested to lead to increased blood pressure, due to the early cessation of the vascular protective effects of endogenous oestrogen[5]. Also, loss of the ovarian function is associated with the activation of the renin-angiotensin-aldosterone system, leading to downstream endothelial dysfunction[6]. However, findings from epidemiological studies on the association between age at natural menopause and blood pressure are scarce and inconclusive[5 7]. Also, most of the studies available in the literature that explore the association between age of menopause and hypertension, have a cross-sectional design, therefore causality cannot be determined. Although increased blood pressure has been proposed as consequence of menopause, the alternative hypothesis, that fluctuations in blood pressure in premenopausal women may promote early menopause, has also been suggested[8]. Hypertension is associated with changes in vasculature that may affect the ovarian blood flow, which in turn could result in follicle loss and therefore influencing the age at which natural menopause occurs[9-11].

In the Mendelian randomization (MR) approach, causality is inferred from associations between genetic variants of a predictor variable and the outcome of interest. If there is indeed a causal effect of age of natural menopause on blood pressure, genetic determinants related to age at natural menopause should be associated with blood pressure[12]. Conversely, if blood pressure leads to onset of age at menopause, then genetic variants associated with higher blood pressure should be related to menopause onset. These associations, unlike the directly observed associations for age at natural menopause/ blood pressure, are less prone to confounding and free from reverse causation as genetic variants are invariant and assigned at random before conception[13].

We investigated the association between age at natural menopause (ANM) and blood pressure in the Rotterdam Study. Furthermore, we evaluated potential causal effect by using genetic variants as instruments in bi-directional Mendelian randomization analyses.

METHODS

Population for Analyses

The Rotterdam Study (RS) is a population-based cohort study of individuals 45years and over living in the Ommoord district of Rotterdam, the Netherlands. The rationale and design of RS is described elsewhere[14]In brief, all inhabitants of the Ommoord district aged 55 years or older were invited to participate (n = 10,215). At baseline (1990-1993), 7,983 participants were included (RS-I). In 2000, an additional 3011 participants were enrolled (RS-II), consisting of all persons living in the study district who had become 55 years of age. A second extension of the cohort was initiated in 2006, in which 3,932 participants aged 45 years or older were included (RSIII). Follow-up visits were held every 3-5years. The present study includes data from postmenopausal women from the third visit of the first cohort of RS (RS I-3), and from the first visits of the second (RSII-1) and third cohort (RSIII-1). The RS has been approved by the medical ethics committee according to the Population Study Act Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. A written informed consent was obtained from all participants.

Assessment of Age at Menopause

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. For women with natural menopause, age at menopause was defined as self-reported age at the time of last menstruation. Postmenopausal women were defined women who reported absence of menstrual periods for 12 months.

Assessment of Blood Pressure

At the research centre, after a resting period of 5 minutes, blood pressure was measured twice in a single visit using a random-zero sphygmomanometer (cuff size of 32×17) on the right arm of participants in sitting position by a trained research assistant. Systolic BP was recorded at the appearance of sounds (first-phase Korotkoff) and diastolic BP at the disappearance of sounds (fifth-phase Korotkoff). Systolic and diastolic BP were calculated as the average of the 2 measurements. Hypertension was defined as a systolic BP ≥140 mm Hg, a diastolic BP ≥90 mm Hg, or the use of antihypertensive medication. At the research centre, a physician ascertained the indication for which the medication had been prescribed. [15].

Assessment of Covariates

Information on current health status, medical history, smoking behaviour, and education was obtained by trained research assistants. Participants were classified as current, and former/never smokers. 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. 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. Diabetes mellitus was defined as the use of blood glucose–lowering medications or a random nonfasting glucose >11.1 mmol/L[16]. Estimated glomerular filtration rate was calculated using the simplified Modification of Diet in Renal Disease equation[17]. Medication use information was based on home interview. Antihypertensive medication use was defined as diuretics, β blockers, angiotensin-converting enzyme inhibitors, and calcium channel blockers. 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. Total cholesterol was measured on the COBAS 8000 Modular Analyser (Roche Diagnostics GmbH). Total estradiol levels were estimated in duplicate using the ultrasensitive RIA.

Genotyping

Genotyping was conducted, in self-reported white participants, using the Illumina 550K array. Participants were excluded if they had excess autosomal heterozygosity, mismatch between called and phenotypic sex, or recognized as being outlier with identical-by-state clustering analysis. Moreover, SNPs with allele frequency ≤1%, Hardy–Weinberg equilibrium P<10–5, or SNP call rate ≤90% were excluded. Imputation was done with reference to HapMap release 22 CEU (Utah residents of northern and western European ancestry) using the maximum likelihood method implemented in Markov Chain based haplotyper (version 1.0.15).

Population For Analyses

Age at natural menopause and blood pressure traits

There were 6760 women eligible for the analysis. 731 participants were excluded because they were premenopausal and 1464 women had non-natural menopause, surgical or unknown type of menopause. Also, 97 participants did not have information on age at menopause and 97 women had menopause before 40 years and after 60 years old, therefore were not included in the analyses. Data on blood pressure traits were not available for 528 participants, leaving 3843 women for the cross-sectional analyses on ANM and blood pressure traits (Supplemental material).

Genetic risk score of age at natural menopause and blood pressure traits

Among 4371 women who experienced natural menopause between age 40 and 60, 528 women had no information on blood pressure traits and 550 women had no genetic data, leaving 3293 women for the analysis on genetic risk score of age at menopause and blood pressure traits (Supplemental material).

Genetic risk score of systolic and diastolic blood pressure and age at natural menopause

Among 4371 postmenopausal women, 849 participants were excluded because of no information data on blood pressure SNPs. Hence, 3522 were included in the final analyses on genetic risk scores of blood pressure traits and ANM (Supplemental material).

Statistical Analyses

Multivariable linear and logistic regression models were used to assess the association between ANM (continuous), systolic blood pressure (SBP), diastolic blood pressure (DBP) and presence of hypertension (HTA). We also compared levels of blood pressure and prevalence of HTA by categories of ANM (early; 40-44 years, intermediate; 45-49 years, normal: 50 to 54 years (reference) and late menopause ≥ 55 years). We used genetic variants related to ANM, SBP and DBP and to compute genetic risk scores. We selected SNPs previously reported to have an association with systolic blood pressure from a GWAS of >200 000 European ancestry individuals[18] and with ANM from a GWAS of 40 000 women of European descent[3 19]. GRS were compiled using 24 SNPs associated with systolic blood pressure, 23 with diastolic blood pressure and 16 with age at menopause. We calculated a weighted GRS by multiplying the number of risk alleles at each locus by the corresponding reported β coefficient from the previous GWAS and then summing the products. The total score was then divided by the average effect size multiplied by 100 to rescale the scores to a range between 0 and 100. To examine the strength of the allele scores as instruments, the F-statistic was approximated from the proportion of variation in the respective phenotype (R^2) explained by the allele score, [F-stat=(R^2×(n−2))/(1−R^2)][20]. We performed linear regression and logistic regression analyses to examine the association between the genetic risk scores and their respective phenotypes. First we adjusted for age, cohort effect, and estimated glomerular filtration rate. In the second model, we additionally adjusted for antihypertensive medications, body mass index, alcohol consumption, prevalent diabetes mellitus, history of cardiovascular diseases, total cholesterol, smoking, statin use, hormone replacement therapy, estradiol and education level. When HTA was the outcomes, we did not adjust for antihypertensive medications. Also, we did not adjusted for age when examining the associations between genetic risk scores of blood pressure traits and observed ANM. To minimize the possibility of pleiotropic associations influencing results, we performed sensitivity analyses excluding SNPs with a more significant association with ANM/blood pressure traits than expected

by chance. As a further sensitivity analysis, for the factor (ANM, SBP or DBP) that showed evidence of a causal association with the corresponding outcome ($p < 3.8 \times 10{-}3$), we also performed a "leave one out" analysis to further investigate the possibility that the causal association was driven by a single SNP. Furthermore, we rerun all analysis among participants who had information available on ANM, blood pressure traits and genetic information (N=3293). A P-value lower than 0.05 was considered as statistically significant. To adjust for potential bias associated with missing data from the covariates we used multiple imputation procedure (N=5 imputations). We did not impute ANM, blood pressure traits or genetic risk scores, but we did enter them as predictor variables in our imputation model. All analyses were done using SPSS statistical software (SPSS, version 21.0; SPSS Inc., Chicago, Illinois).

Results

Baseline characteristics are shown in **Table 2.1.1**. Average age of participants was 68.78 years, with mean ANM of 50.08 years and mean SBP and DBP of 140.93 mmHg and 77.56 mmHg respectively. 45% of women were hypertensive and 35% of them were using antihypertensive medications.

Table 2.1.1. Characteristics of the Study Participants

Covariates		**N=3843**
Age at menopause y, mean (SD)		50,18 (3,95)
Systolic Blood Pressure mm Hg, mean (SD)		140,93 (29,03)
Diastolic Blood Pressure mm Hg, mean (SD)		77,56 (23,93)
Hypertension n (%)		1800 (45,1)
Alcohol intake g/d, median (IQR)		1,7 (13,18)
Smoking, Current smoker n (%)		444 (14,2)
Body Mass Index kg/m^2, mean (SD)		27,34 (4,47)
Total cholesterol mmol/L, mean (SD)		5,97 (0,98)
Statin n (%)		437 (10,9)
GFR, mL/min per 1.73 m^2, mean (SD)		74,78 (15,87)
Diabetes mellitus n (%)		308 (10,6)
Prevalent CVD n (%)		345 (8,7)
Hormone replacement therapy n (%)		70 (2,3)
Estradiol median (IQR)		30,75 (1048,65)
Education	Low n (%)	1952 (48,9)
	Primary n (%)	900 (22,4)
	High,n (%)	1105 (27,7)
Antihypertensive medication use, n (%)		1081 (35)

*Was defined as use of the diuretics, β blockers, angiotensin- converting enzyme inhibitors and calcium channel blockers

Association between ANM and blood pressure traits

We observed a non-linear association between ANM and SBP (*p* for non-linearity = 0.026). Early menopause, compared to menopause age 50-54 years, was associated with lower SBP (β = -3.39, 95%CI: -5.8; -1.01) after adjustment for age, cardiovascular risk factors and medication, presence of comorbidity, hormone replacement therapy and estradiol levels. No association was found between ANM and DBP (**Table 2.1.2**). Women who experienced menopause at age 45-49 years or ≥55 years old did not show difference in levels of SBP compared to women who experienced menopause at age 50-54 (**Table 2.1.2**). Early menopause was associated with lower prevalence of HTA (early menopause vs. menopause age 50-54: odds ratio (OR) = 0.72, 95%CI: 0.54-0.95) while no difference was observed for other groups of menopause age (**Table 2.1.2**). Also, younger ANM was associated with lower prevalence of HTA (per 1 year younger age at menopause, OR= 0.97, 95%CI: 0.95-0.99).

Association between ANM genetic risk score and blood pressure traits

Genetic risk of ANM was associated with observed ANM and the variance in ANM explained by genetic risk score of ANM was 3% in our study (Supplemental material). We found no association between genetic risk score of ANM, SBP, DBP or HTA (**Table 2.1.3**).

Association between genetic risk scores of blood pressure traits and age of menopause

Both genetic risk scores of SBP and DBP were associated with their traits (SBP: *p-value=0.012* and DBP: *p-value <0.001*) (Supplemental material). Further adjustment for antihypertensive medications did not affect the association between genetic risk score of SBP and observed SBP, but abolished the association between genetic risk of DBP and observed DBP. The variance in phenotype explained by genetic risk scores was 5.4% for SBP and 4.6% for DBP (Supplemental material). Also, both genetic risk scores of SBP and DBP were associated with higher odds of taking antihypertensive medications, independent of observed SBP and DBP (p<0.001) (Supplemental material). Higher genetic risk scores of SBP and DBP were associated with higher ANM (per 1 mm Hg of SBP, β=0.025; 95%CI: 0.002-0.04; per 1 mm Hg of DBP, β=0.028; 95%CI: 0.01-0.05)(**Table 2.1.4**). Further adjustment of SBP, DBP and antihypertensive medications did not abolish the association.

Sensitivity analyses

To test for pleiotropy , we assessed the association between the SNPs that were used for making the genetic risk score(as independent variable) and the outcome (as dependent). We used the Bonferroni Correction p-value as the threshold of significance and we did not find any association (Supplemental material**).** In the "leave one out" analyses, where

Table 2.1.2. Association of ANM with blood pressure traits.

Age of menopause	SBP				DBP				HTA*			
	Model 1		Model 2		Model 1		Model 2		Model 1**		Model 2	
	B (95% CI)	P-value	B(95% CI)	P-value	B (95% CI)	P-value	B(95% CI)	P-value	OR (95%CI)	P-value	OR (95%CI)	P-value
Continuous	0.180 (0.007-0.353)	0.041	0.115 (-0.055-0.285)	0.184***	0.066 (-0.026-0.159)	0.158	0.018 (-0.073-0.109)	0.391	1.025 (1.007-1.044)	0.006	1.026 (1.007-1.046)	0.008
40-44 years	-3.300 [(-5.719)-(-0.881)]	0.008	-3.388 [(-5.766)-(-1.009)]	0.005	-0.961 (-2.307-0.385)	0.148	-0.870 (-2.143-0.404)	0.181	0.790 (0.615-1.015)	0.065	0.715 (0.540-0.946)	0.019
45-49 years	-0.616 [(-2.255)-(1.022)]	0.461	-0.420 (-2.025-1.185)	0.608	0.014 (-0.862-891)	0.974	0.108 (-0.752-0.967)	0.806	0.989 (0.835-1.172)	0.902	0.982 (0.815-1.184)	0.851
50-54 years	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
≥55 years	-0.786 (-2.885-1.312)	0.477	-1.749 (-3.871-0.373)	0.106	-0.207 (-0.384-0.798)	0.723	-0.442 (-1.578-0.695)	0.447	1.151 (0.919-1.441)	0.221	0.974 (0.758-1.251)	0.837

Model 1: adjusted for age, rs_cohort_2, rs_cohort_3, glomerular filtration rate

Model 2: Model 1 +body mass index, treatment for hypertension , alcohol, smoking, statin use, cholesterol, education (primary, high), prevalent type 2 diabetes and prevalent cardiovascular disease, hormone replacement therapy and estradiol.

*Hypertension was defined as a systolic BP ≥140 mm Hg, a diastolic BP ≥90 mm Hg, or the use of antihypertensive medication.

** Not adjusted for treatment for hypertension.

***The quadratic term in the model was significant *p=0.026*

Table 2.1.3. Association of ANM genetic risk score with blood pressure traits

Genetic risk score of age of menopause	Systolic blood pressure				Diastolic blood pressure				Hypertension*			
	Model 1		Model 2		Model 1		Model 2		Model 1		Model 2	
	B (95% CI)	P-value	B (95% CI)	P-value	B (95% CI)	P-value	B (95% CI)	P-value	OR (95%CI)	P-value	OR (95%CI)	P-value
Continuous	-0.085 (-0.180-0.009)	0.077	-0.083 (-0.177-0.010)	0.080	-0.048 (-0.099-0.003)	0.066	-0.048 (-0.098-0.002)	0.060	0.997 (0.987-1.006)	0.496	0.995 (0.984-1.006)	0.382

Model 1: adjusted for age, rs_cohort_2, rs_cohort_3, glomerular filtration rate

Model 2: Model 1 +treatment for hypertension, body mass index, alcohol, smoking, cholesterol, education (primary, high), prevalent type 2 diabetes, statin use, prevalent cardiovascular disease, hormone replacement therapy and estradiol.

* Not adjusted for treatment for hypertension.

Table 2.1.4. Association between genetic risk scores of blood pressure traits and ANM

GRS	AGE OF MENOPAUSE									
	Continuous		40-44 years		45-49		50-54		>=55	
	B(95% CI)	P-value	OR(95% CI)	P-value	OR(95% CI)	P-value	NA	NA	OR(95% CI)	P-value
Systolic Blood Pressure										
Model 1	0.025 (0.004;0.045)	0.018	0.998 (0.980;1.016)	0.816	0.996 (0.984;1.009)	0.540	Ref.	Ref.	1.026 (1.009;1.043)	0.003
Model 2	0.022 (0.002;0.043)	0.033	0.999 (0.981;1.018)	0.918	0.997 (0.984;1.009)	0.604	Ref.	Ref.	1.025 (1.008;1.043)	0.004
Diastolic Blood Pressure										
Model 1	0.028 (0.008;0.048)	0.006	0.990 (0.972;1.007)	0.230	0.995 (0.983;1.007)	0.400	Ref.	Ref.	1.019 (1.003;1.036)	0.019
Model 2	0.026 (0.007;0.046)	0.009	0.990 (0.972;1.008)	0.274	0.995 (0.983;1.007)	0.427	Ref.	Ref.	1.020 (1.003;1.036)	0.020

Model 1: adjusted for rs_cohort_2, rs_cohort_3.

Model 2: Model 1 + glomerular filtration rate, age, body mass index, alcohol, smoking, cholesterol, statin use, education (primary, high), prevalent type 2 diabetes, prevalent cardiovascular disease, hormone replacement therapy and estradiol.

the genetic risk scores where recalculated excluding SNPs one by one, we didn't find any evidence that the association was driven by a specific SNP (Supplemental material). Restriction of analyses to participants who had information on ANM, blood pressure and genetic data did not affect any of the associations n=3293).

DISCUSSION

In the current study we report higher burden of hypertension with increase age of natural menopause, but we did not find any evidence for a casual effect of ANM on blood pressure. Contrary, using genetic variants associated with blood pressure traits, we found evidence for a potential causal relationship between higher blood pressure and later onset of natural menopause.

Age at menopause has been associated with hypertension [7 21]. However, uncertainties remain over the nature of the association[5 7 21], perhaps complicated by the cross-sectional design of small-scale studies, reliability of age at menopause, especially after a long follow-up, or the ethnicity and age of study participants[5 7 21 22]. A cross-sectional study[23] of 150 Chinese postmenopausal women found an inverse association between age at menopause, SBP and DBP, whereas a large study[22] of 22,426 Japanese women reported that women who experienced later onset of natural menopause were more likely to have hypertension, but it did not remain significant after further adjustment for age. In the current study, after adjustment for a broad range of confounding factors, we found that later onset of natural menopause was associated with higher SBP, DBP and higher odds of having hypertension. However, we did not find any causal effect of age at menopause on blood pressure. In line with our findings, a prospective study of 1288 women did not show any effect of age at menopause on blood pressure changes[24]. Also, a large number of studies, while show an effect on cholesterol levels, have not observed significant differences in blood pressure levels with menopause[10 25].

Contrary to common belief, recent data show that medical conditions, such as hypertension can be related to menopause onset[8]. Hypertension may induce a reduction in the ovarian blood flow, which results in irreversible follicle loss and substantial diminished ovarian reserve with acceleration of the timing of entering menopause[9-11]. Strikingly, as opposed to the current limited literature, our findings suggest that genetically elevated levels of SBP and DBP are not predisposing to early onset of menopause but in fact are delaying the age at which natural menopause occurs. The significant causal role of blood pressure for age at natural menopause is in line with the direction of the observational findings we report for ANM and blood pressure traits. Also, the leave out analysis revealed that the genetic overlap between blood pressure levels and age at natural menopause we observed was not driven by a single SNP. Few studies

examining whether premenopausal hypertension is associated with age at menopause have shown overall no association[8 21]. However, a recent study of 6650 postmenopausal Korean women showed that women with hypertension after the age of 50 years were more likely to undergo a later menopause[21]. Given that blood pressure is a major risk factor for cardiovascular disease[26], our observation that a genetically determined marginal increase in the level of SBP and DBP is likely to delay menopause onset, does not advocate raising blood pressure to delay menopause onset, yet these findings offer intriguing etiological insight. Also, the SNPs associated with SBP overlap extensively with those associated with DBP[18] as well as with pulse pressure and pulse wave velocity[27], so it remains unclear, if the association is causal, which of the components of blood pressure drives the effect.

In line with other studies, we also found that genetically predicted higher SBP and DBP were associated with higher probability of being on antihypertensive medication[28]. Also, in our study, the genetic risk score of SBP was associated with observed SBP independent of use of antihypertensive medications, while the genetic risk score of DBP was not associated with observed DBP after adjustment for antihypertensive medications. Therefore, if antihypertensive medications delay menopause onset, they may confound the association between genetically predicted systolic blood pressure and age at menopause onset. However, in our study, the analysis by use of antihypertensive medication did not show any difference. Furthermore, adjustment for antihypertensive medications did not have any effect on our results. On the other hand, if antihypertensive medications have a causal effect on menopause onset, their effect is likely to be independent on blood pressure, since the variants associated with SBP are associated with SBP independent of treatment with antihypertensive medication. We could not find any study to investigate the association between use of anti-hypertensive medications and onset of age at menopause. It would be of interest for future studies to further investigate whether antihypertensive medications may delay menopause onset independently of their effects on blood pressure.

Major strengths of our study are the large sample size for measurement of both ANM and blood pressure, and a comprehensive assessment of this association using both observational and genetic data. We are the first study to investigate the causal relationship between ANM and blood pressure through the Mendelian randomization approach. Some limitations should be acknowledged. A key advantage of using a genetic approach over a traditional epidemiologic approach to investigate an association such as that between ANM and blood pressure is that, genotypes (because they are randomly distributed at birth) are unlikely to be confounded by lifestyle or environmental factors[29]. Regardless of whether such factors are known or unknown, they can independently affect the exposure and the outcome and lead to a spurious association between them. It is nonetheless possible that the genetic variants themselves affect ANM and

blood pressure through entirely different mechanisms. However, given the big number of variants that we included in the analysis, all of which were selected because of their association with ANM or blood pressure, it is likely that at least some of the processes are shared. A limitation of the MR approach is the limited strength of the SNPs to explain variation in the intermediate traits, restricting statistical power. Therefore, we used multiple SNPs combined into a single genetic instrument to increase the statistical power of our study. The results presented in Supplemental material show that our genetic risk scores are not weak instruments, as indicated by their *F*-values. Another caveat of MR is that developmental compensation might occur, through a genotype being expressed during foetal development that in turn buffers the effects of either environmental or genetic factors, a process called canalization[29 30]. Therefore, buffering mechanisms could hamper the associations between genetic variants and the outcome of interest.

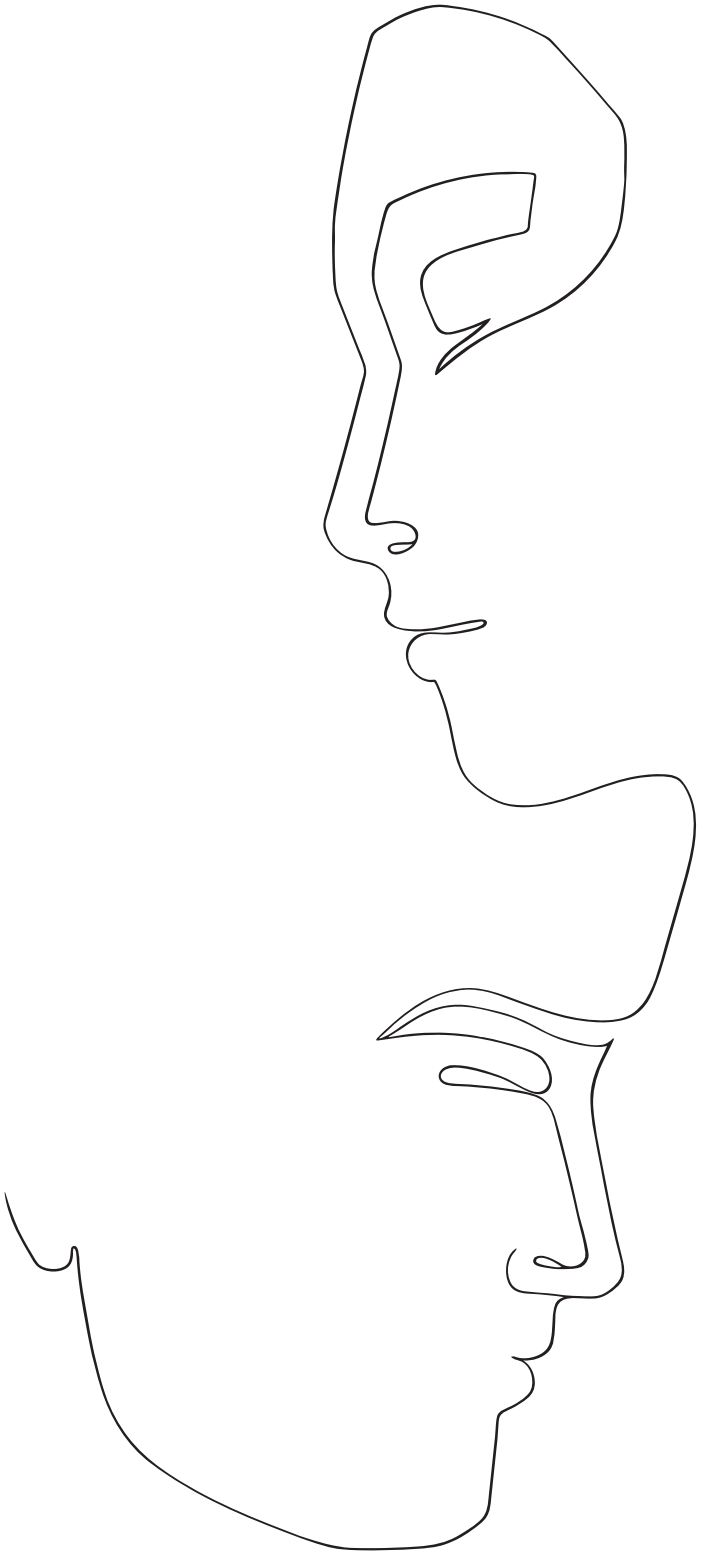
Also the poor biology understanding is another limitation of the MR approach[12 13 29]. Although thousands of SNP-trait associations have been discovered by GWASs, little is typically understood about the underlying biology or mechanisms of association. This limitation can sometimes lead to counterintuitive results[31]. On the other hand, genetic polymorphisms are sometimes associated with multiple aspects or dimensions of a single trait. Even though, we did sensitivity analyses (Supplemental material) further biological investigation is needed to identify the pathways in which these SNPs are involved and avoid the pleiotropic effect.

In conclusion, we found associations between higher genetic risk score of SBP/DBP and later onset of menopause. However, since there is a strong association between higher SBP gene scores and use of antihypertensive treatments, there is a need to evaluate the possible role of some of these medications in delaying the timing of menopause, independent of their effects on blood pressure.

REFERENCES

1. JC. D. Population Projections of the United States by Age, Sex, Race, and Hispanic Origin: 1995 to 2050. US Bureau of the Census, Current Population Reports, P25-1130, US Government Printing Office, Washington, DC 1996.
2. Gong DD, Sun J, Zhou YJ, et al. Early age at natural menopause and risk of cardiovascular and all-cause mortality: A meta-analysis of prospective observational studies. Int J Cardiol 2016;**203**:115-19.
3. Burt VL, Whelton P, Roccella EJ, et al. Prevalence of hypertension in the US adult population. Results from the Third National Health and Nutrition Examination Survey, 1988-1991. Hypertension 1995;**25**(3):305-13.
4. Wassertheil-Smoller S, Anderson G, Psaty BM, et al. Hypertension and its treatment in postmenopausal women: baseline data from the Women's Health Initiative. Hypertension 2000;**36**(5):780-9.
5. Izumi Y, Matsumoto K, Ozawa Y, et al. Effect of age at menopause on blood pressure in postmenopausal women. Am J Hypertens 2007;**20**(10):1045-50.
6. Zhao Z, Wang H, Jessup JA, et al. Role of estrogen in diastolic dysfunction. Am J Physiol Heart Circ Physiol 2014;**306**(5):H628-40.
7. Maas AHEM, Franke HR. Women's health in menopause with a focus on hypertension. Neth Heart J 2009;**17**(2):68-72.
8. Kok HS, van Asselt KM, van der Schouw YT, et al. Heart disease risk determines menopausal age rather than the reverse. J Am Coll Cardiol 2006;**47**(10):1976-83.
9. Xiangying H, Lili H, Yifu S. The effect of hysterectomy on ovarian blood supply and endocrine function. Climacteric 2006;**9**(4):283-9.
10. Matthews KA, Meilahn E, Kuller LH, et al. Menopause and Risk-Factors for Coronary Heart-Disease. New Engl J Med 1989;**321**(10):641-46.
11. Trabuco EC, Moorman PG, Algeciras-Schimnich A, et al. Association of Ovary-Sparing Hysterectomy With Ovarian Reserve. Obstet Gynecol 2016;**127**(5):819-27.
12. Smith GD, Ebrahim S. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? Int J Epidemiol 2003;**32**(1):1-22.
13. Lawlor DA, Harbord RM, Sterne JAC, et al. Mendelian randomization: Using genes as instruments for making causal inferences in epidemiology. Stat Med 2008;**27**(8):1133-63.
14. Hofman A, Darwish Murad S, van Duijn CM, et al. The Rotterdam Study: 2014 objectives and design update. Eur J Epidemiol 2013;**28**(11):889-926.
15. van Leeuwen R, Ikram MK, Vingerling JR, et al. Blood pressure, atherosclerosis, and the incidence of age-related maculopathy: The Rotterdam Study. Invest Ophth Vis Sci 2003;**44**(9):3771-77.
16. Gavin JR, Alberti KGMM, Davidson MB, et al. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 1997;**20**(7):1183-97.
17. Perrone RD, Madias NE, Levey AS. Serum Creatinine as an Index of Renal-Function - New Insights into Old Concepts. Clin Chem 1992;**38**(10):1933-53.
18. Ehret GB, Munroe PB, Rice KM, et al. Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. Nature 2011;**478**(7367):103-09.
19. Stolk L, Perry JRB, Chasman DI, et al. Meta-analyses identify 13 loci associated with age at menopause and highlight DNA repair and immune pathways. Nat Genet 2012;**44**(3):260-U55.
20. JA R. Expected values. Mathematical statistics and data analysis. 2nd edition. Pacific Grove (California): Duxbury Press 1995.

21. Lim HS, Kim TH, Lee HH, et al. Hypertension and age at onset of natural menopause in Korean postmenopausal women: Results from the Korea National Health and Nutrition Examination Survey (2008-2013). Maturitas 2016;**90**:17-23.
22. Lee JS, Hayashi K, Mishra G, et al. Independent Association between Age at Natural Menopause and Hypercholesterolemia, Hypertension, and Diabetes Mellitus: Japan Nurses' Health Study. J Atheroscler Thromb 2013;**20**(2):161-69.
23. Izumi Y, Ozawa Y, Jumabay M, et al. Effect of age at menopause on blood pressure in postmenopausal women. J Hypertens 2006;**24**:96-96.
24. Akahoshi M, Soda M, Nakashima E, et al. Effects of age at menopause on serum cholesterol, body mass index, and blood pressure. Atherosclerosis 2001;**156**(1):157-63.
25. Akahoshi M, Soda M, Nakashima E, et al. Effects of menopause on trends of serum cholesterol, blood pressure, and body mass index. Circulation 1996;**94**(1):61-66.
26. Lewington S, Clarke R, Qizilbash N, et al. Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. Lancet 2002;**360**(9349):1903-13.
27. Wain LV, Verwoert GC, O'Reilly PF, et al. Genome-wide association study identifies six new loci influencing pulse pressure and mean arterial pressure. Nat Genet 2011;**43**(10):1005-11.
28. Ostergaard SD, Mukherjee S, Sharp SJ, et al. Associations between Potentially Modifiable Risk Factors and Alzheimer Disease: A Mendelian Randomization Study. Plos Med 2015;**12**(6).
29. Smith GD, Ebrahim S. Mendelian randomization: prospects, potentials, and limitations. Int J Epidemiol 2004;**33**(1):30-42.
30. Jansen H, Samani NJ, Schunkert H. Mendelian randomization studies in coronary artery disease. Eur Heart J 2014;**35**(29):1917-+.
31. Juul K, Tybjaerg-Hansen A, Marklund S, et al. Genetically reduced antioxidative protection and increased ischemic heart disease risk - The Copenhagen City Heart Study. Circulation 2004;**109**(1):59-65.



2.2

Age at natural menopause and risk of type 2 diabetes: a prospective cohort study

ABSTRACT

Objective

We aimed to examine the association between age at natural menopause (ANM) and the risk of type 2 diabetes (T2D), and to assess whether this association is independent of potential mediators.

Methods

We included 3,639 postmenopausal women from the prospective population-based Rotterdam Study. ANM was self-reported retrospectively and was treated continuously and in categories (premature, <40 years; early, 40-44 years; normal, 45 to 55 years and late menopause > 55 years (reference)). T2D events were diagnosed on the basis of medical records and glucose measurements from Rotterdam Study visits. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated using Cox proportional hazards models, adjusted for confounding factors and, in another model, additionally adjusted for potential mediators, including total estradiol, androgen levels, obesity, C-reactive protein, glucose and insulin.

Results

During a median follow-up of 9.2 years, we identified 348 incident cases of T2D. After adjustment for confounders, HRs of T2D were 3.1 (95% CI 1.5–6.4), 2.1 (1.2–4.0), and 1.60 (0.9–2.7) for women with premature, early and normal menopause, respectively, relative to those with late menopause (*P*-trend = 0.001). The HR for T2D per one year older age at menopause was 0.96 (0.94–0.99). Further adjustment for body mass index, glycaemic traits, metabolic risk factors, C-reactive protein, endogenous sex hormone levels or shared genetic factors did not affect this association.

Conclusion

Early onset of ANM is an independent marker for T2D in postmenopausal women.

INTRODUCTION

Menopause marks a major life transition for women, resulting in the loss of ovarian follicle development[1]. Although menopause is a universal phenomenon among women, the timing of the final menstrual period differ greatly between women[1 2], and is considered a marker of ageing and cardiovascular health[2]. Women with early onset of menopause (<45 years) have an increased risk of cardiovascular disease (CVD) and overall mortality, whereas menopause onset at age 50-54 years is linked to reduced risk of CVD and mortality[3]. The increased risk of CVD and mortality is believed to be due to the adverse effects that early onset of menopause has on CVD risk factors , but the influence of age at menopause on levels of cardiovascular risk factors remains unclear[3].

Type 2 diabetes (T2D) is a major risk factor for CVD and it remains unclear whether age at menopause is associated with risk of T2D[3 4]. Data from cross-sectional studies examining the association between age at menopause and T2D are conflicting, with few studies reporting no association and some other reporting higher odds of having T2D with early onset of menopause[5-7]. Recently, a nested case-cohort study presented an increased risk of T2D associated with early onset of menopause, but it lacked adjustment for estradiol and other endogenous sex hormone-levels[8]. Hormonal changes associated with the menopause transition, in particular the decline in oestrogen levels and the relative androgen excess[9], have been postulated as mediators of the adverse cardiometabolic health profile observed with early onset of menopause[3 10 11]. Also, estradiol and sex hormone-binding globulin are associated with risk of type 2 diabetes in postmenopausal women[12]. Therefore, it is not clear whether the observed association between early onset of menopause and risk of T2D is explained by differences in sex hormones levels between women who experience early and late menopause. Also, no study has examined whether potential intermediate factors such as obesity, glucose metabolism, insulin or shared genetic factors can explain the association between age at menopause and risk of T2D. Menopause transition is associated with weight gain and an increase in visceral fat, as well as with impairment of glucose homeostasis, important risk factors for T2D[13-15].

This study aims to investigate the association between age at natural menopause (ANM) and risk of developing T2D, and to assess whether this association is independent of endogenous sex hormone levels and intermediate risk factors for T2D.

METHODS

Study Population

The Rotterdam Study is a population based, prospective cohort study in the Netherlands. This project was initiated in 1990-1993 in the Ommoord district of the port city of Rotterdam. Design and rationale of the Rotterdam study have been described in detail elsewhere[16]. In summary, all inhabitants of this district aged 55 years and over were invited to participate, leading a baseline cohort of 7,983 subjects (RS-I). Over the years, two more allocation rounds were held, one in 2000-2001 for all inhabitants aged 55 years and over, leading to an additional 3,011 participants (RS-II)[16]. A second extension was initiated in 2006, in which 3,932 participants aged 45 years and over were included (RS-III)[16]. For follow-up, examinations were scheduled every 3-5 years[16]. The Rotterdam Study has been approved by the medical ethics committee according to the Population Study Act Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. All participants provided written informed consent to obtain and process data from their treating healthcare providers.

Population for 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) and third cohort (RSIII-1). There were 6,816 women eligible for the analysis. Of those, 2,053 women were excluded because (i) there was no information on their menopause status (n=9); (ii) were non-postmenopausal women (n=732); (iii) age at menopause was not known (n=145); (iv) did not give inform consent for T2D follow-up (n=56); (v) had prevalent T2D (n=609); and (vi) no information on incident T2D was available (n=502) (**Figure 2.2.1)**. Furthermore, 1,124 women were excluded because experienced non-natural menopause (n=1,109) or type of menopause was not known (n=15), leaving 3639 women for final analysis (**Figure 2.2.1)**.

Assessment of Age at Menopause

Menopausal status was evaluated using a subsection in the home interview questionnaire. 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 past 12 months, and the age at last period for women who had no period for at least 12 months. Postmenopausal women were defined as women who reported absence of menstrual periods for 12 months. For women with natural menopause, age at menopause was defined as self-reported age at the time of last menstruation. For all women reporting menopause after gynaecologic surgery or radiation therapy and for those reporting any other operations before age 50 that might have led to menopause, information on the exact date and type of operation was verified using general practitioners' records, which included correspondence from medical specialists.

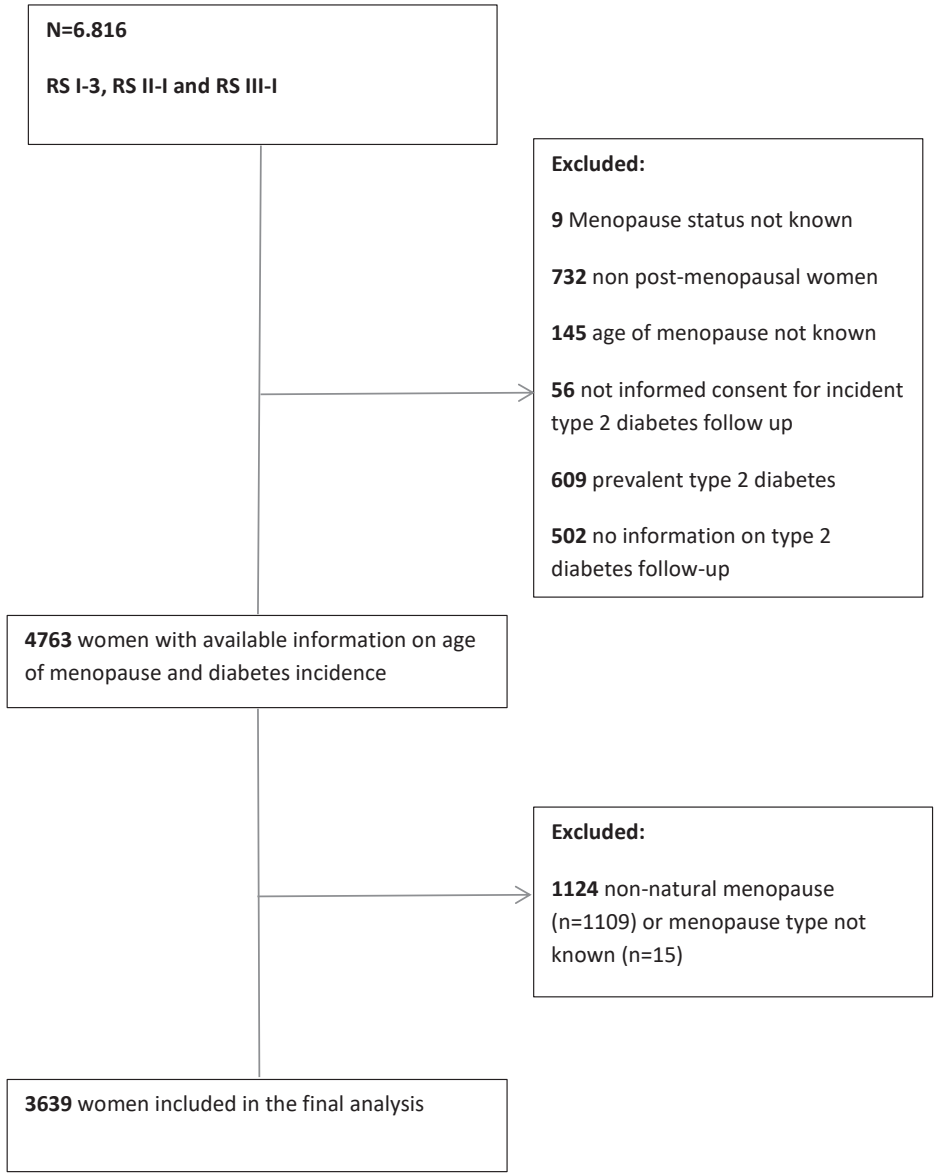


Figure 2.2.1. Flow Chart of Participants in the Study, the Rotterdam Study

Ascertainment of Type 2 Diabetes

The participants were followed from the date of baseline centre visit onwards. At baseline and during follow-up, prevalent and incident 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[17]. Prevalent and incident T2D was defined according to recent WHO guidelines, as a fasting blood glucose ≥ 7.0 mmol/L, a non-fasting blood glucose ≥ 11.1 mmol/L (when fasting samples were absent), or the use of blood glucose lowering medication[18]. Information regarding the use of blood glucose lowering medication was derived from both structured home interviews and linkage to pharmacy records[17]. 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.

Potential Confounding Variables

Information on current health status, medical history, medication use, smoking behaviour, socioeconomic status and other factors was obtained at baseline (RSI-3, RSII-1 and RSIII-1). Education was defined as low (primary education), intermediate (secondary general or vocational education), or high (higher vocational education or university). Data on age at menarche were collected by asking women, "How old were you when you had your first menstrual period?", The retrospective data on self-reported number of pregnancies of at least 6 months and use of hormone replacement therapy were collected by a questionnaire during the home interview. 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), heart failure and stroke, 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. The mean of two consecutive measurements was taken. Medication use information was based on home interview. Antihypertensive medication use was defined as use of diuretics, β blockers, angiotensin-converting enzyme inhibitors, and calcium channel blockers. All biochemical parameters were assessed in fasting serum. Thyroid stimulating hormone (TSH) was measured on the Vitros Eci (Ortho Diagnostics). Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG) and C-reactive protein (CRP) were measured on the COBAS 8000 Modular Analyser (Roche Diagnostics GmbH). Low density lipoprotein cholesterol (LDL-C) levels were estimated indirectly from measurements of TC, HDL, and TG by means of the Friedewald equation[19]. The corresponding interassay coefficients of variations are the following: TSH<13.2%, lipids <2.1%, and CRP <16.9%. Physical activity was assessed using the LASA Physical Activity Questionnaire (LAPAQ) and is expressed in METhours/week [20].

Potential Intermediate Variables

All intermediate variables were assessed at baseline (RSI-3, RSII-1 and RSIII-1). 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).). Fasting insulin and glucose were measured on the COBAS 8000 Modular Analyser (Roche Diagnostics GmbH). The interassay coefficients of variations are <8% and <1.4% for insulin and glucose respectively. Total estradiol levels were measured with a radioimmunoassay and sex hormone-binding globulin (SHBG) with the Immulite platform (Diagnostics Products Corporation Breda, the Netherlands). The minimum detection limit for estradiol was 18.35 pmol/L. Serum levels of total testosterone were measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS). The corresponding interassay coefficients of variations for total estradiol, SHBG and total testosterone are <7%, <5%, and <5%. Serum levels of dehydroepiandrosterone (DHEA), and dehydroepiandrosterone (DHEAS) were estimated in 12 batches by coated-tube or double-antibody radioimmunoassays, purchased from Diagnostic Systems Laboratories (Webster, TX, USA). Genotyping was conducted, in self-reported white participants, using the Illumina 550K array. We selected 54 SNPs previously reported to have an association with ANM from a GWAS of 70,000 women[21]. We calculated a weighted GRS by multiplying the number of risk alleles at each locus by the corresponding reported β coefficient from the previous GWAS and then summing the products. The total score was then divided by the average effect size multiplied by 100 to rescale the scores to a range between 0 and 100.

Statistical Analysis

Main analyses

Person years of follow-up were calculated from study entrance (March 1997- December 1999 for RSI-3, February 2000-December 2001 for RSII-1, and February 2006-December 2008 for RSIII-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 hazards models were used to evaluate whether ANM as continues and in categories (premature menopause: <40 years; early: 40-44 years, normal; 45-55 years and late menopause > 55 years (reference)) was associated with risk of T2D. Hazard ratios (HR) and 95% confidence intervals (95% CIs) were reported. The proportional hazard assumption of the Cox model was checked by the visual inspection of log minus log plots, and by performing a test for heterogeneity of the exposure over time. There was no evidence of violation of the proportionality assumption in any of the models (P for time-dependent interaction terms >0.05). To study the relations across increasing categories of ANM, trend tests were computed by entering the categorical variables as continues variables in multivariable Cox's proportional hazard models. To achieve normal distribu-

tions, skewed variables (DHEAS, testosterone, SHBG, CRP, TSH, TG and insulin) were natural log transformed. In the base model (Model 1), we adjusted for age, cohort (I, II and III), use of hormone replacement therapy, and reproductive factors (age at menarche and number of pregnancies of at least 6 months). To examine whether the relations of ANM with risk of T2D were independently of potential intermediate factors, model 2 included terms of model 1, body mass index (BMI) (continuous), glucose (continuous), and insulin (continuous). Model 3 included all covariates in model 2 and further potential confounding factors or intermediate factors: 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), smoking status (current vs. former/never) and physical activity (continuous)), education level (low, intermediate and high), and prevalent coronary heart disease (yes vs. no), and CRP (continuous). Moreover, to explore whether a nonlinear association was present, a quadratic term of the ANM (continuously) was tested.

Potential mediators

Levels of estradiol and other endogenous sex hormone levels, TSH and SHBG, as well as shared genetic (as assessed by genetic risk score of ANM) are suggested to explain the association between ANM and chronic diseases, including T2D[10]. Therefore, the models were further adjusted for these factors.

Sensitivity analyses

We performed a series of sensitivity analyses. Since waist circumference is a better measure of visceral adiposity, an important risk factor for diabetes, and because menopause is associated with accumulation of abdominal fat, we performed a sensitivity analysis substituting BMI with waist circumference[13]. To account for the specific effects of lipid particles on diabetes, we substituted total cholesterol with HDL-C, TG, and LDL-C. Also, we restricted the analysis among participants who did not report use of lipid-lowering medication. Parental history of diabetes was collected by trained research assistants during home visits at RSI and RSII, but not at RSIII. Therefore we further adjusted in the multivariable model for parental history of diabetes restricting the analysis in the first two cohorts of the RS (RSI-3 and RSII-1). Since smoking and hormone replacement therapy are important determinant of ANM and are associated with risk of T2D[22 23], we restricted the analysis among women who were not current smokers and did not report use of hormone replacement therapy. To explore the potential of survival bias, we stratified the analysis by baseline age (< 65 years and ≥65 years old). Also we rerun the analysis by excluding the first three years of follow up and by excluding the participants with prevalent cardiovascular disease. Moreover, we included women with non-natural menopause or menopause type non-known in the analysis to investigate the role of

both age at natural and non-natural menopause no the risk of T2D. There were missing values on one or more covariates (**Table 2.2.1**). Because the missing values were likely to be missing at random and for avoidance of loss in efficiency, missing values were imputed using a multiple imputation technique (5 imputation sets). No significant differences were observed in ANM or in incidence cases of T2D in subjects with complete information on all covariates (n=1884) compared to subjects who had missing values on at least one of the covariates included in model 3 (n=1755). Rubin's method was used for the pooled coefficients (HR) and 95% Confidence Intervals[24]. A *p*-value of less than 0.05 was considered as statistically significant. All analyses were done using SPSS statistical software (SPSS, version 21.0; SPSS Inc, Chicago, Illinois).

RESULTS

Table 2.2.1 summarizes the baseline characteristics of the women included in the analysis. The mean (standard deviation) age at entry in the study was 69.6 (9.6) years. The mean ANM was 50.0 (4.4) years and 2.3% and 7.6% of women experienced menopause before age of 40 and between ages 40 to 44, respectively (**Table 2.2.2**).

Of the 3,639 postmenopausal women without diabetes at baseline, 348 women developed incident T2D over a median follow-up of 9.2 years. Premature and early onset of natural menopause was associated with higher risk of T2D (**Table 2.2.2**). In model 1, the HRs for the association between ANM and T2D were 3.43 (95% CI 1.65–7.12), 2.00 (1.08–3.70), and 1.41 (0.82–2.41) for women with menopause at ages <40, 40–44, and 45–55, respectively, relative to those with menopause at age >55 years (*P*-trend <0.001, **Table 2.2.2**). The HR for T2D per one year older ANM was 0.96 (0.94–0.98) (**Table 2.2.2**). Controlling for BMI, glycaemic traits, metabolic risk factors, lifestyle factors, inflammatory markers, and prevalent cardiovascular disease did not affect this association (**Table 2.2.2**). Also, no evidence of nonlinear relationship was observed (*P*-quadratic term > 0.05, **Table 2.2.2**). In the analysis of potential mediators, further adjustment for endogenous sex hormones levels, SHBG, TSH or genetic risk score of ANM did not affect the association (**Table 2.2.3**).

Sensitivity analysis

In sensitivity analyses, substituting BMI with waist circumference as a measure of adiposity, substituting total cholesterol for other blood lipids, restricting analysis to subjects who did not report use of lipid-lowering medications, adjusting further for physical activity, serum TSH, total estradiol and other endogenous sex hormone levels, SHBG, or parental history of diabetes, excluding subjects with prevalent cardiovascular disease, and excluding the first three years of follow up did not affect the association between ANM and risk of T2D (**Table 2.2.3**). Also, results did not change when the analysis were

restricted only to women who were no current smokers or did not use hormone replacement therapy (**Table 2.2.3**). Furthermore, stratification by age did not show any difference in the results (**Table 2.2.3**). Although the results were attenuated after inclusion of women with non-natural menopause, the association between early age at (natural and non-natural) menopause and risk of T2D remained significant.

Table 2.2.1 Selected Characteristic of Study Participants, the Rotterdam Study.

	Women (N=3,639)
Age (years)	66.9 ± 9.6
Age of menopause (years)	50.0 ± 4.4
Number of pregnancies of at least 6 months	2.2 ± 1.4
Age of menarche, (years)	13.4 ± 1.7
Current smokers, n (%)	718 (19.7)
Alcohol intake g/day	2.9 (13.0)[a]
Low education, n (%)	544 (14.9)
Intermediate education, n (%)	2714 (74.6)
High education, n (%)	381 (10.5)
Body mass index (kg/m^2)	27.0 ± 4.4
Waist circumference (cm)	89.2 ± 11.6
Prevalent cardiovascular disease, n (%)	189 (5.2)
Physical activity (METhours/week)	82.5 ± 50.7
Total Estradiol (pmol/l)	30.2 (36.3)[a]
Total testosteron (nmol/l)	0.8 (0.5) [a]
Sex-hormon binding globuline (nmol/l)	60.7 (39.2) [a]
Dehydroepiandrosterone sulfate (nmol/l)	1649 (1533.8) [a]
Dehydroepiandrosterone (nmol/l)	9.6 (8.7) [a]
Androstenedione (nmol/l)	2.3 (1.4) [a]
Thyroid-stimulating hormone (mU/l)	2.0 (1.7)[a]
Hormone replacement therapy, n (%)	95 (2.6)
Insulin (pmol/l)	68 (47)[a]
Glucose (mmol/l)	5.4 ± 0.6
C-reactive protein (mg/ml)	1.6 (2.7)[a]
Total cholesterol (mmol/l)	6.0 ± 1.0
Low density lipoprotein cholesterol (mmol/l)	5.1 ± 1.2
High density lipoprotein cholesterol (mmol/l)	1.5 ± 0.4
Lipid-lowering medication use, n (%)	502 (13.8)
Triglycerides (moml/l)	1.3 (0.75) [a]
Systolic Blood pressure (mm/Hg)	139.1 ± 21.4
Antihypertensive medications, n (%)	1131 (31.1)
Incident type 2 diabetes, n (%)	348 (9.6)

Plus minus values are mean ± SD [a] Median (interquartile range)

Table 2.2.2 Associations of age at natural menopause with the risk of type 2 diabetes in postmenopausal women with natural menopause, the Rotterdam Study (N=3639)

Age at menopause	Women at risk/ Incident type 2 diabetes cases	Model 1 HR (95% CI)	Model 2 HR (95% CI)	Model 3 HR (95% CI)
Continuous	3639/348	0.96 (0.94; 0.98)	0.96 (0.94; 0.98)	0.96 (0.94; 0.99)
Premature menopause (<40 years)	83/15	3.65 (1.76; 7.6)	3.23 (1.56; 6.73)	3.06 (1.47; 6.37)
Early menopause (40-44 years)	298/39	2.37 (1.47; 3.84)	2.20 (1.19; 4.1)	2.13 (1.15; 3.95)
Normal menopause (45-55 years)	3015/280	1.62 (0.95; 2.78)	1.65 (0.96; 2.83)	1.60 (0.93; 2.74)
Late menopause (>55 years)	243/14	Reference	Reference	Reference
P-trend	3639/348	<0.001	<0.001	0.001
P-quadratic	3639/348	0.40	0.68	0.50

Model 1 included age at natural menopause (continuous or in categories), age (continuous), Rotterdam Study cohort (I, II and III), hormone replacement therapy (yes vs. no), age at menarche (continuous), number of pregnancies of at least 6 months (continuous). Model 2 included all variables in Model 1 and body mass index (continuous), glucose (continuous) and insulin (continuous). Model 3 included all variables of model 2 and total cholesterol (continuous), lipid lowering medications (yes vs. no), systolic blood pressure (continuous), antihypertensive medications (yes vs. no), alcohol intake (continuous), smoking (current vs. former/never), education (low, intermediate and high), prevalent cardiovascular disease (presence vs. non-present), physical activity (continuous) and C-reactive protein (continuous).

DISCUSSION

In this large population based study of postmenopausal women free of T2D at baseline, we showed that early onset of natural menopause is associated with increased risk of T2D, independently of potential intermediate risk factors for T2D, including body mass index, glucose, insulin, levels of estradiol and other endogenous sex hormone, and levels of SHBG. Also, we showed that shared genetic factors could not explain the association between ANM and risk of T2D.

While majority of studies have studied age at menopause with cardiovascular outcomes, reporting increased risk of cardiovascular disease associated with early onset of menopause, few studies have examined age at menopause with risk of T2D[3]. Cross-sectional studies examining the association between age at menopause and T2D have yielded conflicting results, showing no association or increased prevalence of T2D among women who experience early onset of menopause[5-7]. Similar to our findings, Brand and colleagues, in a nested case-cohort study, showed an increased risk of T2D with early onset of age at menopause, reporting similar size effects as the current investigation (HR of 0.93 per one year older age at menopause) [8]. However, we further extended their findings and showed that this association was independent of potential mediators, including endogenous sex hormone levels.

Table 2.2.3 Sensitivity analysis of age at natural menopause and the risk of type 2 diabetes in postmenopausal women, the Rotterdam Study.

	Age at natural menopause				
	Continuous[a]	**Premature menopause (<40 years)**	**Early menopause (40-44 years)**	**Normal menopause (45-55 years)**	**Late menopause (>55 years)**
	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
Multivariable model[b]	0.96 (0.94; 0.99)	2.98 (1.43; 3.84)	2.08 (1.12; 3.84)	1.55 (0.91; 2.67)	Reference
Multivariable model + waist circumference	0.96 (0.94; 0.99)	3.1 (1.49; 6.4)	2.11 (1.14; 3.92)	1.59 (0.93; 2.73)	Reference
Multivariable model + HDL + TG + LDL	0.96 (0.94; 0.98)	3.65 (1.59; 6.93)	2.17 (1.17; 4.03)	1.62 (0.94; 2.78)	Reference
Multivariable model + serum thyroid stimulating hormone	0.96 (0.94; 0.98)	3.04 (1.46; 6.33)	2.09 (1.13; 3.99)	1.60 (0.93; 2.74)	Reference
Multivariable model + DHEA	0.96 (0.94; 0.99)	3.25 (1.56; 6.77)	2.05 (1.10; 3.81)	1.56 (0.91; 2.69)	Reference
Multivariable model + total estradiol, total testosterone, sex hormone-bindings globulin	0.96 (0.94; 0.98)	3.08 (1.48; 6.42)	2.08 (1.12; 3.85)	1.60 (0.93; 2.75)	Reference
Multivariable model + DHEAS and androstenedione	0.97 (0.94; 0.99)	3.03 (1.45; 6.31)	2.12 (1.14; 3.93)	1.58 (0.92; 2.71)	Reference
Multivariable model + genetic risk score of age of natural menopause	0.97 (0.94; 0.99)	2.83 (1.34; 5.98)	2.00 (1.07; 3.75)	1.54 (0.89; 2.65)	Reference
Multivariable model excluding subjects with prevalent cardiovascular disease	0.96 (0.94; 0.99)	3.07 (1.43; 6.61)	1.86 (0.99; 3.48)	1.37 (0.79; 2.35)	Reference
Multivariable model excluding the first 3 years of follow-up	0.97 (0.95; 0.996)	3.05 (1.28; 7.24)	2.13 (1.03; 4.43)	1.75 (0.92; 3.31)	Reference
Multivariable model + parental history of diabetes[c]	0.97 (0.94; 0.99)	2.57 (1.16; 5.69)	2.06 (1.08; 3.91)	1.52 (0.87; 2.66)	Reference
Smoking status					
Former/never (n=2921)	0.97 (0.95; 0.997)	2.42 (1.01; 5.76)	2.25 (1.14; 4.42)	1.57 (0.88; 2.82)	Reference
Hormone replacement therapy					
Non-User (n=3544)	0.97 (0.94; 0.99)	3.00 (1.44; 6.25)	2.03 (1.09; 3.79)	1.57 (0.91; 2.69)	Reference
Lipid-lowering medication					
Non-User (n=3139)	0.97 (0.95-0.995)	2.68 (1.20; 5.95)	1.68 (0.87; 3.22)	1.41 (0.80; 2.49)	Reference
Baseline age of women					
<65 years old (n=1876)	0.95 (0.92; 0.99)	7.35 (2.11; 25.55)	3.86 (1.23; 12.15)	2.75 (1.00; 7.56)	Reference
≥ 65 years old (n=1763)	0.97 (0.94; 0.996)	1.89 (0.70; 5.02)	1.74 (0.83; 3.66)	1.29 (0.68; 2.46)	Reference

DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate;HDL, high density lipoprotein cholesterol; TG, triglycerides; LDL, low density lipoprotein cholesterol.

[a] Values are per 1 unit increase.

[b] Analysis restricted to third round of the first cohort and first round of second cohort (n=2541, 311 incident cases of type 2 diabetes); there were 218 participants reporting parental history of diabetes.

[c] Multivariable model included the following variables (model 3 in Table 2): age at natural menopause (continuous or in categories), age (continuous), Rotterdam Study cohort (I, II and III), hormone replacement therapy (yes vs. no), age at menarche (continuous), number of pregnancies of at least 6 months (continuous), body mass index (continuous), glucose (continuous) and insulin (continuous), total cholesterol (continuous), lipid lowering medications (yes vs. no), systolic blood pressure (continuous), antihypertensive medications (yes vs. no), alcohol intake (continuous), smoking (current vs. former/never), education (low, intermediate and high), prevalent cardiovascular disease (presence vs. non-present), physical activity (continuous) and C-reactive protein (continuous).

Early onset of natural menopause has been suggested to increase the risk of cardiometabolic diseases, including T2D, due to the early cessation of the protective effects of endogenous oestrogen[5]. Data from animal studies show that estradiol decreases adipose tissue and have a protective role in glucose metabolism[25 26]. Also, trials in postmenopausal women link oral oestrogen therapy with a lower risk of T2D among postmenopausal women[27-29]. In contrast, observational data do not support a protective effect of oestrogen in cardiometabolic health. In postmenopausal women, higher endogenous estradiol levels have been associated with higher levels of glucose and insulin, and an increase rather than a decrease in diabetes risk[30-33]. Moreover, an early start of oestrogen exposure (i.e., an early age at menarche) and a state of high endogenous estradiol have been linked with insulin resistance and increased risk of T2D[34-36]. This evidence, which is also supported by our study, suggests that other menopause-related factors may explain the association between age at menopause and risk of T2D. In the current study, we showed that neither levels of SHBG, nor androgen levels, both of which are associated with menopause and with T2D, could explain the association between early onset of natural menopause and risk of T2D. A possible explanation for the observed association between ANM and risk of T2D could be the disruption of the hypothalamus-pituitary-ovarian axis resulting in increased release of the gonadotropins and follicle-stimulating hormone by the pituitary gland. In our study we did not have levels of follicle-stimulating hormone. However, observational studies show that low levels of follicle-stimulating hormone rather than high levels, are associated with increased risk of T2D in postmenopausal women[10 37]. Also, lifestyle factors such as smoking and alcohol consumption are closely linked to age at menopause; e.g. smokers reach menopause on average 2 years earlier than non-smokers[22 38]. Therefore, the relationship between age at menopause and T2D is likely confounded by these factors. However, in our analysis we adjusted for both smoking and alcohol consumption, and restriction of analysis to women who did not currently smoke, did not have any impact on our results.

Moreover, we found that ANM was associated with T2D independently of glucose and insulin levels. Therefore, the mechanisms linking ANM with risk of T2D remain unclear and future studies are needed to explore potential pathways.

Recent data show that early ANM may be a marker of premature ageing and is related to pathways linked to longevity[21]. Also, ANM is associated with DNA damage repair, which is linked to risk of T2D[21 39 40]. Menopause, therefore, might be a marker of ageing of the soma[15]. In women equipped with the less efficient DNA repair and maintenance genes, soma might age faster compared to women with the more efficient repair and maintenance genes[15]. Hence, early menopause might be a consequence of ageing acceleration of the soma and might be a very good predictor of general health in later life, including T2D[15]. However, in our analysis we adjusted for shared genetic factors and the results did not change. Nevertheless, genome-wide association studies have identified approximately 56 single-nucleotide polymorphism (SNP) across human genome that explain only a minor fraction of the inter-individual variation in the age at menopause[21]. Epigenetic modifications - such as DNA methylation of cytosine residues in CpG dinucleotides and histone modifications might constitute an additional pathway leading to menopause onset and T2D. Future studies should explore epigenetic marks related to menopause onset and whether epigenetic signatures can explain the association between ANM and risk of T2D.

Strengths of our study include its prospective design, the long follow-up and adequate adjustments for a broad range of confounders and possible intermediate risk factors for T2D. Also, the diagnosis of incident diabetes was done by standardized blood glucose measurements at the repeated study centre visits and electronic linkage with pharmacy dispensing records in the study area. However, there are several limitations that need to be taken into account. A limitation is the reliance on retrospective self-report of ANM, which is subject to fault memory and reporting bias, particularly in older women. However, the results did no differ when we stratified by age at enrolment. Also, because the outcome (T2D incidence) was assessed prospectively, the subjective measure of ANM would likely lead to non-differential misclassification with respect to the outcome, and therefore would likely bias the estimates toward the null. Furthermore, there are studies reporting that the validity and reproducibility of self-reported age at menopause is fairly good[3 41]. Despite the prospective design, we cannot rule out that the observed associations may partially reflect unmeasured residual confounding or that diabetes can lead to early onset of menopause as suggested recently[42]. Survival bias may be present since women included in our study may represent survivors of early menopause who did not develop T2D or die prior to enrolment. Also, there is a time difference since stepping into menopause and the start of Rotterdam Study (mean age of years since menopause for women included in the analysis is 15.3 years). However, when we stratified by age of enrolment, we did not find any difference in the results. Furthermore, if survival bias would

be present, the true point estimate for the relationship between early menopause and T2D may be larger than we observed. Furthermore, all confounding factors and mediators considered in the current investigation were assessed years after menopause and not at time when menopause started, and estradiol was measured using an immunoassay with a detection limit of 18.35 pmol/L, which is considered suboptimal particularly in postmenopausal women. Therefore our results should be considered with caution. Also, the Rotterdam Study mainly includes individuals from European Ancestry (98%). Thus, our findings may not be extended to non-Caucasian ethnicities.

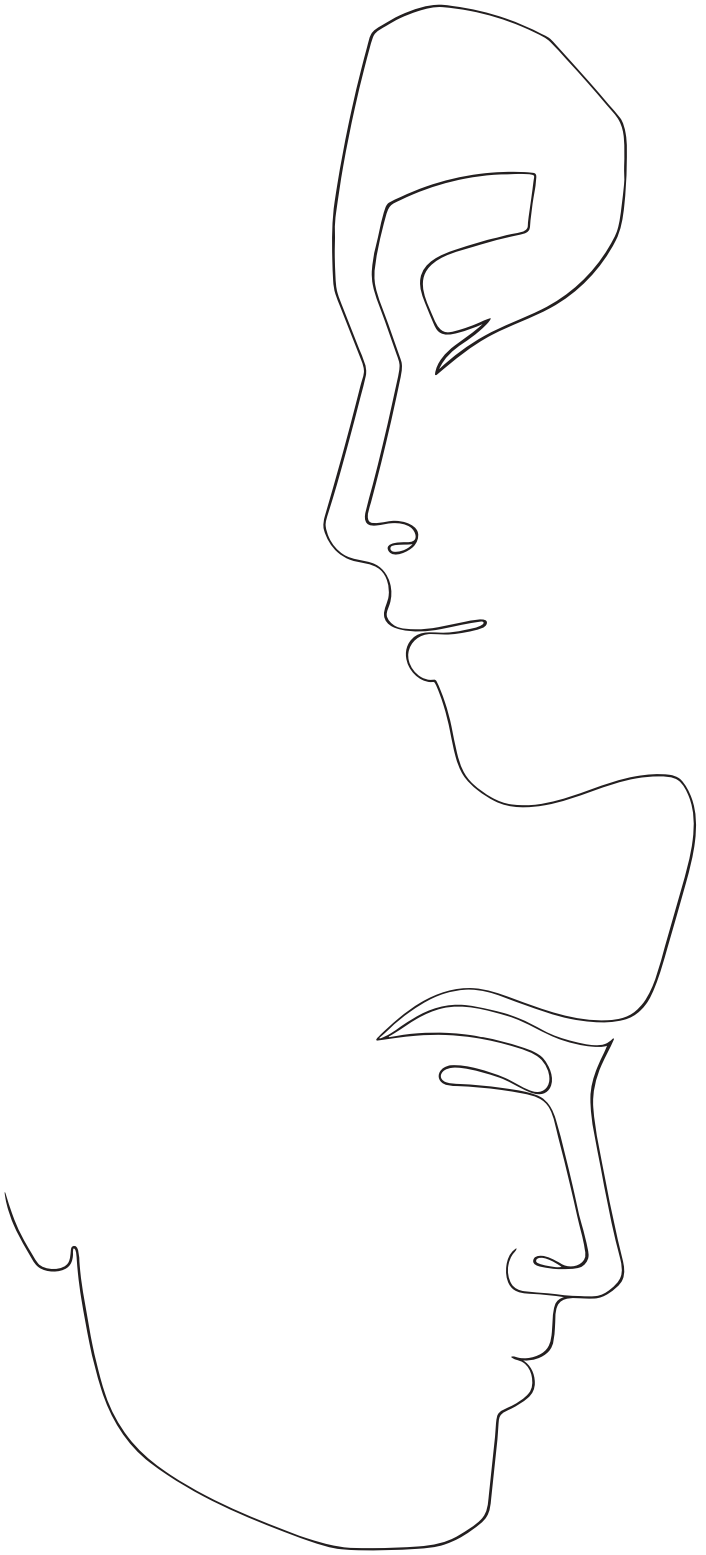
Early onset of ANM is an independent marker for T2D in postmenopausal women. Future studies are needed to examine the mechanisms behind this association and explore whether timing of natural menopause has any added value in diabetes prediction and prevention.

REFERENCES

1. Jaspers L, Daan NM, van Dijk GM, et al. Health in middle-aged and elderly women: A conceptual framework for healthy menopause. Maturitas 2015;**81**(1):93-8.
2. Gold EB. The timing of the age at which natural menopause occurs. Obstetrics & Gynecology Clinics of North America 2011;**38**(3):425-40.
3. Muka T, Oliver-Williams C, Kunutsor S, et al. 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. In press. Jama Cardiology 2016.
4. Canto JG, Iskandrian AE. Major risk factors for cardiovascular disease: debunking the "only 50%" myth. JAMA 2003;**290**(7):947-9.
5. Malacara JM, Huerta R, Rivera B, et al. Menopause in normal and uncomplicated NIDDM women: physical and emotional symptoms and hormone profile. Maturitas 1997;**28**(1):35-45.
6. Di Donato P, Giulini NA, Bacchi Modena A, et al. Risk factors for type 2 diabetes in women attending menopause clinics in Italy: a cross-sectional study. Climacteric 2005;**8**(3):287-93.
7. Luborsky JL, Meyer P, Sowers MF, et al. Premature menopause in a multi-ethnic population study of the menopause transition. Hum Reprod 2003;**18**(1):199-206.
8. Brand JS, van der Schouw YT, Onland-Moret NC, et al. Age at menopause, reproductive life span, and type 2 diabetes risk: results from the EPIC-InterAct study. Diabetes Care 2013;**36**(4):1012-9.
9. Liu Y, Ding J, Bush TL, et al. Relative androgen excess and increased cardiovascular risk after menopause: a hypothesized relation. Am J Epidemiol 2001;**154**(6):489-94.
10. Rocca WA, Shuster LT, Grossardt BR, et al. Long-term effects of bilateral oophorectomy on brain aging: unanswered questions from the Mayo Clinic Cohort Study of Oophorectomy and Aging. Womens Health (Lond) 2009;**5**(1):39-48.
11. Jaspers L, Dhana K, Muka T, et al. Sex Steroids, Sex Hormone-Binding Globulin and Cardiovascular Health in Men and Postmenopausal Women: The Rotterdam Study. J Clin Endocr Metab 2016;**101**(7):2844-52.
12. Muka T, Nano J, Jaspers L, et al. 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. . Diabetes, In press 2016.
13. Gambacciani M, Ciaponi M, Cappagli B, et al. Prospective evaluation of body weight and body fat distribution in early postmenopausal women with and without hormonal replacement therapy. Maturitas 2001;**39**(2):125-32.
14. Wu SI, Chou P, Tsai ST. The impact of years since menopause on the development of impaired glucose tolerance. Journal of Clinical Epidemiology 2001;**54**(2):117-20.
15. Laven JS, Visser JA, Uitterlinden AG, et al. Menopause: Genome stability as new paradigm. Maturitas 2016;**92**:15-23.
16. Hofman A, Brusselle GG, Darwish Murad S, et al. The Rotterdam Study: 2016 objectives and design update. Eur J Epidemiol 2015;**30**(8):661-708.
17. Leening MJ, Kavousi M, Heeringa J, et al. Methods of data collection and definitions of cardiac outcomes in the Rotterdam Study. Eur J Epidemiol 2012;**27**(3):173-85.
18. Wabnitz AM, Derdeyn CP, Fiorella DJ, et al. Hemodynamic Markers in the Anterior Circulation as Predictors of Recurrent Stroke in Patients With Intracranial Stenosis. Stroke 2018:STROKEAHA118020840.

19. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972;**18**(6):499-502.
20. Stel VS, Smit JH, Pluijm SM, et al. Comparison of the LASA Physical Activity Questionnaire with a 7-day diary and pedometer. Journal of Clinical Epidemiology 2004;**57**(3):252-8.
21. Day FR, Ruth KS, Thompson DJ, et al. Large-scale genomic analyses link reproductive aging to hypothalamic signaling, breast cancer susceptibility and BRCA1-mediated DNA repair. Nature Genetics 2015;**47**(11):1294-303.
22. Sun L, Tan L, Yang F, et al. Meta-analysis suggests that smoking is associated with an increased risk of early natural menopause. Menopause 2012;**19**(2):126-32.
23. Gold EB, Bromberger J, Crawford S, et al. Factors associated with age at natural menopause in a multiethnic sample of midlife women. Am J Epidemiol 2001;**153**(9):865-74.
24. Rubin, B. D. Multiple Imputation for Nonresponse in Surveys. Investigative Radiology 1987.
25. Godsland IF. Oestrogens and insulin secretion. Diabetologia 2005;**48**(11):2213-20.
26. Stubbins RE, Najjar K, Holcomb VB, et al. Oestrogen alters adipocyte biology and protects female mice from adipocyte inflammation and insulin resistance. Diabetes Obes Metab 2012;**14**(1):58-66.
27. Kanaya AM, Herrington D, Vittinghoff E, et al. Glycemic effects of postmenopausal hormone therapy: the Heart and Estrogen/progestin Replacement Study. A randomized, double-blind, placebo-controlled trial. Ann Intern Med 2003;**138**(1):1-9.
28. Margolis KL, Bonds DE, Rodabough RJ, et al. Effect of oestrogen plus progestin on the incidence of diabetes in postmenopausal women: results from the Women's Health Initiative Hormone Trial. Diabetologia 2004;**47**(7):1175-87.
29. Bonds DE, Lasser N, Qi L, et al. The effect of conjugated equine oestrogen on diabetes incidence: The Women's Health Initiative randomised trial. Diabetologia 2006;**49**(3):459-68.
30. Ding EL, Song Y, Manson JE, et al. Plasma sex steroid hormones and risk of developing type 2 diabetes in women: a prospective study. Diabetologia 2007;**50**(10):2076-84.
31. Kalyani RR, Franco M, Dobs AS, et al. The association of endogenous sex hormones, adiposity, and insulin resistance with incident diabetes in postmenopausal women. J Clin Endocrinol Metab 2009;**94**(11):4127-35.
32. Golden SH, Dobs AS, Vaidya D, et al. Endogenous sex hormones and glucose tolerance status in postmenopausal women. J Clin Endocrinol Metab 2007;**92**(4):1289-95.
33. Goodman-Gruen D, Barrett-Connor E. Sex differences in the association of endogenous sex hormone levels and glucose tolerance status in older men and women. Diabetes Care 2000;**23**(7):912-8.
34. Livingstone C, Collison M. Sex steroids and insulin resistance. Clin Sci (Lond) 2002;**102**(2):151-66.
35. Lakshman R, Forouhi N, Luben R, et al. Association between age at menarche and risk of diabetes in adults: results from the EPIC-Norfolk cohort study. Diabetologia 2008;**51**(5):781-6.
36. He C, Zhang C, Hunter DJ, et al. Age at menarche and risk of type 2 diabetes: results from 2 large prospective cohort studies. Am J Epidemiol 2010;**171**(3):334-44.
37. Wang N, Kuang L, Han B, et al. Follicle-stimulating hormone associates with prediabetes and diabetes in postmenopausal women. Acta Diabetol 2016;**53**(2):227-36.
38. Taneri PE, Kiefte-de Jong JC, Bramer WM, et al. Association of alcohol consumption with the onset of natural menopause: a systematic review and meta-analysis. Hum Reprod Update 2016;**22**(4):516-28.

39. Jackson SP, Bartek J. The DNA-damage response in human biology and disease. Nature 2009;**461**(7267):1071-8.
40. Shimizu I, Yoshida Y, Suda M, et al. DNA damage response and metabolic disease. Cell Metab 2014;**20**(6):967-77.
41. den Tonkelaar I. Validity and reproducibility of self-reported age at menopause in women participating in the DOM-project. Maturitas 1997;**27**(2):117-23.
42. Brand JS, Onland-Moret NC, Eijkemans MJ, et al. Diabetes and onset of natural menopause: results from the European Prospective Investigation into Cancer and Nutrition. Hum Reprod 2015;**30**(6):1491-8.



2.3

Age at natural menopause and life expectancy with and without type 2 diabetes

ABSTRACT

Objective

Effective interventions of future health-care require a better understanding of the health risks associated with early onset of menopause and diabetes, but the necessary data are scarce. Little quantitative information is available about the combined association of early menopause and diabetes on life expectancy and the number of years lived with and without diabetes.

Methods

We included 3,650 postmenopausal women aged 45+ years from the Rotterdam Study, a prospective population-based cohort study. Age at menopause categories were defined as: early (≤44 years old), normal (45-54 years old) and late (≥55 years old). For life table calculations, we used prevalence, incidence rates and hazard ratios for three transitions (free of diabetes to diabetes, free of diabetes to death and diabetes to death) stratifying by age at menopause categories and adjusting for confounders.

Results

Compared to late menopause, the difference in life expectancy for women who experienced early menopause was -3.5 95%CI: -6.6,-0.8 years overall and -4.6 95%CI: -8.9,-0.9 years without diabetes. Compared to age at normal menopause, the difference in life expectancy for women who experienced early menopause was -3.1 95%CI: -5.1,-1.1 years overall and -3.3 95%CI: -6.0,-0.6 years without diabetes.

Conclusion

Women who experienced early menopause lived less long and spent fewer years without diabetes than women who experienced normal or late menopause.

INTRODUCTION

Diabetes is one of the major causes of premature illness and death in most countries and imposes a substantial financial burden to society, especially in women [1 2]. Almost 1 in 2 women will develop type 2 diabetes (T2D) during their lifetime, and recent data has shown that the age-standardised prevalence of diabetes among adult women has increased in the past 30 years from 5.0% to 7.9% [3 4].

In women, T2D often manifests during mid-life and thus coincides with the timing of the menopausal transition [5]. Emerging evidence shows an association between age at menopause and diabetes with studies reporting almost a 2-fold increased risk of T2D with early onset of menopause [6 7]. Also, it is well established that early onset of menopause is associated with early death[8-10]. This could be important because, while mortality rates for women with non-T2D have declined over time [11], mortality rates for women with T2D may have instead increased [11].

Effective interventions and accurate projections of future health-care costs require a better understanding of the health risks associated with early onset of menopause, but the relevant data are scarce. To our knowledge, no study up to date has quantified (calculating the number of years lived with and without diabetes) the combined association of early menopause and T2D with life expectancy. Previous estimates reflecting the association of age at menopause with diabetes have been limited to absolute risks or lifetime risk without combining information about the number of the remaining years lived with or without diabetes, raising a gap in the intuitive understanding of risk and impact communicated among doctors and patients [6 7 12].

Two studies have examined the association of age at menopause with total life expectancy [13 14]. Ossewaarde and colleagues, in a breast cancer-screening cohort, reported that both premature and early onset of menopause were associated with a decrease in life expectancy of approximately 1 to 2 years [13]. Snowdon et al. concluded that each-one year decrease in age at menopause was associated with a 0.47-year decrease in the age at death in women with natural menopause before the age of 47 years [14]. However, these studies did not distinguish between life expectancy with and without diabetes and did not provide a direct observation of a well-defined population, as the results were obtained through modelling and simulation using nation-wide mortality data.

In a large population of postmenopausal women, we aimed to calculate and compare the association of age at natural menopause with total life expectancy and the number of years lived with and without T2D.

METHODS

Population for analyses

The Rotterdam Study (RS) is a population-based prospective cohort study of individuals aged 45years and over, living in Rotterdam, the Netherlands. The rationale and design of the RS have been previously described [15].

The present study used data from the third visit of the first cohort, RSI-3 (March 1997-December 1999) and the baseline examinations of the second, RSII-1 (February 2000-December 2001) and third cohort, RSIII-1 (February 2006-December 2008). Information about the visits and participants used for this study are presented in Supplemental Digital Content Figure S1. A total of 6816 women were eligible for the analysis. Women who were not postmenopausal (N=741), had missing information on age of menopause (N=145), experienced non-natural (N=1408) or unknown type of menopause (N=20) were excluded from the analyses. Furthermore, we excluded 434 women who reported using oral contraceptives during the menopause transition, since these may mask or influence the onset of menopause [16] and are associated with an increased risk of T2D in postmenopausal women [17]. The remaining 3650 postmenopausal women were eligible for the analyses (**Figure 2.3.1**).

Study population

The Rotterdam Study (RS) is a population-based prospective cohort study ongoing since 1990 in the city of Rotterdam in The Netherlands. Potential participants aged 55 years and over were invited in random clusters. Names and addresses were drawn from the municipal register which is reliable, complete and up to date. The baseline cohort (RSI) included 7983 participants (78% of 10,215 invitees)[18]. Over the years, two more rounds were held. The first, in 2000–2001, included all inhabitants aged 55 years and over, recruiting 3011 participants (out of 4472 invitees) (RSII)[18]. The second extension initiated in 2006, included 3932 participants aged 45 years and over, out of 6057 invited (RSIII)[18]. Detailed information about the visits and participants of the Rotterdam study are presented in Supplemental Digital Content Figure S1. The overall response figure for all three cycles was 72.0% (14,926 of 20,744). RS complies with the Declaration of Helsinki and has been approved by the Medical Ethics Committee of the Erasmus Medical Centre and complies with the Dutch Ministry of Health, Welfare and Sport. All participants provided written informed consent to obtain and process data from their treating healthcare providers.

Assessment of age at menopause

The self-reported age at menopause was assessed during the baseline interview using a questionnaire. Age at menopause was defined in retrospect as the age at final menstrual

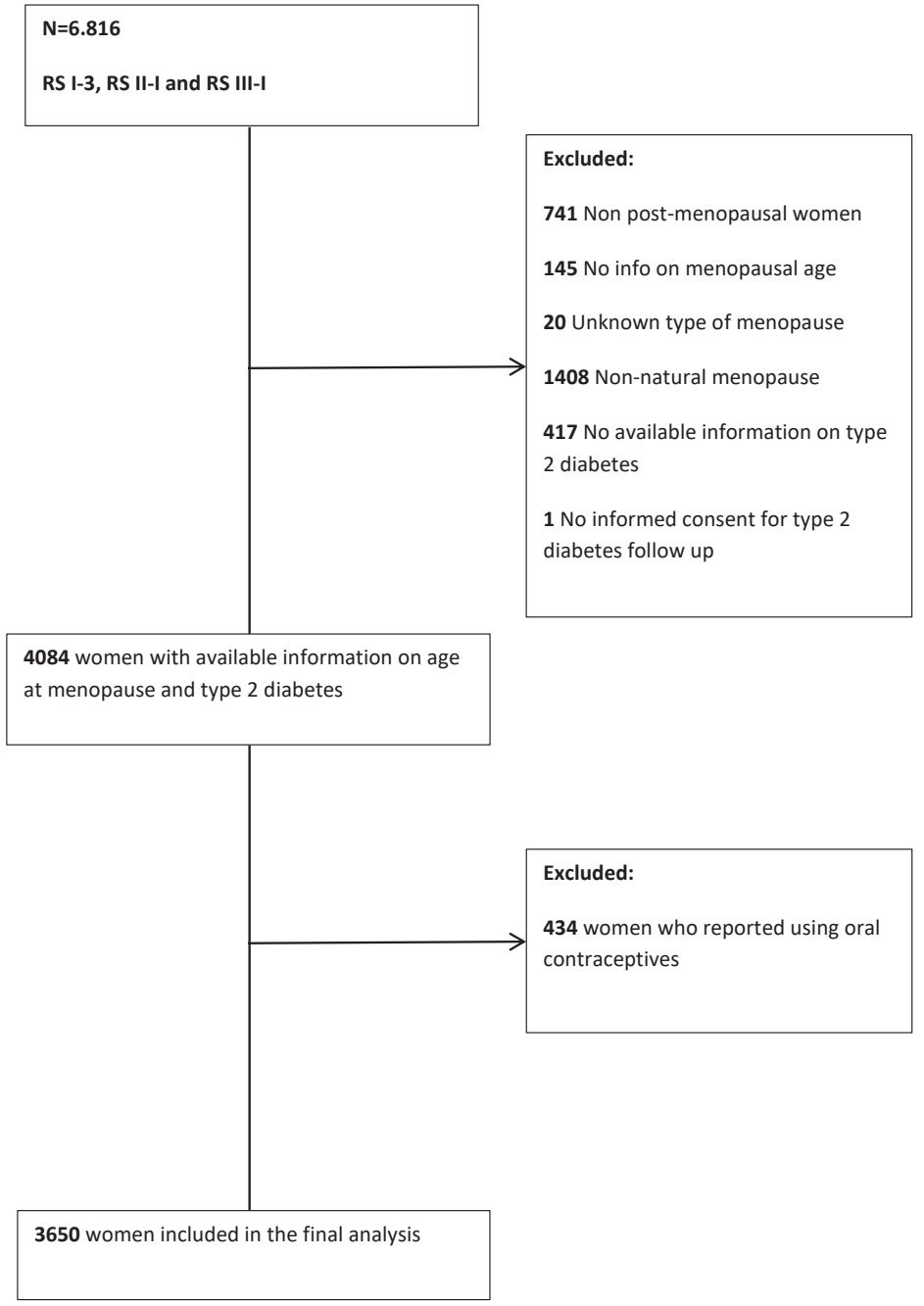


Figure 2.3.1. Flow Chart of Participants in the Study, the Rotterdam Study (1997-2012).

period, which was followed by cessation of menses that lasted at least 12 months [19]. In addition, to define the nature of menopause, women were asked to report if bleeding was ceased naturally or was ceased because of other reason. For all women reporting menopause after gynaecologic surgery or radiation therapy and for those reporting any other operations that might have led to menopause, information on the exact date and type of operation was verified using general practitioners' records.

Ascertainment of type 2 diabetes

The participants were followed from the date of baseline centre visit onwards. Cases of T2D were ascertained at baseline and during follow-up through: (i) active follow-up using general practitioners' records, (ii) hospital discharge letters and (iii) glucose measurements from RS visits that took place approximately every 4 years [20]. T2D was defined according to recent WHO guidelines, as a fasting blood glucose ≥ 7.0 mmol/L, a non-fasting blood glucose ≥ 11.1 mmol/L (when fasting samples were absent), or the use of glucose-lowering medication. Information regarding the use of glucose-lowering medication was derived from both structured home interviews and linkage to pharmacy records [20]. Two study physicians independently adjudicated all potential events of T2D. In case of disagreement, consensus was sought with an endocrinologist. Follow-up data were through January 1st 2012.

Follow-up for mortality

Mortality data were obtained by notification from the municipal administration. Participants were followed from the first day they entered the study till the day of death, the day of lost to follow-up or the last date of contact, whichever came first. Data on all-cause mortality and living status were updated biweekly until August 1st 2016. The method applied requires all outcomes to have the same end date. In our data, the follow up for diabetes was until January 1st 2012, therefore all the analyses were truncated accordingly.

Assessment of potential confounders

Based on previous literature[7 10 16 17 21-24], biological plausibility and data availability in the RS, potential confounding variables (including age, smoking, alcohol, education level, hormone therapy, physical activity, age at menarche, number of pregnancies and oral contraceptive use) were selected for the analyses. Also, to account for any potential effect of the cohorts in the RS, we additionally adjusted all our analyses for cohort (I, II and III). Information on current health status, medical history, medication use and smoking behaviour was obtained at baseline (RSI-3, RSII-1 and RSIII-1). Participants were asked whether they were currently smoking cigarettes, cigars, or pipes. Alcohol intake was assessed in grams of ethanol per day. Education was defined according to the standard

international classification of education as low (primary education), intermediate (secondary general or vocational education), or high (higher vocational education or university). Data on age at menarche were collected by asking women, "How old were you when you had your first menstrual period?". The retrospective data on self-reported number of pregnancies and use of hormone therapy were collected by a questionnaire during the home interview. Physical activity was assessed using the LASA Physical Activity Questionnaire (LAPAQ) and is expressed in METhours/week.

Data analysis

Data are presented as mean (± SD) for normally distributed continuous variables and median (range) for continuous variables that are not normally distributed. When fitting the models and checking for multicollinearity, in our analyses variance inflation factor (VIF) was lower than 3, suggesting no evidence of collinearity. The One-way ANOVA test (for continuous variables) and $\chi 2$ (for categorical variables) were used to compare parameters between the groups. We created population-based multistate life tables to calculate life expectancy and years lived with and without T2D in early (≤44 years old), normal (45-54 years old) and late menopause (≥55 years old, reference) categories. The multistate life table is a demographic tool that allows the experience of individuals in different health states to be combined in order to calculate the total life expectancy and the amount of years that individuals could expect to live in the different health states. We considered 3 different health states: free of T2D, T2D, and death. Participants could experience the following transitions: from free of T2D to T2D or death and from T2D to death. No backflows were allowed (eg. from having T2D to not having T2D), and only the first entry into a state was considered.

To obtain transition rates, we first calculated the overall age-specific rates for each transition. Next, we calculated the prevalence of early, normal, and late menopause by 10-year age groups, and separately for women with and without diabetes. Hazard ratios (HRs) comparing women who experienced early and normal menopause to women who experienced late menopause were calculated using Poisson regression ("Gompertz" distribution) in two models. In model 1 we adjusted for age and cohort. In model 2 we additionally adjusted for potential confounders including: smoking status (current smokers vs former/ever smokers), alcohol intake (continuous), education level (low, intermediate and high), physical activity (continuous), use of oral contraceptives (yes vs. no), use of hormone therapy, and reproductive factors (age at menarche and number of pregnancies of at least 6 months). Finally, we calculated three sets of transition rates for each menopausal age category using (i) the overall transition rates, (ii) the adjusted HRs for T2D and mortality, and (iii) the prevalence of age at natural menopause categories by presence of T2D. Similar calculations have been described previously [25 26]. Considering the age range of participants in RS and the small number of participants between 45 to

50 years old (N=34) we a priori decided to start the multistate life tables at the age of 50 years. We used Monte Carlo simulation (parametric bootstrapping) with 10,000 runs to calculate the 95 % confidence intervals of our life expectancy estimates with @RISK software (Palisade Corporation, Ithaca, New York) runs [27]. To deal with missing values, we used multiple imputation in SPSS (IBM SPSS Statistical for Windows, Armonk, New York: IBM). To calculate the HRs and the transition rates we used STATA version 12 for Windows (StataCorp, College Station, Texas).

Sensitivity analyses

Several sensitivity analyses were performed. To examine the impact of women who reported use of oral contraceptive at onset of menopause, we included them in the analyses (n=434). Also, to investigate both, the association of natural and non-natural menopause with T2D risk and life expectancy, we included in the analyses women with non-natural menopause. Furthermore, to explore whether there were significant differences between early and normal age at menopause in risk of T2D and mortality, life expectancy, and years lived with and without T2D, we repeated the analysis using normal age at menopause category as reference.

RESULTS

Baseline characteristics

Mean age at menopause in the early, normal and late categories were respectively 41years (SD = 3.0), 50 years (SD=2.5) and 56 years (SD=1.6) (**Table 2.3.1**). Compared to women who experienced late menopause, early menopausal women had lower education levels, drank less and were more likely to smoke (**Table 2.3.1**).

Diabetes events and death

Of the 3240 postmenopausal women free of diabetes at baseline, 305 women developed incident T2D over a median follow-up of 9.2 years (**Table 2.3.2**). Among women free of diabetes, 489 women died during the follow-up (median= 6.9 years), whereas 164 women died among women with T2D (median follow-up= 4.9 years). Both models yielded similar estimates; therefore, we further report the results of the most adjusted model (model 2). Compared to late menopause, early (HR=1.42, 95% CI: 1.01, 2.00), but not age at normal menopause (HR=1.13, 95% CI: 0.85, 1.49) was associated with an increased risk of mortality among women free of diabetes. Among diabetic women with early and normal menopause, the HRs for mortality were 1.64 95% CI: 0.93, 2.88, and 0.85 95% CI: 0.52, 1.39 respectively, relative to those with late menopause (**Table 2.3.2**).

Table 2.3.1. Baseline Characteristics of 3.650 Women by Age at Menopause Categories, the Rotterdam Study (1997-2012).

	Categories of age at menopause*		
Characteristics	**Early (n= 414)**	**Normal (n=2787)**	**Late (n= 449)**
Age, mean (SD), y	69.0 (10.6)	67.4 (9.8)	67.9 (8.6)
Age at menopause, mean (SD)	40.8 (3.0)	50.2 (2.5)	56.0 (1.6)
Type 2 Diabetes, Yes (%)	54 (13.0)	304 (10.9)	52 (11.6)
Age at menarche, mean (SD)	13.4 (1.9)	13.4 (1.7)	13.6 (2.0)
Education, No. (%)			
Primary	97 (23.4)	427 (15.3)	55 (12.2)
Lower	200 (48.3)	1451 (52.1)	234 (52.1)
Intermediate	88 (21.3)	622 (22.3)	108 (23.0)
Higher/university	29 (7.0)	287 (10.3)	52 (11.6)
Smoking Status, Yes. (%)	119 (28.7)	539 (19.3)	54 (12.0)
Alcohol, mean (SD), g/day	8.7 (11.9)	9.5 (12.5)	11.4 (13.6)
Physical activity (METhours/week)	81.4 (44.4)	85.3 (50.4)	83.5 (43.7)
Hormone replacement therapy (HRT), Yes (%)	9 (2.2)	73 (2.6)	9 (2.0)
Oral contraceptive use, yes (%)	190 (45.9)	1464 (52.5)	230 (51.2)
Number of pregnancies, mean (SD)	2.5 (2.3)	2.3 (1.9)	2.3 (1.8)
Total cholesterol, mean (SD), mg/dl	5.9 (1.0)	5.9 (0.9)	6.0 (1.0)
Statin use, Yes (%)	57 (13.8)	420 (15.1)	59 (13.1)
Systolic blood pressure (SBP), mean (SD)	138.8 (21.2)	140.3 (21.6)	140.7 (21.7)
Anti-hypertensive medication, Yes (%)	160 (38.6)	919 (33.0)	173 (3805)
Estradiol, median (IQR)	32.7 (323)	33.6 (1066.9)	36.3 (442.1)
Total testosterone, median (IQR)	0.8 (5.7)	0.8 (12.5)	0.8 (18.5)
SHBG, median (IQR)	57.1 (190.8)	59.8 (189.7)	58.8 (157.6)
DHEA, median (IQR)	8.2 (50.7)	9.5 (67.3)	9.6 (82.5)
TSH, median (IQR)	2.0 (58.7)	2.0 (70.6)	2.0 (74.4)
Glucose, mean (SD)	5.8 (1.6)	5.8 (1.4)	5.8(1.3)
Insulin, mean (SD)	105.0 (157)	91.5 (101.6)	96.6 (106.3)
Body mass index, BMI, mean (SD), kg/m^2	27.6 (4.9)	27.3 (4.5)	28.1 (4.8)
C-reactive protein, CRP, mean (SD)	3.7 (6.1)	3.1 (5.3)	3.3 (7.1)
Prevalent Cancer, Yes (%)	39 (9.4)	282 (10.1)	44 (9.8)
Prevalent COPD, Yes (%)	33 (8.0)	148 (5.3)	15 (3.3)
Prevalent CVD, Yes (%)	40 (9.7)	215 (7.7)	26 (5.8)

*Menopause categories are defined as: Early ≤44 years old, Normal 45-54 and Late ≥55years old.

Table 2.3.2. Hazard Ratios by Transition Among Categories of Age at Menopause.*

Transition	No. Of Cases	Person-years	Age at Menopause Categories	HR†	95% CI	HR‡	95% CI
Incident T2D	305	28448	Late	1.00		1.00	
			Normal	1.22	0.84,1.78	1.14	0.78,1.67
			Early	1.89	1.20,2.97	1.69	1.07,2.67
No T2D to Death	489	26229	Late	1.00		1.00	
			Normal	1.16	0.88,1.54	1.13	0.85,1.49
			Early	1.49	1.05,2.09	1.42	1.01,2.00
T2D to Death	164	5213	Late	1.00		1.00	
			Normal	0.91	0.56,1.48	0.85	0.52,1.39
			Early	1.73	0.99,3.00	1.64	0.93,2.88

Abbreviations: HR, Hazard Ratio; CI, Confidence Interval; T2D, Type 2 Diabetes.
*Age 50 and over at start of follow-up
† Adjusted for age and cohort
‡ Adjusted for age, cohort, smoking, alcohol, education, PA, OC, HRT, menarche, nr of pregnancies.

Table 2.3.3. Differences of age at menopause categories on life expectancy.

Age at menopause categories	Difference in total LE		Difference in LE free of DM		Difference in LE with DM	
	HR	95% CI	HR	95% CI	HR	95% CI
Early vs. Late	-3.5	-6.6,-0.8	-4.6	-8.9,-0.9	1.1	-1.8,4.4
Normal vs. Late	-0.5	-2,1,1.3	-1.3	-4.3,0.8	0.8	-1.3,2.4
Early vs. Normal	-3.1	-5.1,-1.1	-3.3	-6.0,-0.6	0.2	-2.0,2.8

Total life expectancy and life expectancy with and without type 2 diabetes

The association of early, normal, and late menopause with the risk of each health/disease/death transition was translated into the number of years lived with and without diabetes (**Figure 2.3.2**). Total life expectancy at the age of 50 years was lower in women who had an early menopause and higher in women who had a late menopause. Compared to women with late menopause, the difference in life expectancy for women with normal and early age at menopause was 0.5 95% CI: -2.1, 1.3 and 3.5 95% CI: -6.6, -0.8 years, respectively. Compared to women with late menopause, the difference in life expectancy for women with normal and early age at menopause was 1.3 95% CI: -4.3, 0.8 and 4.6 95% CI: -8.9,-0.9 years without T2D as well as 0.8 years 95% CI: -1.3, 2.4 and 1.1 years 95% CI: -1.8, 4.4 years with T2D, respectively.

Sensitivity analyses

Total life expectancy and the number of years lived with and without T2D did not significantly differ after including women who reported using oral contraceptives. These results are presented in Supplemental Digital Content Table S1, Table S2, Figure S2. The

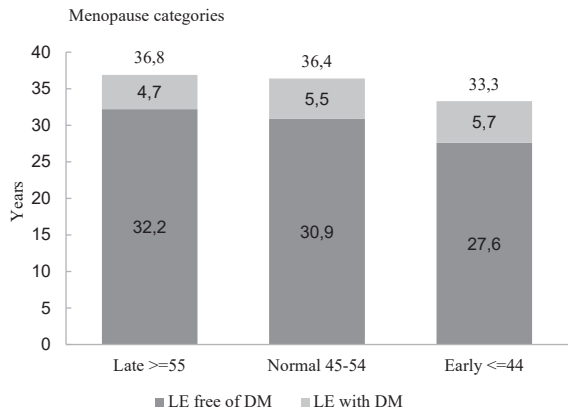


Figure 2.3.2. LE at 50 Years, Among Women With Late, Normal and Early Menopause.
Abbreviations: HR, hazard ratio; LE life expectancy; T2D type 2 diabetes
*Results after adjusted for: age, cohort, education levels, alcohol, smoking, menarche, HRT, physical activity, number of pregnancies and OC use.
**Late menopause group is the reference category.

results were attenuated, but did not substantially change after the inclusion of women with non-natural menopause (results are shown in Supplemental Digital Content, Table S4 and Figure S3). Furthermore, the difference in life expectancy for women who experienced early menopause, compared to women with normal age at menopause was -3.1 95% CI: -5.1,-1.1) and -3.3 95% CI: -9.5, -0.6 years overall and without T2D respectively, and 0.2 95% CI: -2.0,2.8 years with T2D, although the latter was not a statistically significant difference. Results using the normal menopause group as the reference category are presented in Supplemental Digital Content.

DISCUSSION

At the age of 50 years, women who experienced early menopause lived less years and spent fewer years without diabetes than women who experienced normal or late menopause. Compared to women with normal or late age at menopause, women with early menopause lived at least 3.1 years fewer overall and at least 3.3 years fewer without diabetes, respectively.

The decreased life expectancy without diabetes among women with early menopause might be due to the increased risk of T2D and mortality associated with early menopause. The higher risk of diabetes in women with early age at natural menopause might reflect an earlier diagnosis of diabetes across the lifespan and therefore, a decreased life expectancy without T2D, although the difference in years lived overall and without T2D did not differ significantly. Furthermore, early menopause was also associated with an

increased mortality risk among participants without T2D, resulting in a further decrease in total life expectancy and number of years lived without T2D. The number of years lived with diabetes was a result of incident diabetes risk and mortality risk among those with diabetes. In our study, no significant association was observed of age at menopause with mortality among women with diabetes, which could be due to the small number of cases in this transition (transition 3). Life expectancy with diabetes was at least 0.2 and 1.1 years more for women with early age at menopause compared with those who experienced normal and late menopause respectively. This reflects two opposing results: 1) higher incidence of diabetes in women who experienced early menopause increasing the time spent with diabetes; and 2) increased mortality associated with early menopause in diabetic patients, decreasing the time spent with diabetes. The net result is that women who experienced early menopause lived shorter and did not spend more years with diabetes.

In our study, total life expectancy in women aged 50 years and over was very similar between normal and late menopause and significantly decreased in the early menopause group. Consistent with our findings, data from a breast cancer screening cohort reported that a later menopause is associated with a longer overall survival and higher life expectancy [13]. In that study, women who experienced menopause at the age of 55 years or after lived 2 and 1 years longer than those who experienced menopause before the age of 40 and at age 40-44 respectively, which is slightly less than our findings. This discrepancy could be explained by differences in: (i) age of participants; (ii) categorization of age at menopause and (iii) in the calendar time of baseline measurements. Also, our study included only women who experienced natural menopause, whereas the previous study included women experiencing natural and not-natural menopause. Furthermore, in the previous study, the calculations for the life tables were made using a hypothetical population of women aged 50 years, obtaining estimates by modelling and simulation. In contrast, our study calculated the life expectancy with and without diabetes from direct observation of a well-defined population using multistate life tables.

Previous studies have reported that among major modifiable risk factors, never smoking was associated with the largest gain in total life expectancy in women (up to 4.1 years), followed by high physical activity (3.4 years) and normal weight (1.0 year) [28]. In our study, adjustment for these factors to rule out their influence on the association between age at menopause and T2D did not materially change the results. Previous studies investigating the association of age at menopause and mortality or diabetes, have also reported no changes in estimates when adjusting for smoking [7 13], suggesting that smoking habits could not explain our findings. However, it would be of interest to explore whether smoking and early menopause are related through epigenetic mechanisms which can further affect women's health later in life.

To our knowledge, this is the first study that investigated the association of age at natural menopause with life expectancy with and without T2D. Other major strengths include the prospective design with a long-term follow-up; the large number of participants and the adjustments for a broad range of potential confounders.

Several limitations of this study should be addressed. Age at menopause was assessed by questionnaires that could be subject to some measurement error. Another limitation was the reliance on retrospective self-report of age at menopause that could be subject to memory and reporting bias, particularly in older women. In addition, inaccuracy from reporting bias would have been non-differential in relation to the menopausal categories. Hence, it is unlikely that this would have created any difference between groups. However, because T2D and mortality were assessed prospectively, the subjective measure of age at menopause would likely lead to non-differential misclassification with respect to the outcome, and therefore would likely bias our estimates toward the null. Furthermore, studies have reported that the validity and reproducibility of self-reported age at menopause is fairly good [8 29].

Moreover, diabetes might have been subclinical at baseline and because age at menopause was retrospectively self-reported, the possibility of reverse causality should be considered. However, all women in our study were already postmenopausal years before incident diabetes occurred. Also, all women in RS had an assessment of fasting plasma glucose which would have helped to detect subclinical diabetes and therefore reverse causality is less likely to have happened.

Further, a time difference was observed between the start of the RS and the onset of menopause, which might have introduced immortal-time bias. Nevertheless, if immortal-time bias was present, the true point estimate for the relationship between early menopause, T2D and mortality may be larger than we observed. Also, all population-based cohorts involving active participation are subject to the healthy volunteer effect [30] therefore the mortality rates in our study might be lower than those in the general population, thus leading to underestimation of the results [31]. Finally, the generalizability of our findings can be limited to middle-aged and older white European populations, and therefore, our results need confirmation in other populations.

These results support findings of prior studies that suggest that age at natural menopause might be a risk factor for mortality. Another hypothesis might be that both early menopause and premature death could be associated by a third factor, which may also partly explain the underlying mechanisms of the greater risk of T2D in women with early menopause. Epigenetic modifications such as DNA methylation of cytosine residues in CpG dinucleotides histone modification and micro RNAs might constitute an additional pathway linking the timing when menopause occurs with longevity and type 2 diabetes [32]. Future studies should explore epigenetic modifications associated with age at natural menopause and whether epigenetic signatures can explain the association between

the onset of natural menopause with mortality and type 2 diabetes risk. In addition, our results provide supporting evidence for public health interventions aiming at delaying in menopause to reduce the incidence of T2D, postpone mortality and prolong total life expectancy as well as the years lived free of diabetes. Studies have shown that various lifestyle factors [22] such as smoking cessation [23 33], low to moderate alcohol consumption [23], better nutrition and lower body mass index [21 34], better socioeconomic status [22], are associated with a later menopause. Considering the observational nature of the studies so far, it is unclear whether these factors have a causal effect on age of menopause. Therefore, more research is needed to understand the direction of these associations to help define better health policies. Also, our findings might be important for the health care sector since diabetes poses a significant financial burden on healthcare and nations' welfare budget [35].

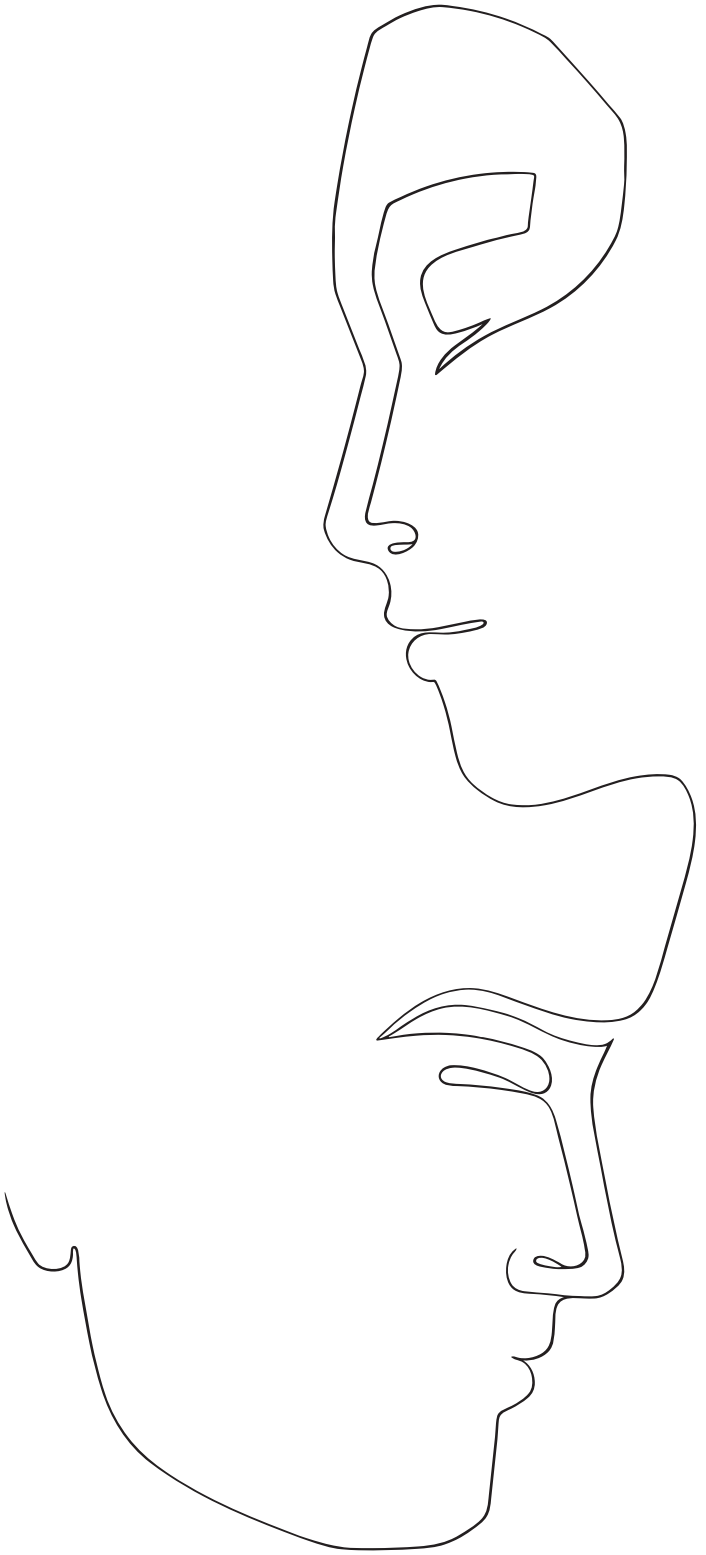
CONCLUSIONS

In our study, women who experienced early menopause lived fewer years overall and spent less years without diabetes than women who experienced normal or late menopause. Future studies are needed to examine the mechanisms behind the association of age at natural menopause with type 2 diabetes and mortality, in order to tailor prevention and treatment strategies to improve women's health across all age-categories of menopause.

REFERENCES

1. Williams JS, Bishu K, Dismuke CE, et al. Sex differences in healthcare expenditures among adults with diabetes: evidence from the medical expenditure panel survey, 2002-2011. Bmc Health Serv Res 2017.
2. Zhuo XH, Zhang P, Barker L, et al. The Lifetime Cost of Diabetes and Its Implications for Diabetes Prevention. Diabetes Care 2014;**37**(9):2557-64.
3. Narayan KM, Boyle JP, Thompson TJ, et al. Lifetime risk for diabetes mellitus in the United States. JAMA 2003;**290**(14):1884-90.
4. Zhou B, Lu Y, Hajifathalian K, et al. Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4.4 million participants. Lancet 2016;**387**(10027):1513-30.
5. Karvonen-Gutierrez CA, Park SK, Kim C. Diabetes and Menopause. Curr Diab Rep 2016;**16**(4):20.
6. Brand JS, van der Schouw YT, Onland-Moret NC, et al. Age at menopause, reproductive life span, and type 2 diabetes risk: results from the EPIC-InterAct study. Diabetes Care 2013;**36**(4):1012-9.
7. Muka T, Asllanaj E, Avazverdi N, et al. Age at natural menopause and risk of type 2 diabetes: a prospective cohort study. Diabetologia 2017;**60**(10):1951-60.
8. Muka T, Oliver-Williams C, Kunutsor S, et al. Association of Age at Onset of Menopause and Time Since Onset of Menopause With Cardiovascular Outcomes, Intermediate Vascular Traits, and All-Cause Mortality: A Systematic Review and Meta-analysis. JAMA Cardiol 2016;**1**(7):767-76.
9. Jacobsen BK, Knutsen SF, Fraser GE. Age at natural menopause and total mortality and mortality from ischemic heart disease: The Adventist health study. J Clin Epidemiol 1999;**52**(4):303-07.
10. Gold EB. The Timing of the Age at Which Natural Menopause Occurs. Obstet Gyn Clin N Am 2011;**38**(3):425-+.
11. Preis SR, Hwang SJ, Coady S, et al. Trends in All-Cause and Cardiovascular Disease Mortality Among Women and Men With and Without Diabetes Mellitus in the Framingham Heart Study, 1950 to 2005. Circulation 2009;**119**(13):1728-U65.
12. Epstein RM, Alper BS, Quill TE. Communicating evidence for participatory decision making. JAMA 2004;**291**(19):2359-66.
13. Ossewaarde ME, Bots ML, Verbeek ALM, et al. Age at menopause, cause-specific mortality and total life expectancy. Epidemiology 2005;**16**(4):556-62.
14. Snowdon DA. Early natural menopause and the duration of postmenopausal life. Findings from a mathematical model of life expectancy. J Am Geriatr Soc 1990;**38**(4):402-8.
15. Hofman A, Brusselle GGO, Murad SD, et al. The Rotterdam Study: 2016 objectives and design update. European Journal of Epidemiology 2015;**30**(8):661-708.
16. de Vries E, den Tonkelaar I, van Noord PAH, et al. Oral contraceptive use in relation to age at menopause in the DOM cohort. Human Reproduction 2001;**16**(8):1657-62.
17. Kim SW, Jeon JH, Lee WK, et al. Long-term effects of oral contraceptives on the prevalence of diabetes in post-menopausal women: 2007-2012 KNHANES. Endocrine 2016;**53**(3):816-22.
18. Ikram MA, Brusselle GGO, Murad SD, et al. The Rotterdam Study: 2018 update on objectives, design and main results. Eur J Epidemiol 2017;**32**(9):807-50.
19. Harlow SD, Gass M, Hall JE, et al. Executive summary of the Stages of Reproductive Aging Workshop + 10: addressing the unfinished agenda of staging reproductive aging. J Clin Endocrinol Metab 2012;**97**(4):1159-68.
20. Leening MJ, Kavousi M, Heeringa J, et al. Methods of data collection and definitions of cardiac outcomes in the Rotterdam Study. Eur J Epidemiol 2012;**27**(3):173-85.

21. Dorjgochoo T, Kallianpur A, Gao YT, et al. Dietary and lifestyle predictors of age at natural menopause and reproductive span in the Shanghai Women's Health Study. Menopause 2008;**15**(5):924-33.
22. Hardy R, Kuh D. Social and environmental conditions across the life course and age at menopause in a British birth cohort study. Bjog-Int J Obstet Gy 2005;**112**(3):346-54.
23. Kinney A, Kline J, Levin B. Alcohol, caffeine and smoking in relation to age at menopause. Maturitas 2006;**54**(1):27-38.
24. Olsson L, Ahlbom A, Grill V, et al. High Levels of Education Are Associated With an Increased Risk of Latent Autoimmune Diabetes in Adults Results from the Nord-Trondelag Health Study. Diabetes Care 2011;**34**(1):102-07.
25. Franco OH, de Laet C, Peeters A, et al. Effects of physical activity on life expectancy with cardiovascular disease. Arch Intern Med 2005;**165**(20):2355-60.
26. Franco OH, Steyerberg EW, Hu FB, et al. Associations of diabetes mellitus with total life expectancy and life expectancy with and without cardiovascular disease. Arch Intern Med 2007;**167**(11):1145-51.
27. Rindskopf D. An introduction to the bootstrap - Efron, B, Tibshirani, RJ. Journal of Educational and Behavioral Statistics 1997;**22**(2):245-45.
28. Nusselder WJ, Franco OH, Peeters A, et al. Living healthier for longer: Comparative effects of three heart-healthy behaviors on life expectancy with and without cardiovascular disease. Bmc Public Health 2009;**9**.
29. denTonkelaar I. Validity and reproducibility of self-reported age at menopause in women participating in the DOM-project. Maturitas 1997;**27**(2):117-23.
30. Leening MJ, Heeringa J, Deckers JW, et al. Healthy volunteer effect and cardiovascular risk. Epidemiology 2014;**25**(3):470-1.
31. Pizzi C, De Stavola B, Merletti F, et al. Sample selection and validity of exposure-disease association estimates in cohort studies. J Epidemiol Community Health 2011;**65**(5):407-11.
32. Muka T, Nano J, Voortman T, et al. The role of global and regional DNA methylation and histone modifications in glycemic traits and type 2 diabetes: A systematic review. Nutr Metab Cardiovas 2016;**26**(7):553-66.
33. Hayatbakhsh MR, Clavarino A, Williams GM, et al. Cigarette smoking and age of menopause: A large prospective study. Maturitas 2012;**72**(4):346-52.
34. Tao XY, Jiang AR, Yin LP, et al. Body mass index and age at natural menopause: a meta-analysis. Menopause 2015;**22**(4):469-74.
35. Muka T, Imo D, Jaspers L, et al. The global impact of non-communicable diseases on healthcare spending and national income: a systematic review. Eur J Epidemiol 2015;**30**(4):251-77.



2.4

Sex steroids, sex hormone-binding globulin and levels of NT-proBNP in postmenopausal women

ABSTRACT

Objective

Amino-terminal pro-B-type natriuretic peptide (NT-proBNP) has a well-documented prognostic value for cardiovascular disease and sex-hormones are suggested to modulate NT-proBNP levels. Therefore, our aim was to examine whether endogenous sex-hormones and sex hormone-binding globulin (SHBG) are associated with NT-proBNP levels in postmenopausal women free of clinical cardiovascular diseases.

Methods

Total estradiol (E_2), total testosterone (TT), androstenedione (AD), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), sex hormone-binding globulin (SHBG) and NT-proBNP were assessed in 4,112 postmenopausal women free of cardiovascular diseases from the prospective population-based Rotterdam Study. Free androgen index (FAI) was calculated as ratio of TT to SHBG concentration. TT, AD, DHEA(S), SHBG, FAI and NT-proBNP were natural log transformed. Regression coefficients and 95% Confidence Intervals (CI) were calculated using multivariable linear regression models adjusting for confounders.

Results

In models adjusted for multiple confounders (age, reproductive, life style and cardiovascular risk factors) higher SHBG (per 1 SD increase, β= 0.15, 95% CI=0.12, 0.18), and lower levels of TT (per 1 SD increase, β= -0.05, 95%CI=-0.08, -0.02), FAI (per 1 SD increase, β= -0.13, 95%CI=-0.15, -0.09), DHEAS (per 1 SD increase, β= -0.06, 95% CI=-0.09, -0.04) and DHEA (per 1 SD increase, β= -0.06, 95%CI=-0.09, -0.04) were associated with higher levels of NT-proBNP. However, no association was found between E_2 and AD and NT-proBNP levels. Additionally, stratification by BMI did not affect any of observed associations.

Conclusion

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

INTRODUCTION

After menopause, sex differences in coronary heart disease (CHD) risk gradually disappear resulting in a similar incidence of CHD by the sixth decade in women as compared to men [1].

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

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

METHODS

Study population

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

Population for Analyses

The present study includes data from postmenopausal women from the third visit of the first cohort of the RS (RS I-3), and from the first visits of the second (RSII-1) and the third cohort (RSIII-1). There were 6,760 women eligible for the analysis. Of those, 2,648 women were excluded because (i) they were non-postmenopausal or due to no information on their menopausal status (n=731); (ii) they did not have information on sex steroids (n=1296) or on NT-proBNP levels (n=18); (iii) had prevalent cardiovascular disease (CHD, stroke or heart failure) (n=267); (iv) there was no available information on the presence of cardiovascular disease (n=31), (v) used postmenopausal hormone therapy (n=168) or (vi) there was no available information on hormone therapy use (n=137) (**Figure 2.4.1**). Therefore, 4112 postmenopausal women were included in the final analysis.

Assessment of Exposure, Outcome and Covariates can be found in supplemental material.

Statistical analyses

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

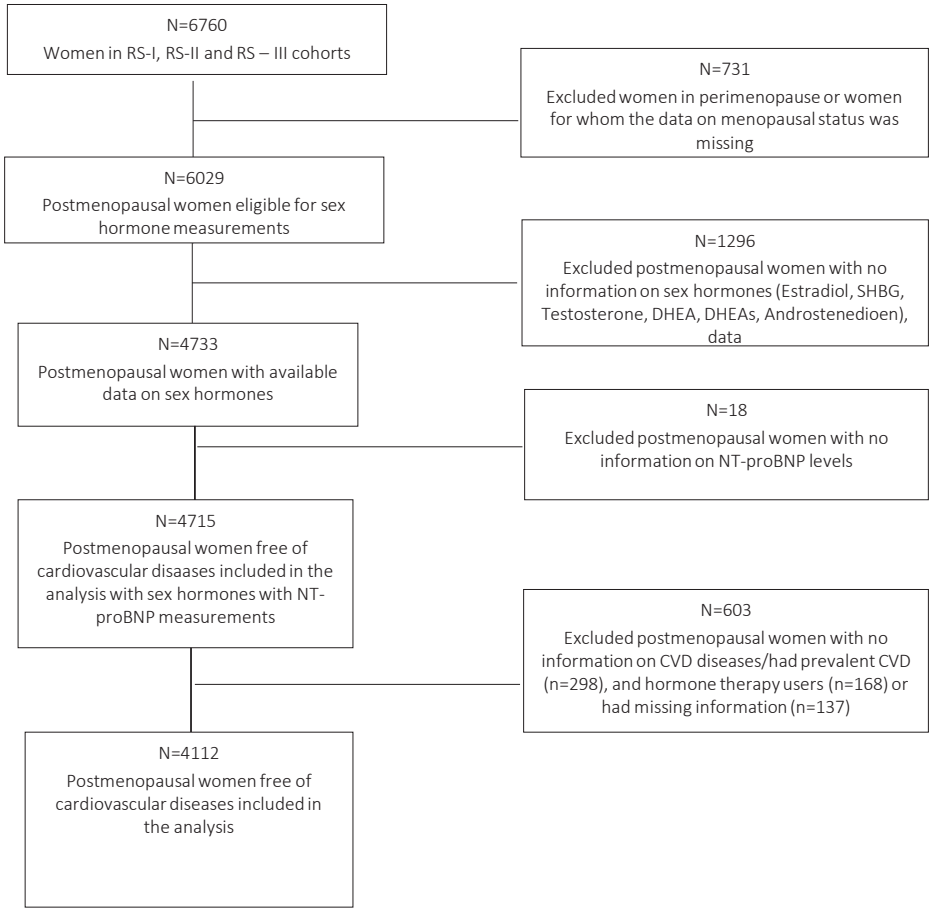


Figure 2.4.1. Flowchart for selection of study participants.

(yes vs. no), glomerular filtration rate (eGFR) (continuous), C-reactive protein (CRP) (continuous). We also controlled (in Model 2) for upstream precursor hormones which may have acted as confounders. Collinearity analysis demonstrated high correlation between DHEA and DHEAS (variance inflation factor, VIF>3), and therefore, when applicable, we did not adjust for DHEA in model 2, but only for DHEAS. There were missing values on one or more covariates (**Table 2.4.1**). Because the missing values were likely to be missing at random and for avoidance of loss in efficiency, missing values were imputed using a multiple imputation technique (5 imputation sets). Rubin's method was used for the pooled coefficients (β) and 95% Confidence Intervals [16]. In total, 21 variable has been imputed. Percentage of missing values for the majority of imputed variables (n=19) was below 5%. However, we imputed 28.4% of missing data on alcohol consumption and 11.2 of missing data on physical activity. A P-value lower than 0.05 was considered as statistically significant, but as sensitivity analysis, to account for multiple testing,

we adjusted the p-value from 0.05 to 0.007 by applying the Bonferroni correction for the number of exposures studied (N=7). All analyses were done using SPSS statistical software (SPSS, version 21.0; SPSS Inc, Chicago, Illinois).

Table 2.4.1. Characteristics of the Study Population

Age at baseline, mean (SD), y	65.9±9
Education	
Primary	612 (14.9%)
Lower/intermediate or lower vocational	2143 (52.8%)
Intermediate vocational or higher general	912 (22.2%)
Higher vocational or university	431(10.5%)
BMI, kg/m^2	27.5 ±4.6
Waist to hip ratio	0.7 (0.9)
Smoking	
yes	568 (13.8%)
no	3526 (85.8%)
Alcohol intake g/day	2.1 (0.01-11.4)
Health indicators	
Systolic BP, mmHg	139.5±21.8
Diastolic BP, mmHg	77.9 ±11.3
Antihypertensive therapy with indication, yes	1022 (24.9%)
Total cholesterol, mmol/l	5.9 ± 0.9
LDL, mmol/l	4.1 (0.9)
HDL, mmol/l	1.5 ±0.4
Triglycerides, mmol/l	1.3 (1-1.8)
Fasting blood glucose, mmol/l	5.7±1.4
Insulin, pmol/l	72 (51-104)
Serum lipid lowering medication, yes	610 (14.8)
Prevalent diabetes mellitus	426 (10.4%)
CRP mg/l	1.6 (0.7-3.4)
eGFR	77.1±15
Physical activity, total MET hours	78.5 (53-111)
Hormones	
Estradiol, pmol/l	31.4 (18.4-56.5)
Testosterone, nmol/l	0.8 (0.6-1.1)
SHBG, nmol/l	57.5 (41.6-79.4)
FAI	1.4 (0.9-2.1)
DHEA, nmol/l	9.5 (6-.14.7)
DHEAS, nmol/l	1669.7 (1025.8-2582.3)
Androstenedione nmol/l	2.3 (1.7-3.2)

Table 2.4.1. Characteristics of the Study Population (continued)

Age at baseline, mean (SD), y	65.9±9
TSH mU/l	2 (1.3-3)
NT-proBNP, pmo/l	9.2 (5.3-16.8)
NT-proBNP, ng/l	77.8 (44.8-142.1)
Women-specific variables	
Age at menopause, years[1]	48.7±5.6
Years since menopause	17.2±10.2
Menopause type, natural menopause[1]	2803 (68.2%)
Age at menarche, years	13.4 ±1.7
Number of pregnancies	2 (1-3)

Values are reported as number 9percentage) for categorical variables, and mean ± SD or median (25^{th}-75^{th} quartile) for continuous variables

[1]Age at menopause and type of menopause were not available for all women, the present values are based on 4425 and 4497 respectively

* body mass index (BMI), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), glomerular filtration rate (eGFR), free androgen index (FAI), High-density lipoprotein L (HDL), low-density lipoprotein L (LDL), amino-terminal pro-brain natriuretic peptide (NT-proBNP), sex hormone binding globulin (SHBG), thyroid stimulating hormone (TSH), high-sensitivity C reactive protein (CRP)

Sensitivity Analysis

We performed a series of sensitivity analyses using imputed data. Firstly, we compared baseline characteristics of postmenopausal women who were not included in our study due to missing data on exposure and outcome (n=922) with women included in analyses. Since waist circumference is a better measure of visceral adiposity, an important determinant of sex steroid levels and SHBG after menopause, and because NT-proBNP levels correlate with abdominal fat, we performed a sensitivity analysis substituting BMI with waist circumference. To account for the specific effects of lipid particles on NT-proBNP levels, we substituted total cholesterol (TC) with high-density lipoprotein cholesterol (HDL-C), triacylglycerol (TG) and low-density lipoprotein (LDL) . Thyroid stimulating hormone (TSH), physical activity, number of pregnancies, age of menarche and type of menopause (non-natural vs. natural) are associated with sex hormone levels, therefore, the models were further adjusted for these factors. Since DHEA showed collinearity with DHEAs, we performed a sensitivity analysis substituting DHEAs with DHEA. Furthermore, we restricted the analysis among women (i) who had NT-proBNP levels within age specific value for the diagnosis of heart failure as proposed by Januzzi et al, i.e. 50 to 75 years, 108 pmol/L; >75 years, 216 pmol/L) [17] and (ii) who had NT-proBNP levels > 125 pg/ml/14.78 pmol/l (30.7 % of our population) because this portion of women might have heart failure with preserved ejection fraction (HFpEF) or heart failure with mid-range ejection fraction (HFmrEF) according to the 2016 ESC Guidelines [18]. Also, to explore whether the associations were independent of downstream hormones, we

further adjusted for hormones including the downstream metabolites that might be casual intermediates. Effect modifications of sex hormones by BMI, age and years since menopause were tested by adding an interaction term in the final multivariable model in addition to performing stratified analysis. We preformed stratified analysis excluding women who had diabetes or were on antihypertensive therapy. Furthermore, to show the clinical relevance, we showed the associations of endogenous sex hormones and SHBG with NT-proBNP levels in tertiles for the 2nd Model. To study the relations across increasing tertiles, trend tests were computed by entering the categorical variables as continuous variables in the linear regression models. Additionally, we run the main analysis among subjects with available information on all covariates including 2582 postmenopausal women.

RESULTS

The mean age of the study population was 65.9 years (SD 9). Women were on average 17.2 years (SD 10.2) into menopause, and the majority of women (68.2%%) experienced natural menopause (**Table 2.4.1**). There was strong positive correlation between DHEA and DHEAS (Spearman's correlation coefficient: r_s=0.73) and between DHEA and androstenedione (r_s =0.67), and moderate negative correlation between FAI and SHBG (r_s =-0.58), FAI and TT (r_s =0.62), and FAI and androstenedione (r_s =0.41) (**Supplemental Table 2**). In addition, there was a weak negative correlation between SHBG and E2 (r_s =-0.15) and a weak positive correlation between SHBG and testosterone (r_s =0.21).

Estradiol and NT-proBNP levels

After adjusting for potential confounders and intermediate factors (Model 2) we did not observe significant associations between E_2 and NT-proBNP levels (per 1 SD increase in estradiol levels, β= 0.014, 95% CI=-0.013, 0.040) **(Table 2.4.2)**.

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

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

Table 2.4.2. Associations of androgens, oestrogen, and sex-hormone binding globulin with the level of serum NT-proBNP in postmenopausal women free of CVD, the Rotterdam Study (N=4112)

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

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

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

**results remain significant after Bonferroni correction p<0.007*

Values shown in the table are continuous per 1 SD

Significant results are bold (p<0.05)

Abbreviations: CRP: c-reactive protein, DHEA: dehydroepiandrosterone , DHEAS: dehydroepiandrosterone sulfate, eGFR: glomerular filtration rate, FAI: free androgen index, SHBG: sex hormone binding globulin

tions of TT, FAI, DHEA, DHEAs and SHBG with NT-proBNP, but abolished the association between androstenedione and NT-proBNP levels (**Table 2.4.2**).

Sensitivity Analysis

The associations between TT, SHBG, FAI, DHEA and DHEAS and NT-proBNP levels remained significant after we applied the Bonferroni correction ($p<0.007$). There were significant differences in age, systolic blood pressure, BMI, hsCRP, prevalent T2D, statin and alcohol use, and smoking status among women included in our analysis and women that were excluded because of incomplete data on sex hormones and NT-proBNP. In sensitivity analyses, substituting BMI with waist circumference as a measure of adiposity, total cholesterol for other blood lipids, adjustment for DHEAs with DHEA in Model 2, adjusting further for serum TSH, number of pregnancies, age of menarche and type of menopause, or further adjustment for downstream sex hormones and excluding women who reported use of HRT, or exclusion of women who came non-fasting in the visit center did not affect the associations of sex steroid and SHBG with NT-proBNP levels. Also, the results did not change after exclusion of (i) 329 postmenopausal women with an NT-proBNP level above the age-specific cutoff value for the diagnosis of heart failure; (ii) 1234 women who had NT-proBNP levels > 125 pg/ml/14.78 pmol/l, (iii) 426 women who had diabetes; and (iv) 1022 women who used antihypertensive medications. In the stratified analysis, no significant interactions were found for sex steroids and SHBG with BMI, age or years since menopause. The results of endogenous sex hormones and SHBG in tertiles provided same conclusions as the analysis of sex steroids and SHBG as continuous variables (**Figure 2.4.2A and Figure 2.4.2B**). Furthermore, when restricting the analysis to women who had available information on all covariates he results did not change. Estimates for testosterone and DHEA had the same direction, however, were not significant probably due to loss of power.

DISCUSSION

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

Androgens and NT-proBNP

Several studies suggest that testosterone has suppressive effect on natriuretic plasma levels. In line with our results on an inverse association between androgens and NT-

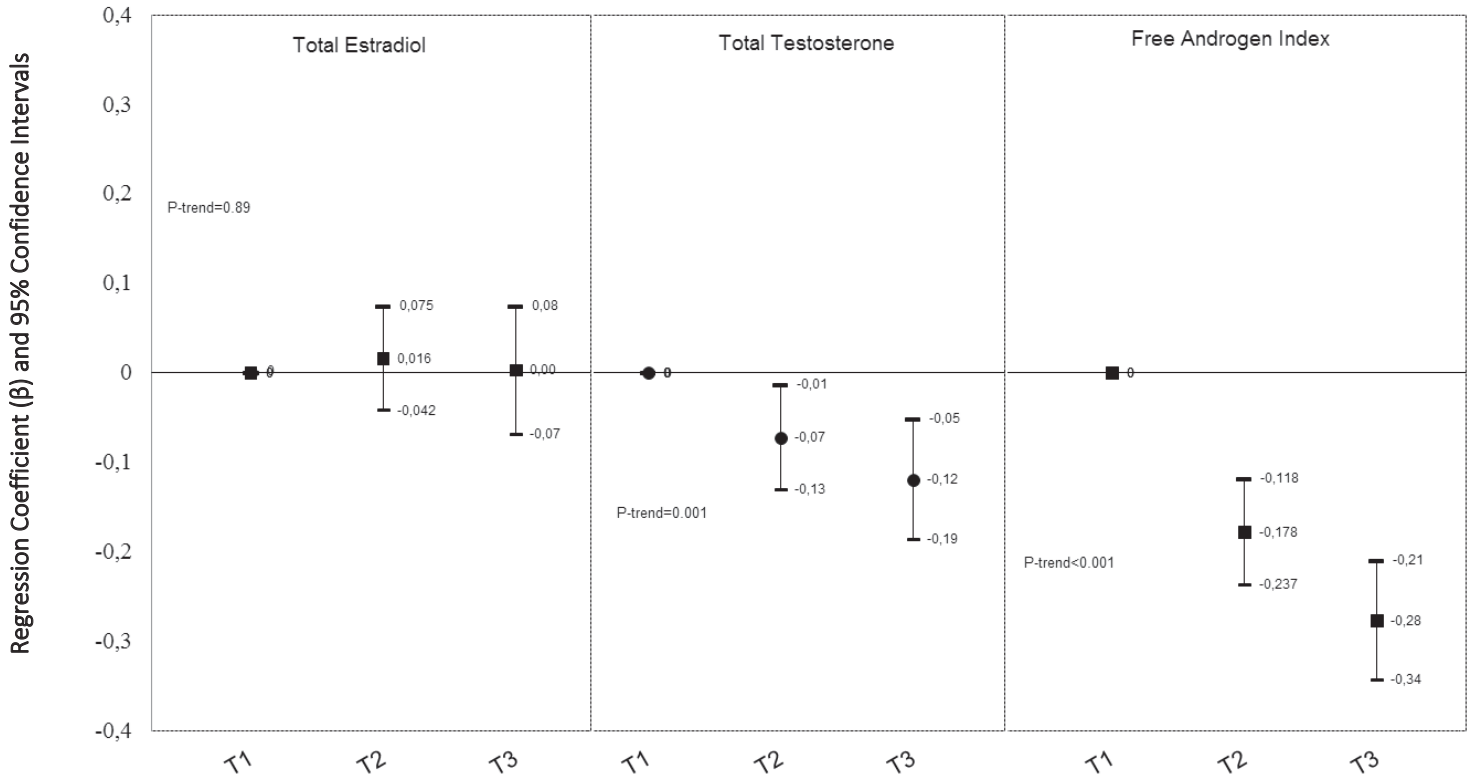


Figure 2.4.2A. Association of NT-proBNP and hormone-specific tertiles sex-hormone binding globulin (SHBG), Estradiol, Testosterone, and free androgen index Estradiol : T1: 18.35 pmol/l (minimum detection limit), T2: 18.530-48.91 pmol/l. T3: 48.91-321.10 pmol/l; Testosterone: T1: 0.007-0.66 nmol/l T2:0.67-1.00 nmol/l, T3: 1.01-2.94 nmol/l; FAI: T1: 0.16-1.069, T2; 1.070-1.81, T3: 1.82-6.07.

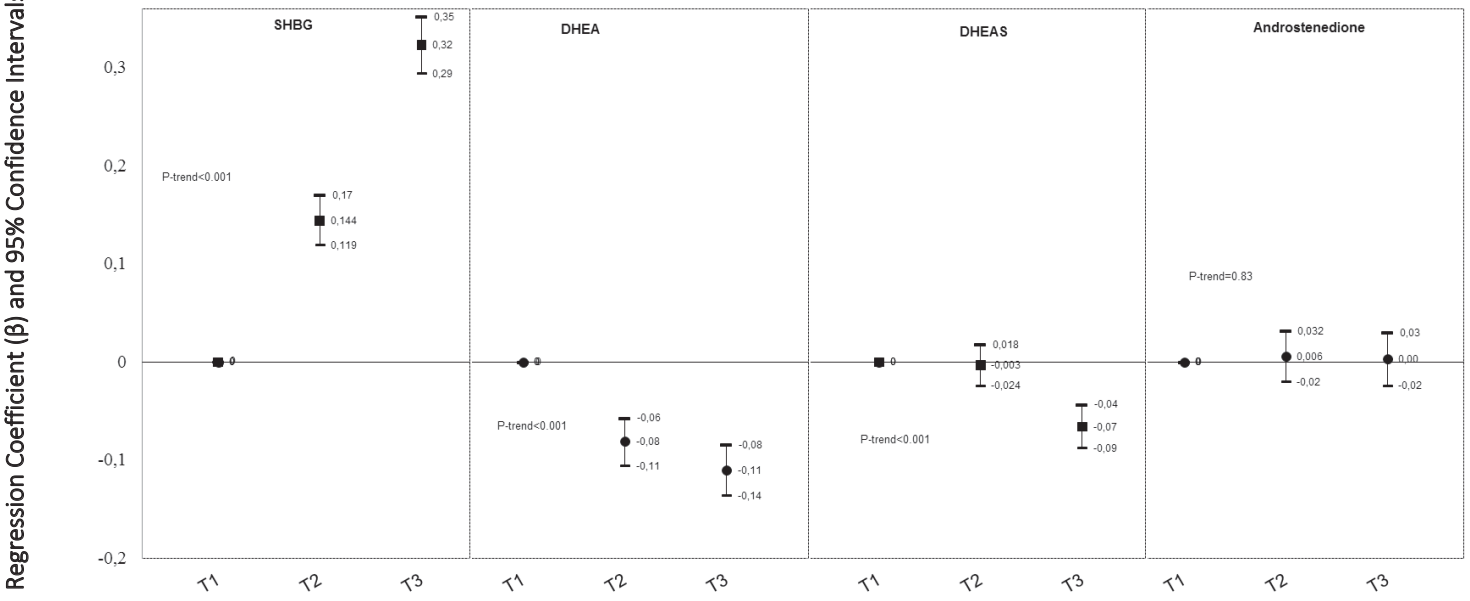


Figure 2.4.2B. Association of NT-proBNP and hormone-specific tertiles sex-hormone binding globulin (SHBG), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS) and androstenedione.
SHBG: T1: 15.47-47.20 nmol/l, T2: 47.29-72.20 nmol/l, T3:72.20-150.20 nmol/l, DHEA: T1: 2.20-7.20

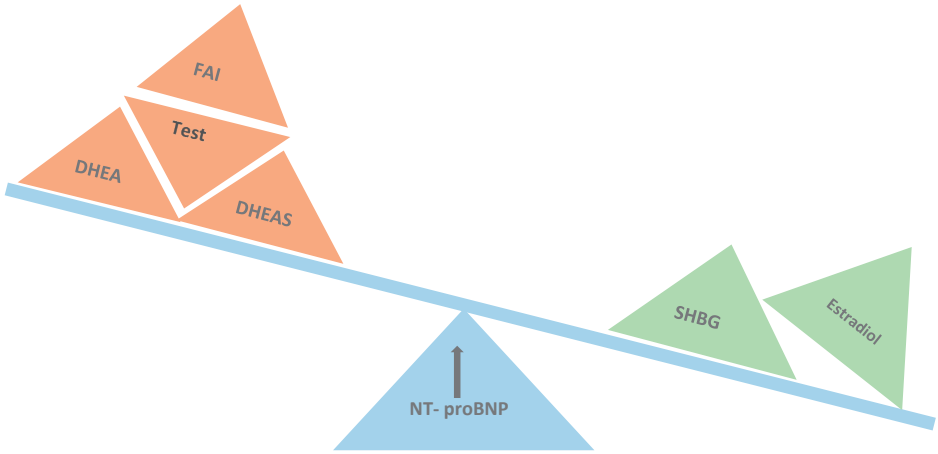


Figure 2.4.3. Summary of study results
Androgens (testosterone, DHEA, DHEAS, FAI) were negatively associated with NT-proBNP levels (lower levels of androgens higher NT-proBNP levels), SHBG was positively associated with NT-proBNP (higher levels of SHBG, higher levels of NT-pro-BNP), Estradiol was positively associated with NT-pro-BNP, however, the association was not statistically significant.

proBNP, in a clinical trial of 51 women with hypoandrogenemia due to hypopituitarism, levels of NT-proBNP decreased after transdermal application of testosterone [19]. Also, Chang et al., in a study of 682 young adult women age 35 to 49 years reported that free testosterone was inversely associated with NT-proBNP levels [6]. Furthermore, Lam et al. in a study of 1798 premenopausal women and 181 postmenopausal women reported inverse associations between FAI and NT-proBNP [9]. Unlike previous population based-studies, which were included mainly young adult women, our study extends these findings to postmenopausal women and shows that the association between testosterone and NT-proBNP is independent of other sex steroids, including estradiol which in a downstream hormone and might be in the pathway between testosterone and NT-proBNP.

We show that other androgens such as DHEA and its derivate DHEAs are inversely associated with NT-proBNP levels in postmenopausal women, supporting the hypothesis that androgens have inhibitory effect on NPs in older women. In a neonatal rat cardiocyte culture system, DHEA significantly inhibited BNP mRNA levels [12]. Similarly, studies in men have reported inverse correlations between DHEAs and BNP. DHEA and its sulphate conjugate DHEAS are the major secretory steroidal products of the human adrenal glands [20]. In either gender, serum level peaks of DHEA and DHEAs occur by the second decade and then declines steadily by an average of about 10%/decade [21]. Mechanisms of action of DHEA are still to be described. DHEA(S) is converted to testosterone or 17β-estradiol and therefore it is unclear whether DHEA directly exerts its

effects or if it acts after conversion to these hormones. However, in our study we corrected for levels of estradiol and testosterone, supporting an independent role of DHEA on NT-proBNP levels. Recent evidence shows that there are specific DHEA-bindings sites in the cardiovascular system, including the heart tissue [22 23]. Therefore, DHEA might have a direct effect in the vascular system, and might play a role in the development of cardiovascular disease independent of its derivate. However, to date, little is known about the role of DHEA and DHEAs in the risk cardiovascular disease, including the risk of developing heart failure. In a sample of 942 postmenopausal women, although higher DHEAS levels were associated with several major cardiovascular risk factors, such as elevated total cholesterol and blood pressure, they were unrelated to the risk of fatal cardiovascular disease [23]. Additional studies should be undertaken to further elucidate the exact mechanisms of how DHEA(S) might affects the levels of NT-proBNP and the risk of developing heart failure.

SHBG and NT-proBNP

We found positive association between SHBG and NT-proBNP levels, independent of potential confounding factors. The main role of SHBG is sex steroids transport within the blood stream to extravascular target tissues. Testosterone have higher SHBG binding affinity than estradiol, thus SHBG regulates balance between bioavailable testosterone and oestrogens. It has been hypothesized that SHBG plays an indirect role in rising NT-pro BNP levels, by binding more testosterone which have negative effect on natriuretic peptides. Findings from Framingham Heart Study showed that each unit increase in log SHBG was associated with a 19% increase in NT-proBNP among men, and a 40% increase in NT-proBNP among young adult women, adjusting for clinical covariates [9]. However, in our study, positive association between SHBG and NT-proBNP remains significant after adjustment for potential confounders but also for TT, DHEA, DHEAS and E_2, implicating that SHBG does not modify only the balance between circulating steroids, but might directly influence NT-proBNP levels. Indeed, in recent years, it has been shown that SHBG may directly mediate cell-surface signalling, cellular delivery and biologic action of sex hormones via activation of a specific plasma receptor [24-26].

Low levels of SHBG have been associated with increased cardiovascular risk in women, irrespective of menopause status [13 14]. Our data and other evidence show that lower SHBG levels are associated with lower NT-proBNP levels in both pre- and postmenopausal women. Natriuretic peptides have antiproliferative and vasodilator effects, as well as antagonism of the renin-angiotensin- aldosterone and adrenergic axes [27]. Therefore, given the cardioprotective effects on NPs, future studies should explore whether lower natriuretic peptide concentrations may explain, in part, the excess cardiovascular risk associated with low SHBG concentrations.

Estradiol and NT-proBNP

This is the first study to examine the association between endogenous estradiol levels and NT-proBNP in postmenopausal women, showing no association. To our knowledge, no study has examined whether endogenous estradiol levels are associated with NT-proBNP in menopausal women, which would shed more light whether the decline in oestrogen levels after menopause would be, in part, responsible for the increased risk of cardiovascular disease observed after menopause. Female hormones are considered as important determinants of the lower risk of CVD observed in premenopausal women, while lack of oestrogens disadvantages men with regard to CVD risk. Therefore, menopause and drop in endogenous oestrogens, suggested that HRT might have an important cardio protective role in women [28].

In line with this hypothesis and in contrast with our findings, a clinical trial of 22 healthy postmenopausal women, reported that administration of hormone replacement therapy with transdermal estradiol produced a rise in plasma levels of BNP [29]. Also, a study conducted in female rats reported that treatment with estradiol and progesterone stimulated atrial NP gene expression[30]. Oral oestrogen leads to an increase in SHBG, which binds more testosterone and therefore leads to an increase of NT-proBNP levels [4]. In contrast to this, a population based study in 682 women showed no association between oral oestrogen use and NT-proBNP levels [6]. Also, results of Women's Health Initiative showed that HRT in postmenopausal women was not cardio protective [7].

Body composition, sex steroids and NT-proBNP

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

Strengths and limitations

To the best of our knowledge, this is the first and most comprehensive study to examine the associations of estradiol, androgens and SHBG with NT-proBNP levels in a large sample of postmenopausal women, with consistent findings. Also, androgens are measured using chromatography-tandem mass spectrometry, which is at the moment considered to be a gold standard method [31]. However, there are several limitations that need to be

taken into account. First, the cross-sectional design does not allow us to address the temporality of the observed associations. Therefore, we cannot draw any conclusions with regard to the causality of the observations. Second, we did not have measures of bioavailable estradiol in the RS, which could have strengthened our results. Also, E_2 was measured using an immunoassay with a detection limit of 18.35 pmol/L, which is considered suboptimal particularly in postmenopausal women. In our population 1502 women (33.18 %) had values of E_2 lower than 18.35 pmol/l. However, we performed sensitivity analysis using E_2 tertiles instead of E_2 as continuous variable, which provided similar results. Third, free T levels were not measured directly in the blood and therefore have to be interpreted with caution. Nevertheless, free T levels in this study were derived from the ratio of T to SHBG, which is considered a precise proxy for bioavailable T [32]. Furthermore, NT-proBNP is hormonally inactive N-terminal portion of its pro-hormone, and we do not have measurement of BNP which is the active hormone. However, recent systematic reviews and meta-analyses demonstrated that both BNP and NT-proBNP have similar diagnostic and prognostic accuracy in CVDs [2]. Finally, we found differences in baseline characteristics between participants included in our analysis and participants that were not. However, it has been shown that using a selected source population for a cohort study usually leads to bias toward the null, but may affect the generalizability of our results regarding mean sex hormone levels and NT-proBNP [33].

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

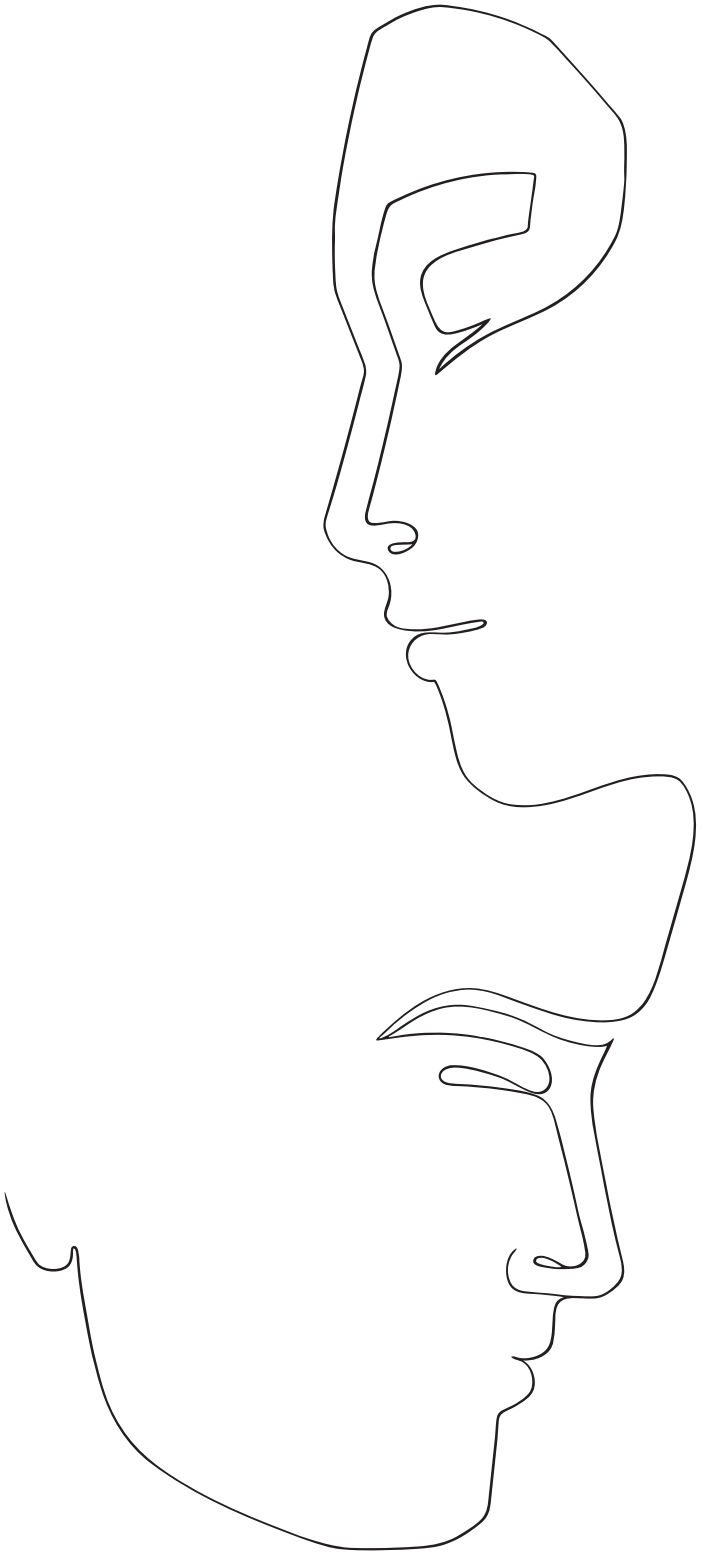
Clinical Perspectives

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

REFERENCES

1. Witteman JC, Grobbee DE, Kok FJ, et al. Increased risk of atherosclerosis in women after the menopause. BMJ 1989;**298**(6674):642-4.
2. Clerico A, Giannoni A, Vittorini S, et al. The paradox of low BNP levels in obesity. Heart Fail Rev 2012;**17**(1):81-96.
3. Kavousi M, Elias-Smale S, Rutten JH, et al. Evaluation of newer risk markers for coronary heart disease risk classification: a cohort study. Ann Intern Med 2012;**156**(6):438-44.
4. Rutten JH, Mattace-Raso FU, Steyerberg EW, et al. Amino-terminal pro-B-type natriuretic peptide improves cardiovascular and cerebrovascular risk prediction in the population: the Rotterdam study. Hypertension 2010;**55**(3):785-91.
5. Pfister R, Sharp S, Luben R, et al. Mendelian randomization study of B-type natriuretic peptide and type 2 diabetes: evidence of causal association from population studies. PLoS Med 2011;**8**(10):e1001112.
6. Chang AY, Abdullah SM, Jain T, et al. Associations among androgens, estrogens, and natriuretic peptides in young women: observations from the Dallas Heart Study. Journal of the American College of Cardiology 2007;**49**(1):109-16.
7. Grimes DA, Lobo RA. Perspectives on the Women's Health Initiative trial of hormone replacement therapy. Obstet Gynecol 2002;**100**(6):1344-53.
8. Daan NM, Muka T, Koster MP, et al. Cardiovascular Risk in Women With Premature Ovarian Insufficiency Compared to Premenopausal Women at Middle Age. J Clin Endocrinol Metab 2016;**101**(9):3306-15.
9. Lam CS, Cheng S, Choong K, et al. Influence of sex and hormone status on circulating natriuretic peptides. J Am Coll Cardiol 2011;**58**(6):618-26.
10. Ober C, Loisel DA, Gilad Y. Sex-specific genetic architecture of human disease. Nat Rev Genet 2008;**9**(12):911-22.
11. Jankowski M, Rachelska G, Donghao W, et al. Estrogen receptors activate atrial natriuretic peptide in the rat heart. Proc Natl Acad Sci U S A 2001;**98**(20):11765-70.
12. Nakamura S YM, Nakayama M, Ito T, Mizuno Y, Harada E, Sakamoto T, Saito Y, Nakao K, Yasue H, Ogawa H. Possible Association of Heart Failure Status With Synthetic Balance Between Aldosterone and Dehydroepiandrosterone in Human Heart. Circulation 2004;**110**(13):1787-93.
13. Rexrode KM, Manson JE, Lee IM, et al. Sex hormone levels and risk of cardiovascular events in postmenopausal women. Circulation 2003;**108**(14):1688-93.
14. Sutton-Tyrrell K, Wildman RP, Matthews KA, et al. Sex-hormone-binding globulin and the free androgen index are related to cardiovascular risk factors in multiethnic premenopausal and perimenopausal women enrolled in the Study of Women Across the Nation (SWAN). Circulation 2005;**111**(10):1242-9.
15. Hofman A, Brusselle GG, Darwish Murad S, et al. The Rotterdam Study: 2016 objectives and design update. Eur J Epidemiol 2015;**30**(8):661-708.
16. B.D. R. Multiple Imputation for Nonresponse in Surveys. Investigative radiology. . 1987.
17. Januzzi JL, van Kimmenade R, Lainchbury J, et al. NT-proBNP testing for diagnosis and short-term prognosis in acute destabilized heart failure: an international pooled analysis of 1256 patients: the International Collaborative of NT-proBNP Study. Eur Heart J 2006;**27**(3):330-7.
18. Ponikowski P, Voors AA, Anker SD, et al. 2016 ESC Guidelines for the Diagnosis and Treatment of Acute and Chronic Heart Failure. Rev Esp Cardiol (Engl Ed) 2016;**69**(12):1167.

19. Lin E, McCabe E, Newton-Cheh C, et al. Effects of transdermal testosterone on natriuretic peptide levels in women: a randomized placebo-controlled pilot study. Fertil Steril 2012;**97**(2):489-93.
20. Parker LN, Odell WD. Control of adrenal androgen secretion. Endocr Rev 1980;**1**(4):392-410.
21. Orentreich N, Brind JL, Rizer RL, et al. Age changes and sex differences in serum dehydroepiandrosterone sulfate concentrations throughout adulthood. J Clin Endocrinol Metab 1984;**59**(3):551-5.
22. Altman R, Motton DD, Kota RS, et al. Inhibition of vascular inflammation by dehydroepiandrosterone sulfate in human aortic endothelial cells: roles of PPARalpha and NF-kappaB. Vascul Pharmacol 2008;**48**(2-3):76-84.
23. Barrett-Connor E, Khaw KT, Yen SS. A prospective study of dehydroepiandrosterone sulfate, mortality, and cardiovascular disease. N Engl J Med 1986;**315**(24):1519-24.
24. Catalano MG, Frairia R, Boccuzzi G, et al. Sex hormone-binding globulin antagonizes the anti-apoptotic effect of estradiol in breast cancer cells. Mol Cell Endocrinol 2005;**230**(1-2):31-7.
25. Fortunati N, Catalano MG, Boccuzzi G, et al. Sex Hormone-Binding Globulin (SHBG), estradiol and breast cancer. Mol Cell Endocrinol 2010;**316**(1):86-92.
26. Liu D, Dillon JS. Dehydroepiandrosterone activates endothelial cell nitric-oxide synthase by a specific plasma membrane receptor coupled to Galpha(i2,3). J Biol Chem 2002;**277**(24):21379-88.
27. Rosner W HD, Khan MS, Nakhla AM, Romas NA. Sex hormone-binding globulin mediates steroid hormone signal transduction at the plasma membrane. J Steroid Biochem Mol Biol 1999;**69**(1-6):481-5.
28. Santhanakrishnan R, Lam CS. Natriuretic peptides, gender and cardiovascular risk: what is the link? Maturitas 2012;**71**(2):89-91.
29. Maffei S, Del Ry S, Prontera C, et al. Increase in circulating levels of cardiac natriuretic peptides after hormone replacement therapy in postmenopausal women. Clin Sci (Lond) 2001;**101**(5):447-53.
30. Noubani A, Farookhi R, Gutkowska J. B-type natriuretic peptide receptor expression and activity are hormonally regulated in rat ovarian cells. Endocrinology 2000;**141**(2):551-9.
31. Rosner W, Auchus RJ, Azziz R, et al. Position statement: Utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement. J Clin Endocrinol Metab 2007;**92**(2):405-13.
32. Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. J Clin Endocrinol Metab 1999;**84**(10):3666-72.
33. Pizzi C, De Stavola B, Merletti F, et al. Sample selection and validity of exposure-disease association estimates in cohort studies. J Epidemiol Community Health 2011;**65**(5):407-11.



2.5

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

ABSTRACT

Objective

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

Methods

Serum DHEA, DHEAs and NT-proBNP were assessed in 7,390 men and women free of cardiovascular diseases from the prospective population-based Rotterdam study. DHEA, DHEAS and NT-proBNP were naturally log transformed. Regression coefficients and 95% confidence intervals (CI) were calculated from multivariable linear regression models adjusting for confounders to explore the cross-sectional association of DHEA and DHEAs with NT-proBNP. To investigate the causal association between DHEAs and NT-proBNP, allele score of exposure was used as an instrumental variable to perform a Mendelian Randomization (MR) analysis using two-stage least squares (2SLS) method. Pleiotropic effect was evaluated through Egger plots. A pathway analysis was used to give a complementary information about biological causal association paths among these two hormones.

Results

In models adjusted for multiple confounders (age, sex, lifestyle and cardiovascular risk factors), high levels of DHEA (β=-0.146, 95%CI: -0.190; -0.101, p<0.001) or DHEAs (β=-0.214, 95%CI: -0.262; -0.166, p<0.001) were associated with lower levels of NT-proBNP. Genetic risk score of DHEAs explained 0.75% and 29.39% variance of the circulating levels of NT-proBNP in crude and full adjusted models, respectively. The Mendelian Randomization analysis showed evidence for a causal association between DHEAs and NT-proBNP, with a causal coefficient of -0.450 (95% CI: -0.792; -0.107, p<0.010). Sex differences were observed with significant association only in women. A pathway analysis, to identify common networks, showed inflammatory and immunologic pathways linking genes associated with DHEAs and NT-pro-BNP.

Conclusions

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

INTRODUCTION

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

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

However, due to observational nature of previous studies affected by the possibility of residual confounding and reverse causation, it is not possible to draw conclusion regarding the causal association between DHEAs and NPs. Mendelian randomization (MR) method may be used to study the causal associations in presence of such limitations. The method is considered as a 'natural' randomized control trial since it uses selected common genetic variants related to a specific exposure of interest as an instrumental variable to evaluate causality between exposure and outcome. Since genotypes are assorted randomly during meiosis, MR avoids the issue of reverse causality. In addition, the distribution of genetic variants is thought to be unrelated to confounders, a common source of false positives in epidemiological studies[8]. Although, the physiological function of DHEAs and its importance in maintaining health are poorly understood,

several common single nucleotide polymorphisms (SNPs) were associated with changes in gene expression levels, and the related genes are connected to biological pathways linking DHEAs with ageing[9 10].

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

METHODS

Study Population

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

Population for Analyses

The present study includes data from individuals from the third visit of the first cohort of the RS (RS I-3), and from the first visits of the second (RSII-1) and the third cohort (RSIII-1). There were 11,732 subjects eligible for the analysis. Of those 4,342 participants were excluded because (i) information was not available on NT-proBNP (n=814), DHEAs (n=205) or on the genetic risk score (n=2,318); (ii) they had prevalent cardiovascular disease (coronary heart disease, stroke or HF) (n=947); and (iv) there was no information on history of cardiovascular disease (n=58). Finally, there were 7,390 participants left for the analysis (**Figure 2.5.1**).

Exposure and Outcome Measurement

DHEA and DHEAs were exposure variables. They were measured on a Waters XEVO-TQ-S system (Waters, Milford, MA, USA) using CHS™ MSMS Steroids Kit (Perkin Elmer, Turku, Finland). Inter-assay coefficients of variation of androstenedione, DHEAs and DHEA were

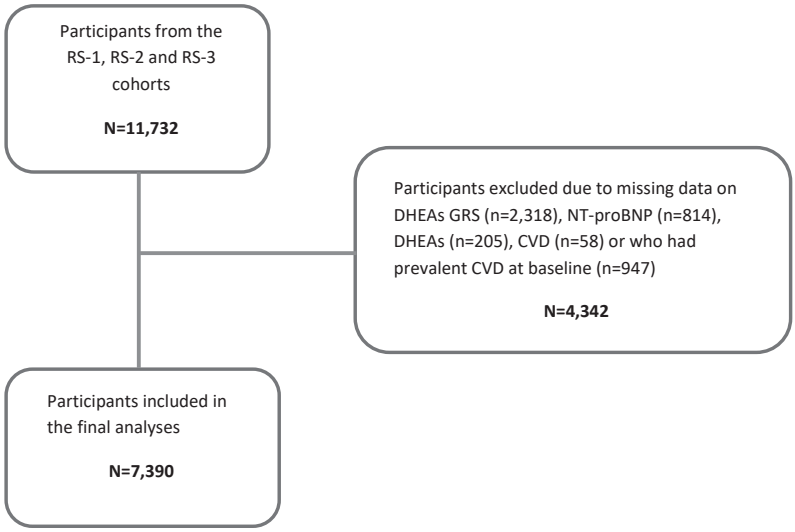


Figure 2.5.1. Flowchart for selection of study participants (n=7,390)

<6.5%. NT-proBNP levels were obtained from serum. After blood collection, samples were left to clot for 30 minutes and then centrifuged for 20 minutes at 3000 rotations per minute at 4ºC. Serum was stored at -80°C. NT-proBNP was measured using a commercially available electrochemiluminescence immunoassay (Elecsys proBNP, F. Hoffman-La Roche Ltd., Basel, Switzerland) on an Elecsys 2010 analyser. Precision, analytical sensitivity and stability characteristics of the system have been previously described[12].

Assessment of Covariates

At baseline interview, all participants provided information on current health status, medical history, medication use, alcohol intake, smoking and physical activity. History of cardiovascular disease was defined as the history of coronary heart disease (myocardial infarction, revascularization, coronary artery bypass graft surgery or percutaneous coronary intervention) and was verified from the medical records of the general practitioner. Diabetes mellitus was defined as the use of blood glucose–lowering medications or a random non-fasting glucose >11.1 mmol/L[13]. Antihypertensive or antidiabetic therapy and statins were collected by questionnaire during home interview. Alcohol intake was assessed in grams of ethanol per day and grouped into 4 categories (0-0.99, 1-19.9, 20-39.9 and ≥40 g/day); smoking status was assessed by asking participants whether they were current smokers of cigarettes, cigars, or pipe and were classified (yes/no). Physical activity was assessed with adapted version of the Zutphen Physical Activity Questionnaire[14]. Every activity mentioned in the questionnaire was attributed a MET-value according to the 2011 Compendium described in detail elsewhere[15]. Blood pressure was measured in sitting position on the right upper arm with a random-zero sphygmoma-

nometer. Body mass index (BMI) was calculated as weight (kg) divided by height square (m^2). Glomerular filtration rate (eGFR) was estimated using the simplified Modification of Diet in Renal Disease (MDRD) equation[16]. Thyroid stimulating hormone (TSH) was measured on the Vitros Eci (Ortho Diagnostics). Insulin, glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triacylglycerol (TG) and C-reactive protein (CRP) were measured on the COBAS 8000 Modular Analyzer (Roche Diagnostics GmbH). Corresponding interassay coefficients of variations are as follows: TSH<13.2%, insulin <8%, glucose <1.4%, lipids <2.1% and CRP <16.9%. LDL-cholesterol level was estimated indirectly from measurements of total cholesterol, HDL and triglycerides by means of the Friedewald equation[17]. Total estradiol (TE) levels were measured with a radioimmunoassay and sex hormone binding globulin (SHBG) by means of the Immulite platform (Diagnostics Products Corporation Breda, the Netherlands). Minimum detection limit for estradiol was 18.35pmol/l. Undetectable estradiol was scored as 18.35pmol/l. Serum levels of total testosterone (TT) were measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Corresponding interassay coefficients of variations for TE, SHBG and TT are <7%, <5%, and <5%. Free androgen index (FAI), calculated as (T/SHBG)*100 is used as a surrogate measure of bioavailable testosterone (BT). All biochemical parameters were assessed in fasting serum.

Genotyping

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

Construction of DHEAs Genetic Risk Score (GRS)

We searched PubMed using key words 'genome-wide association study', 'GWAS', 'DHEAS', 'Dehydroepiandrosterone sulfate', GWAS catalogue, and Genome- Wide Repository of Associations between SNPs and Phenotypes (GRASP). We identified two large genome-wide association studies conducted on >14,846 individuals of European descent[9 10]. Nine SPNs identified from these GWAS were used to build the genetic risk score of DHEAs (rs148982377, rs11761528, rs2637125, rs7181230, rs2497306, rs2185570, rs740160, rs17277546 and rs6738028) (Supplemental Table 1). The effect allele (coded 0–2) was

the DHEAs raising allele. A weighted GRS was calculated by multiplying the number of risk alleles at each locus by the corresponding reported β coefficient from the previous GWAS and then summing the products[18]. The total score was then divided by the average effect size multiplied by 100 to rescale the scores to a range between 0 and 100. We could not identify genome-wide association studies published for DHEA, and therefore we could only build a genetic risk score for DHEAs.

Pathway analysis

To explore the pathways in which the genes identified by GWAS for DHEAs and NT-proBNP may be related, we used Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com/products/ipa/), which is a web-based functional analysis tool to identify the biological mechanisms, pathways, and functions most relevant to the genes of interest. To this end, we uploaded a list of all DHEAs genes and performed a core analysis with the default settings in IPA. We mapped these genes to biological functions or diseases. We further sought to determine whether these genes are enriched in specific networks linking NT-proBNP and DHEAs to heart failure (or cardiovascular disease). The p-values are calculated using the right-tailed Fisher Exact Test and a p-value less than 0.05 indicates a statistically significant, non-random association.

Statistical Analyses

Cross-sectional Analyses

DHEA, DHEAs, NT-proBNP, hsCRP, 17-hydroxyprogesterone and cortisol levels were log-transformed using a natural log to obtain normal distribution. Cross-sectional association between log transformed continuous DHEA/DHEAs and NT proBNP was assessed using ordinal linear regression (OLR) models. Betas were calculated after adjusting for age, sex, interaction term between sex and DHEA/DHEAs (sex*DHEAs p=0.000 and sex*DHEA p=0.002), RS cohort, BMI, physical activity, smoking, alcohol, cholesterol, statin use, glucose, systolic blood pressure, antihypertensive therapy, type 2 diabetes (T2D), eGFR, hsCRP, 17-hydroxyprogesterone and cortisol. Additionally, to explore potential sex differences we run the analysis stratified by gender.

Association of DHEAs Genetic Risk score and NT-proBNP

The MR approach is used to investigate the causality of associations between DHEAs and NT-proBNP. Since no SNPs genome-wide significant for DHEA have been published, we could not assess the causality between DHEA and NT-proBNP. In the current study we used the genetic risk score (GRS) of DHEAs (calculated based on nine publically available SNPs) as an instrumental variable (IV). Valid instrumental variable is a factor that is associated with the exposure, but is not associated with any confounder of the exposure–outcome association, nor is there any pathway by which the IV can influence the

outcome other than via the exposure of interest/no pleiotropy[19] (**Figure 2.5.2**). Given a continuous outcome (NT-proBNP) and assuming the linear associations between DHEAs and NT-proBNP without interaction, we estimated the casual association between GRS of DHEAs and serum NT-proBNP through a 2-stage least squares (2SLS) regression[20]. The 2SLS estimation proceeds by first fitting the regression of DHEAs (exposure) on the GRS of DHEAs (instrument), and the second step assesses the association of DHEAs with NT-proBNP (outcome) on the fitted values from the first-stage regression. Within these models, age, sex, RS cohort, BMI, physical activity, smoking, alcohol, total cholesterol, statin use, glucose, systolic blood pressure, antihypertensive therapy, T2D, glomerular filtration rate (eGFR), hsCRP, 17-hydroxyprogesterone and cortisol were included as covariates in order to generate estimates from the IV analyses that were comparable to those from the observational regressions. We also evaluated the instrument strength using F-statistics from the first-stage regressions, where F-statistics >10 has been used to indicate sufficient strength, and by R^2 (%) as a measure of the percentage contribution of GRS to the variation of NT-proBNP levels. Standard MR analysis assume that genetic instruments only influence the outcome (i.e. NT-proBNP) through the exposure of interest (serum DHEAs), however, DHEAs associated SNPs may influence serum NT-proBNP through pathways other than serum DHEAs concertation. We therefore tested the robustness of our findings by MR Egger regression which helps to control for biases though horizontal pleiotropy. The slope of the weighted regression line provides an estimate of the causal effect of the exposure on the outcome free from the effects of horizontal pleiotropy. While the intercept in the regression is a function of extent of directional pleiotropy in the data aggregated across all the different variants used in the analysis, and statistical tests of the degree to which the intercept differs from zero are using to test the overall presence of directional pleiotropy in the data[21]. In case of significant intercept (and therefore evidence of directional effects) the estimate from the Eggers would be a better estimate, however, if no evidence of directional pleiotropy then the 2SLS is better powered.

To validate the causal estimate derived from the 2SLS method, we obtained MR estimations using the Wald ratio method. As follows, two normal linear regressions were performed: regression of DHEAS on GRS of DHEAS (the first-stage regression) and regression of NT-proBNP on GRS of DHEAs (reduced-form regression). The ratio of these estimates (the Wald estimate) and corresponding confidence intervals were obtained using *suest* and *nlcom* commands in Stata[22], this estimations were adjusted by age, sex and RS cohort. A multiple imputation (chained equations method) was applied for missing data. For most baseline clinical variables, <2% was missing, whereas this was up to 12% and 26% for self-reported variables such as physical activity and alcohol intake, respectively. All statistical analyses were carried out using Stata/IC statistical Software, version 15 and MR package of R software.

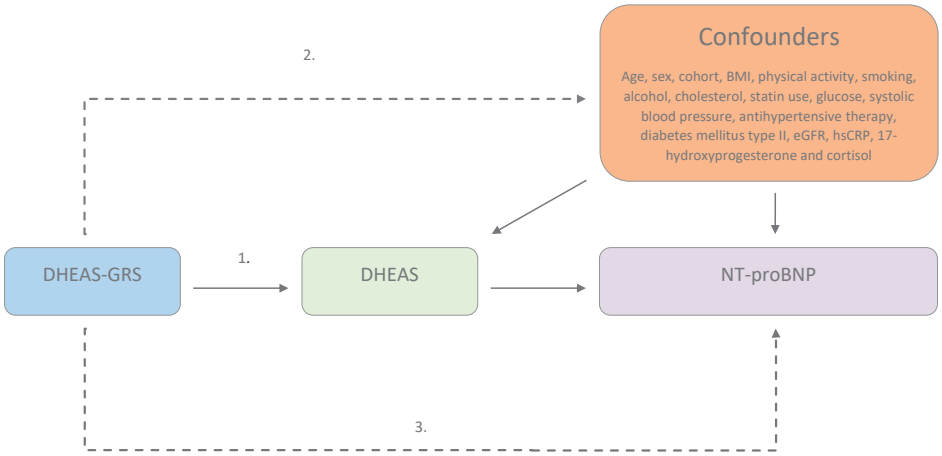


Figure 2.5.2. Assessing the causality of DHEAS and NT-proBNP levels
(1) GRS of DHEAs is associated with DHEAs (exposure)
(2) GRS of DHEAs is not associated with measured or unmeasured confounders
(3) GRS of DHEAs is only associated with NT-proBNP (outcome) through the exposure

RESULTS

Baseline characteristics of the population used for analysis are shown in **Table 2.5.1**. Median age (Q1-Q3) of participants was 63 (58-71) years, and 59.9% of included subjects were women. The median levels of NT-proBNP were 7.9 pmol/l (Q1=4.3; Q3=14.9), DHEA 9.6 nmol/l (Q1=6.2; Q3=15.0), DHEAs 2,099nmol/l (Q1=1,257; Q3=3,365) and DHEAs GRS 48.2 (Q1=45.1; Q3=50.8).

Observational associations between DHEA, DHEAs and NT-proBNP

Based on 7,390 subjects, we observed an inverse association between serum DHEAs levels and NT-proBNP levels. In crude model for each one-point increase in levels of natural log transformed DHEAs, NT-proBNP levels decreased -0.395 (β; 95%CI: -0.423; -0.366; p<0.001). In multivariable linear regression model (adjusted for age, sex, interactions of DHEAs*sex, RS cohort, BMI, physical activity, smoking, alcohol, cholesterol, statin use, glucose, systolic blood pressure, antihypertensive therapy, diabetes mellitus type 2, eGFR, hsCRP, 17-hydroxyprogesterone and cortisol) for each one-point increase in levels of natural log transformed of DHEAs, NT-proBNP levels decreased -0.214 (β; 95%CI: -0.262; -0.166; p<0.001) (Table 2). Stratification by gender did not materially change the results. Among both, men and women in fully adjusted models, high levels of serum DHEAs were associated with low levels of NT-proBNP levels (Supplemental table 2). Furthermore, in fully adjusted model for each one-point increase in levels of natural log transformed DHEA, NT-proBNP levels decreased by -0.146 (β; 95%CI: -0.190; -0.101;

Table 2.5.1. Characteristics of study population.

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

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

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

p<0.001), also, gender stratification did not yield any changes (Supplemental table 3). Assumptions of linearity, homoscedasticity and normality were assessed; but no major violations were observed.

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

A weighted gene score composed of 9 genetic variants for elevating DHEAs was used as the genetic instrument. DHEAs GRS was strongly associated with circulating NT-proBNP levels, explaining on average 0.75% and 29.39% of NT-proBNP variation, in crude and adjusted models, respectively, with F-statistics=58.26 (**Table 2.5.2**), indicating that GRS is unlikely to be affected by weak instrument bias. Neither individual genetic variants

Table 2.5.2. Summary statistics describing observational and causal relationship DHEAs and NT-proBNP (n=7,390)

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

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

nor the gene score were associated with potential confounders including sex, age and BMI (Supplemental Table 4). Also, using ordinal lineal regression model adjusted by age, sex and RS cohort, we investigated the association between individual DHEAs SNPs and NT-proBNP and none of the SNPs was statistically significant at p-value<0.05 (Supplemental Table 5). In the MR analysis, applying 2SLS approach in the entire study group, using DHEAs genetic risk score as the instrumental variable, significant causal association was observed between DHEAs and NT-proBNP levels, either in the crude or adjusted analysis. Genetically predisposed higher levels of DHEAs were associated with decreased serum NT-proBNP levels [crude model β=-0.530 (95%CI:-0.863; -0.198; p=0.002) and adjusted model β=-0.450 (95%CI:-0.792; -0.107; p=0.010)] (Table 2). As in the observational analysis, we found significant interaction between DHEAs and sex we run the 2SLS analysis separate for men and women. After stratification by sex, in both men and women, results were similar to overall findings. However, in men the value of F statistics the GRS of DHEAs was close to value considered as a weak instrument (F <10), which could be due to low power, as we confirmed in the power calculation analysis. We applied an extension of MR, Eggers regression to test for horizontal pleiotropy. The intercept of the MR-Egger regression captures the average pleiotropic effect across all genetic variants. None of the analyses performed had a significant intercept indicating no directional pleiotropy.

Pathways analysis

The IPA core analysis was performed to determine the canonical pathways that link genes associated with NT-proBNP and DHEAs with heart failure and its related phenotypes

(Supplemental Table 7). This analysis indicated that the studied genes are directly or indirectly linked with inflammatory-related pathways and immunological diseases. We further generated common networks linking the genes associated with NT-proBNP and DEHAs to heart failure and its related phenotypes. As shown in supplementary table, these two are both linked by *NPPA* and *NPPB* genes from natriuretic peptide to *ARPC1A* gene from DHEAs indicating potential mechanisms explaining the association between NT-proBNP and DHEAs. Indeed, *NPPA* and *NPPB* are associated with cardiovascular morbidities as acute and chronic heart failure, atrial fibrillation and arrhythmia.

DISCUSSION

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

Our findings complement the preceding publication from the RS, where cross-sectional data from postmenopausal women free of CVD disease, have shown inverse association between DHEA and DHEAs and serum NT-proBNP[7]. Also, our results are in line with previous observational data. Several epidemiological studies have demonstrated an association between low serum levels of DHEAs with elevated CVD risk[23 24], cardiovascular morbidity[25-27], coronary artery disease [28 29] and vascular atherosclerotic disease[30]. Moriyama et al. reported positive association between DHEAs levels and left ventricular ejection fraction (LVEF), as well as inverse association with BNP levels in an Asian population, independently of age and other clinical variables[1]. Also, Kawano et al. showed DHEAs levels to increase upon improvement of ventricular function in patients undergoing HF treatment[31]. It has also been reported that DHEAS can be produced in cardio myocytes of structurally healthy hearts, but not in failing hearts[32].

Despite increasing evidence suggesting its beneficial cardiovascular effects, an intracellular steroid hormone receptor for DHEA has not been identified[3]. Recent reports suggested specific DHEA-binding sites in cardiovascular tissue[33 34], and that this putative receptor is present in the rat heart[34]. However, it is unclear if DHEA directly exerts its effects or if it acts after conversion to testosterone/17β-estradiol, via binding specific receptors for testosterone and 17β-estradiol that are present in the heart[35]. The inverse association between DHEAs and NT-proBNP can be explained by the opposite biological effect they produce. DHEA and DHEAs may play a beneficial role in cardiovascular system through modulation of several processes such as nitric oxide production stimulation, oxidation stress inhibition, prevention of vascular remodelling, stimulated vasodila-

tion[36]. Conversely, increased NPs production at both auricular and ventricular level, and progressively according to ventricular dysfunction, has been previously evidenced in patients with HF, which is in turn associated with increased oxidative stress, that might alter the electron transport mechanism at P450C17 cytochrome level, selectively suppressing 17,20 lyase enzyme activity, resulting in decreased DHEAs serum levels [23].

Recently, nine common genetic variants were associated with serum DHEAs, suggesting its key role in aging mechanisms[37]. Genes at or near these genetic variants include *ARPC1A*, *BCL2L11*, *ZKSCAN5*, *ZNF789*, *TRIM4*, *CYP2C9*, *BMF* and *SULT2A1*. These genes have various associations with steroid hormone metabolism co-morbidities of ageing including type 2 diabetes, lymphoma, actin filament assembly, drug and xenobiotic metabolism, and zinc finger proteins—suggesting a wider functional role for DHEAs than previously thought. Using DHEAs genetic risk score as an IV, our findings suggest that genetically predisposed higher DHEAs concentrations are inversely associated with NT-proBNP levels. Therefore, there might be a causal association between DHEAs and NPs. Still, the common biochemical pathways that link the metabolism of these two hormones are largely unknown, and should further be investigated. However, our pathways analysis shown common biological paths linking DHEAs and NT-pro-BNP in special for *NPPA* and *NPPB* from natriuretic peptide with *ARC1A* from DHEAs, with multiple cardiovascular morbidities. (Supplemental figure 3)

To the best of our knowledge, this is the first study to examine the causal association between DHEAs and NT-proBNP levels in a large population based sample of CVD free men and women. Also, DHEA and DHEAs are measured using chromatography-tandem mass spectrometry, which is at the moment considered to be a gold standard method[38]. Although MR is considered as a flexible and robust statistical method, there is a number of MR limitations which need to be considered, also, the limitations of the observational part of our analysis merits further discussion. First, in the RS, serum BNP levels were not measured, but solely its inactive precursor NT-proBNP. However, recent systematic reviews and meta-analyses demonstrated that both BNP and NT-proBNP have similar diagnostic and prognostic accuracy in CVDs[4]. Second, there were no publically available SNPs on DHEA, therefore, we were not able to calculate GRS of DHEA and we could not study the causal association between DHEA and NT-proBNP. Also, within the RS we did not identify any SNPS associated with serum DHEA. However, DHEAs is more stable sulphate ester of DHEA, and it can be converted back to DHEA by steroid sulfatase, which can be considered a good proxy of the association between DHEA and NT-proBNP as confirmed in our regression analysis (cross-sectional associations between DHEA and DHEAs and NT-proBNP were in line)[39]. Third, calculation of allele score is considered to be a good approach to avoid weak IV bias for reasons and also may increase the power and simplicity[18]. However, due to complex biology, the effects of all the variants in an allele score may not be well known, the instrumental variable assumptions may not

be satisfied for all the variants[18]. Weakly associated instruments (F statistics < 10) can bias causal estimates towards the observational estimate for one-sample MR. Indeed, the strength of the GRS as an instrument, measured by the F statistic was satisfactory overall, and in females, but in men F statistics was close to 10 indicating that in males, DHEAS GRS might be a weak instrument. However, we consider this could be due to that the association of androgens with NT-pro-BNP levels are more robust in women than men, as has been previously reported, and low power, as we confirmed in the power calculation analysis. Fourth, an important assumption of Mendelian randomization is that the genetic variant must mediate its effect on outcome only via the risk factor, i.e., the genetic variant shows no pleiotropic effects. Therefore, this assumption cannot be proven formally in practice because of incomplete knowledge of the underlying biology. However, we applied an extension of MR approach: MR Egger regression, to test for the causal effect free of pleiotropy. In simple words, provided the underlying assumptions are met, the slope of the MR Egger regression analysis should yield an estimate of the causal effect of DHEA on NT-proBNP that is free from any confounding effects due to horizontal pleiotropy. However, it is important to mention that the validity of MR Egger regression rests on the 'INSIDE assumption' (INstrument Strength is Independent of Direct Effect) which states that across all instruments there should be no correlation between the strength with which the instrument proxies the exposure of interest, and its degree of association with the outcome via pathways other than through the exposure[40]. This is a weaker requirement than the exclusion restriction criterion in normal MR which postulates that SNPs may only affect the outcome (NT-proBNP) through the exposure of interest (serum DHEAs), and so MR Egger regression is likely to be more robust to horizontal pleiotropy than standard MR approaches, although this appears to come at the cost of decreased power to detect a causal effect in one sample MR[40]. However, the MR-Egger intercept indicated no presence of horizontal pleiotropy.

Conclusions

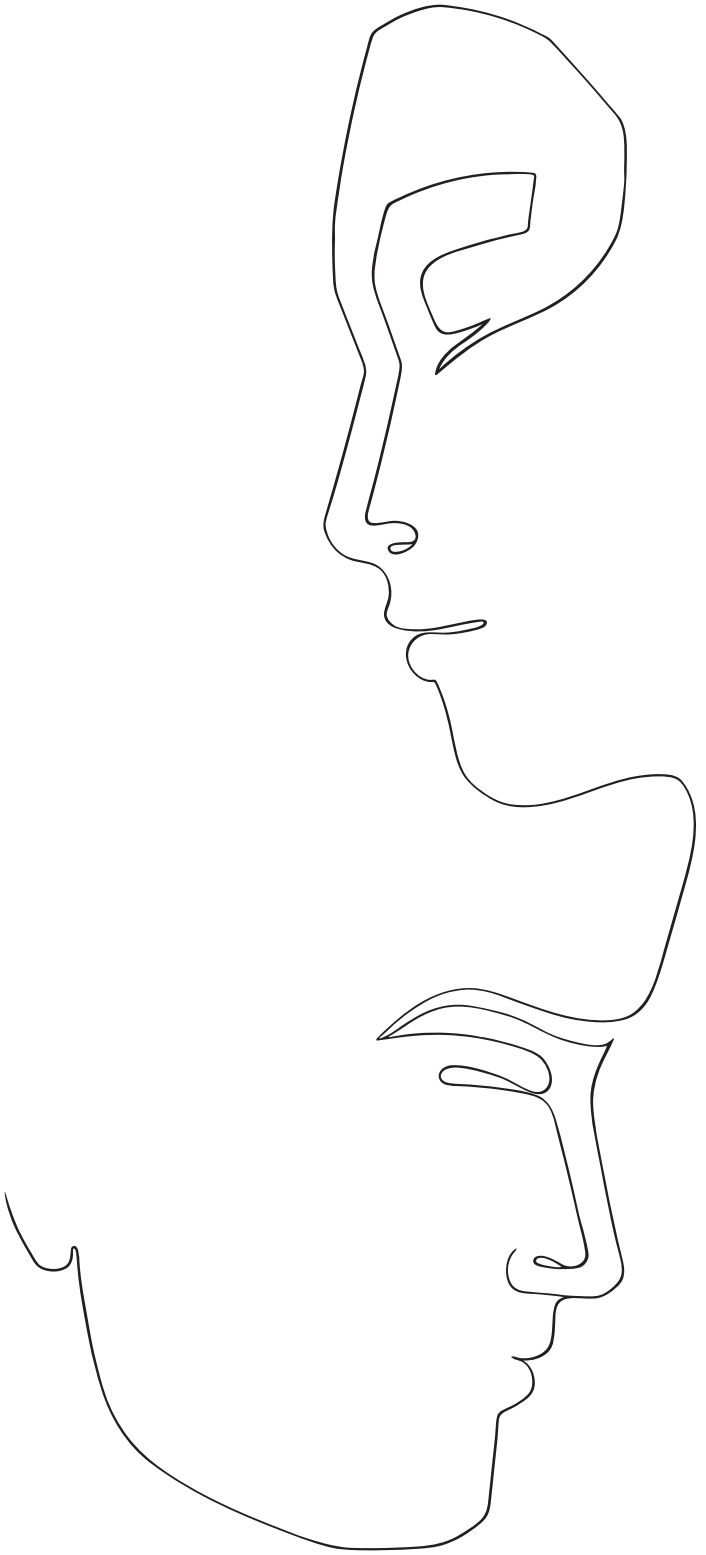
In cross-sectional analysis DHEA and DHEAs were significantly inversely associated with serum NT-ProBNP levels. Causal association we have observed between DHEAs and NT-proBNP suggests a new metabolic pathway linking DHEAs with NT-proBNP, which merits detailed experimental investigation in the future.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

REFERENCES

1. Moriyama Y, Yasue H, Yoshimura M, et al. The plasma levels of dehydroepiandrosterone sulfate are decreased in patients with chronic heart failure in proportion to the severity. J Clin Endocrinol Metab 2000;**85**(5):1834-40.
2. Wu TT, Chen Y, Zhou Y, et al. Prognostic Value of Dehydroepiandrosterone Sulfate for Patients With Cardiovascular Disease: A Systematic Review and Meta-Analysis. J Am Heart Assoc 2017;**6**(5).
3. Nakamura S YM, Nakayama M, Ito T, Mizuno Y, Harada E, Sakamoto T, Saito Y, Nakao K, Yasue H, Ogawa H. Possible association of heart failure status with synthetic balance between aldosterone and dehydroepiandrosterone in human heart. Circulation 2004;**110**(13):1787-93.
4. Clerico A, Giannoni A, Vittorini S, et al. The paradox of low BNP levels in obesity. Heart Fail Rev 2012;**17**(1):81-96.
5. Potter LR YA, Flora DR, Antos LK, Dickey DM. Natriuretic peptides: their structures, receptors, physiologic functions and therapeutic applications. Handb Exp Pharmacol 2009;**191**(341-66).
6. Lam CS, Cheng S, Choong K, et al. Influence of sex and hormone status on circulating natriuretic peptides. J Am Coll Cardiol 2011;**58**(6):618-26.
7. Glisic M, Rojas LZ, Asllanaj E, et al. Sex steroids, sex hormone-binding globulin and levels of N-terminal pro-brain natriuretic peptide in postmenopausal women. Int J Cardiol 2018.
8. Didelez V, Sheehan N. Mendelian randomization as an instrumental variable approach to causal inference. Statistical methods in medical research 2007;**16**(4):309-30.
9. Zhai G, Teumer A, Stolk L, et al. Eight common genetic variants associated with serum DHEAS levels suggest a key role in ageing mechanisms. PLoS Genet 2011;**7**(4):e1002025.
10. Ruth KS, Campbell PJ, Chew S, et al. Genome-wide association study with 1000 genomes imputation identifies signals for nine sex hormone-related phenotypes. Eur J Hum Genet 2016;**24**(2):284-90.
11. Hofman A, Brusselle GG, Darwish Murad S, et al. The Rotterdam Study: 2016 objectives and design update. Eur J Epidemiol 2015;**30**(8):661-708.
12. Yeo KT, Wu AH, Apple FS, et al. Multicenter evaluation of the Roche NT-proBNP assay and comparison to the Biosite Triage BNP assay. Clin Chim Acta 2003;**338**(1-2):107-15.
13. Gavin JR, Alberti KGMM, Davidson MB, et al. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 1997;**20**(7):1183-97.
14. Caspersen CJ, Bloemberg BP, Saris WH, et al. The prevalence of selected physical activities and their relation with coronary heart disease risk factors in elderly men: the Zutphen Study, 1985. Am J Epidemiol 1991;**133**(11):1078-92.
15. Stel VS, Smit JH, Pluijm SM, et al. Comparison of the LASA Physical Activity Questionnaire with a 7-day diary and pedometer. J Clin Epidemiol 2004;**57**(3):252-8.
16. Perrone RD, Madias NE, Levey AS. Serum creatinine as an index of renal function: new insights into old concepts. Clin Chem 1992;**38**(10):1933-53.
17. Fukuyama N, Homma K, Wakana N, et al. Validation of the Friedewald Equation for Evaluation of Plasma LDL-Cholesterol. J Clin Biochem Nutr 2008;**43**(1):1-5.
18. Burgess S, Thompson SG. Use of allele scores as instrumental variables for Mendelian randomization. Int J Epidemiol 2013;**42**(4):1134-44.
19. Lawlor DA, Harbord RM, Sterne JA, et al. Mendelian randomization: using genes as instruments for making causal inferences in epidemiology. Stat Med 2008;**27**(8):1133-63.
20. Greenland S. An introduction to instrumental variables for epidemiologists. Int J Epidemiol 2000;**29**(4):722-9.

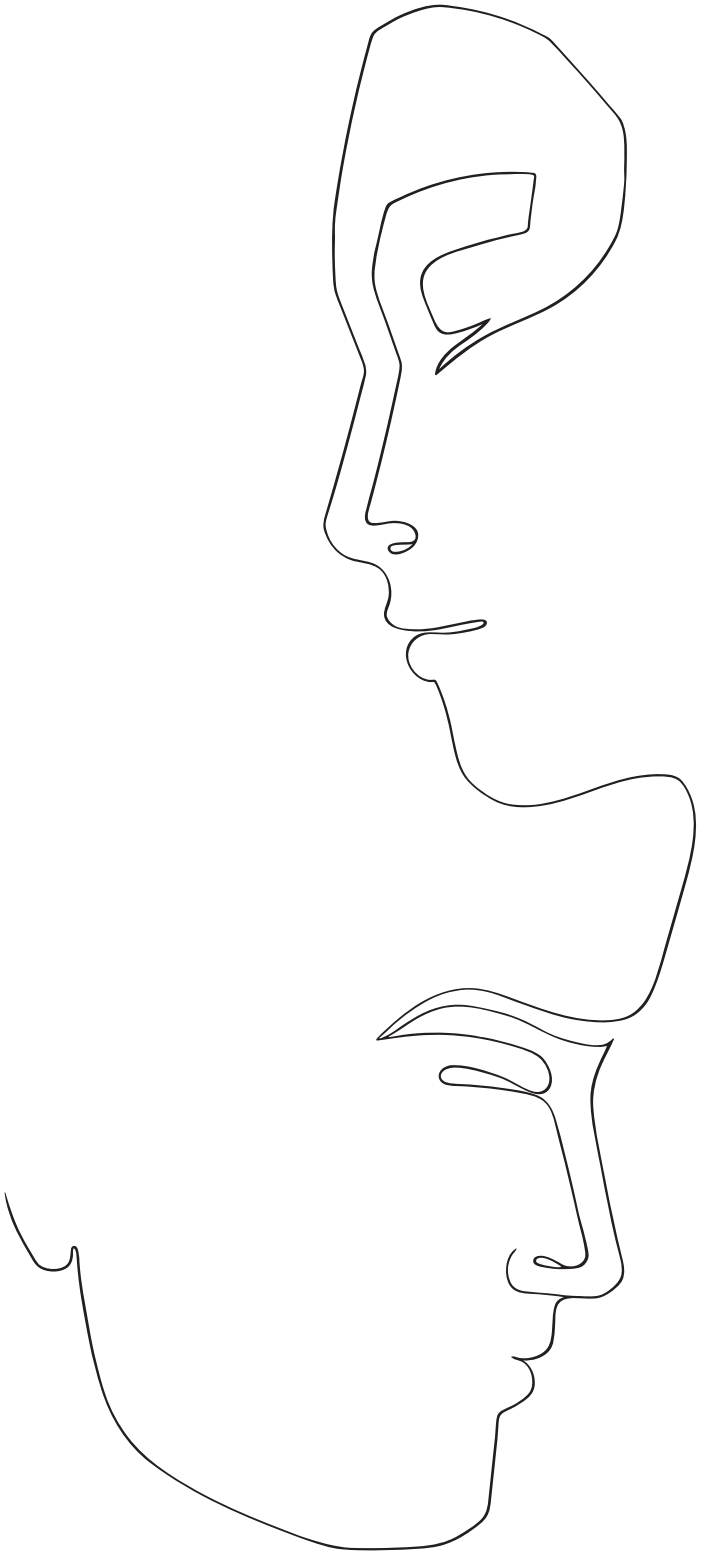
21. Kemp JP, Sayers A, Smith GD, et al. Using Mendelian randomization to investigate a possible causal relationship between adiposity and increased bone mineral density at different skeletal sites in children. Int J Epidemiol 2016;**45**(5):1560-72.
22. Pierce BL, Burgess S. Efficient design for Mendelian randomization studies: subsample and 2-sample instrumental variable estimators. Am J Epidemiol 2013;**178**(7):1177-84.
23. Abbasi A, Duthie EH, Jr., Sheldahl L, et al. Association of dehydroepiandrosterone sulfate, body composition, and physical fitness in independent community-dwelling older men and women. J Am Geriatr Soc 1998;**46**(3):263-73.
24. Shono N, Kumagai S, Higaki Y, et al. The relationships of testosterone, estradiol, dehydroepiandrosterone-sulfate and sex hormone-binding globulin to lipid and glucose metabolism in healthy men. J Atheroscler Thromb 1996;**3**(1):45-51.
25. Alexandersen P, Haarbo J, Christiansen C. The relationship of natural androgens to coronary heart disease in males: a review. Atherosclerosis 1996;**125**(1):1-13.
26. Feldman HA, Johannes CB, Araujo AB, et al. Low dehydroepiandrosterone and ischemic heart disease in middle-aged men: prospective results from the Massachusetts Male Aging Study. Am J Epidemiol 2001;**153**(1):79-89.
27. Trivedi DP, Khaw KT. Dehydroepiandrosterone sulfate and mortality in elderly men and women. J Clin Endocrinol Metab 2001;**86**(9):4171-7.
28. Herrington DM, Gordon GB, Achuff SC, et al. Plasma dehydroepiandrosterone and dehydroepiandrosterone sulfate in patients undergoing diagnostic coronary angiography. J Am Coll Cardiol 1990;**16**(6):862-70.
29. Mitchell LE, Sprecher DL, Borecki IB, et al. Evidence for an association between dehydroepiandrosterone sulfate and nonfatal, premature myocardial infarction in males. Circulation 1994;**89**(1):89-93.
30. Hak AE, Witteman JC, de Jong FH, et al. Low levels of endogenous androgens increase the risk of atherosclerosis in elderly men: the Rotterdam study. J Clin Endocrinol Metab 2002;**87**(8):3632-9.
31. Kawano H, Nagayoshi Y, Soejima H, et al. Dehydroepiandrosterone levels vary according as heart failure condition in patients with idiopathic dilated cardiomyopathy. Int J Cardiol 2008;**125**(2):277-9.
32. Nakamura S, Yoshimura M, Nakayama M, et al. Possible association of heart failure status with synthetic balance between aldosterone and dehydroepiandrosterone in human heart. Circulation 2004;**110**(13):1787-93.
33. Williams MR, Ling S, Dawood T, et al. Dehydroepiandrosterone inhibits human vascular smooth muscle cell proliferation independent of ARs and ERs. J Clin Endocrinol Metab 2002;**87**(1):176-81.
34. Liu D, Dillon JS. Dehydroepiandrosterone activates endothelial cell nitric-oxide synthase by a specific plasma membrane receptor coupled to Galpha(i2,3). J Biol Chem 2002;**277**(24):21379-88.
35. Grohe C, Kahlert S, Lobbert K, et al. Expression of oestrogen receptor alpha and beta in rat heart: role of local oestrogen synthesis. J Endocrinol 1998;**156**(2):R1-7.
36. Savineau JP, Marthan R, Dumas de la Roque E. Role of DHEA in cardiovascular diseases. Biochem Pharmacol 2013;**85**(6):718-26.
37. Zhai G, Teumer A, Stolk L, et al. Eight common genetic variants associated with serum DHEAS levels suggest a key role in ageing mechanisms. PLoS genetics 2011;**7**(4):e1002025.
38. Rosner W, Auchus RJ, Azziz R, et al. Position statement: Utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement. J Clin Endocrinol Metab 2007;**92**(2):405-13.

39. Maninger N, Wolkowitz OM, Reus VI, et al. Neurobiological and neuropsychiatric effects of dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS). Front Neuroendocrinol 2009;**30**(1):65-91.
40. Bowden J, Davey Smith G, Burgess S. Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. Int J Epidemiol 2015;**44**(2):512-25.



3

Lifestyle, cardiometabolic risk and longevity



3.1

Obese ex-smokers live longer overall and in good health, but spend more years with diabetes than normal weight smokers: findings from the Rotterdam Study

ABSTRACT

Objective

We aimed to examine whether total life expectancy and life expectancy with and without type 2 diabetes differs between smokers and non-smokers, and between normal-weight smokers and overweight or obese ex-smokers.

Methods

10 738 participants aged 50+ years old were included from the population-based Rotterdam Study. Multistate life tables were developed to calculate life expectancy for individuals who were (i) current, former and never smokers as well as for (ii) normal-weight current smokers, overweight and obese ex-smokers.

Results

Compared to never smokers, currently smoking men at the age of 50 years, lived 6.3 years fewer overall, of which 0.9 years fewer with type 2 diabetes; whereas current smoker women lived 5. years fewer overall of which 0.4 years more with type 2 diabetes. Obese and overweight former smoker men lived 4.8 and 4.0 years longer than normal-weight current smoking men, of which 4.7 and 1.6 years longer with type 2 diabetes, respectively. For women, obese and overweight ex-smokers lived 9.2 and 8.0 years longer than their current normal-weight counterparts, of which 7.8 and 4.6 years longer with type 2 diabetes, respectively.

Conclusions

In our study, the benefits of quitting smoking outweigh the risk of weight gain in terms of overall life expectancy, but a great part of this increased life expectancy is spent with type 2 diabetes. Due to the increased risk of type 2 diabetes, people who quit smoking and gain weight may be a group to target for proactive type 2 diabetes prevention strategies.

INTRODUCTION

Smoking is the leading avoidable cause of death in the world by killing around 6 million people a year[1]. Besides the well-documented risk for cancer and cardiovascular disease from smoking, a recent meta-analysis reported a 37% increased risk of developing type 2 diabetes (T2D) for current smokers compared to never smokers[2]. Also, studies evaluating the association between smoking and life expectancy have shown that current smokers live shorter compared to nonsmokers, with a reduction of 9 years[3]. However, despite declines in smoking rates in the last decades, more updated scientific evidence is needed, considering that the absolute number of smokers is increasing along with improvements in prevention and treatment of T2D[4].

The beneficial effects of smoking cessation in reducing the risk of disease and in preventing T2D is supported by a large amount of evidence[5 6]. However, smoking cessation is often accompanied by weight gain, with various studies reporting average increases of 4–8 kg, but with 10% to 13% of quitters gaining at least 11 kg [7 8]. Consequently, post smoking cessation weight gain is reported among smokers who have tried to quit as the main cause for their relapse, and among women, as the main reason for not trying to quit[9 10]. This increase in adiposity could blur the benefits of smoking cessation and paradoxically increase the risk of having T2D[11]. In addition, overweight increases the risk of other diseases such as cardiovascular diseases mortality[12]. Therefore, it is of interest to examine which is least detrimental with regard to risk of T2D and mortality; being a normal-weight smoker or to quit smoking and possibly become an overweight or obese ex-smoker? Previous studies have reported that, compared to current normal-weight smokers, overweight or obese ex-smokers are at lower risk of mortality[13-15]. No study to date has examined how incidence of diabetes is different between normal-weight smokers and overweight or obese ex-smokers. Further, there is no information about the burden of T2D associated with smoking cessation and obesity, and their combined net effect on mortality and life expectancy, which would help to shape health policy, effective interventions and accurate projections of future health-care costs.

Using data from a population-based study of subjects 45+years, we evaluated the impact of smoking status on overall life expectancy and years lived with and without T2D. Further, we investigated whether risk of T2D and mortality, and consequently total life expectancy and life expectancy with and without T2D differed between normal-weight smokers and overweight/obese ex-smokers.

METHODS

Assessment of Smoking Status and Obesity

Information on smoking behaviour was obtained using a computerized questionnaire during the home visit. Participants were classified as current smokers, former smokers, or never smokers. Current smokers were participants who answered yes to the question: "are you currently smoking?" Former smokers were participants who answered no to this question but who positively answered the question: "are you a former smoker?" Height and weight at baseline were measured in our research center. BMI at baseline was calculated as weight in kg/height in m^2. We defined three BMI categories based on World Health Organization guidelines[16]: normal weight, BMI of 18.5 to 24.9 kg/m^2; overweight, BMI of 25 to 29.9 kg/m^2; and obese, BMI greater than or equal to 30 kg/m^2.

Ascertainment of Type 2 Diabetes

The participants were followed from the date of baseline centre visit onwards. Cases of T2D were ascertained at baseline and during follow-up through: (i) active follow-up using general practitioners' records, (ii) glucose hospital discharge letters and (iii) glucose measurements from RS visits that take place approximately every 4 years[17]. T2D was defined according to recent WHO guidelines, as a fasting blood glucose ≥ 7.0 mmol/L, a non-fasting blood glucose ≥ 11.1 mmol/L (when fasting samples were absent), or the use of blood glucose lowering medication. Information regarding the use of blood glucose lowering medication was derived from both structured home interviews and linkage to pharmacy records[17]. At baseline, more than 95% of the population was covered by the pharmacies in the study area. Two study physicians independently adjudicated all potential events of T2D. In case of disagreement, consensus was sought with an endocrinologist. Follow-up data was complete until January 1st 2012.

Follow up for Mortality

Mortality data were obtained by notification from the municipal administration. Data on all-cause mortality and living status were updated until August 1st 2016. Participants were followed from the first day they entered the study till the day of death, the day of lost to follow-up or the last date of contact, whichever came first.

Information on *Study Population, Population for Analyses* and *Assessment of Potential Confounders and Intermediates* is presented in the supplementary material.

Data Analysis

We created multistate life tables to calculate the differences in life expectancy and years lived with and without T2D by sex in: (i) current, former and never smokers, as well for (ii) normal-weight smokers, overweight ex-smokers and obese ex-smokers. We considered

three different health states: free of T2D, T2D, and death. Participants could experience the following transitions: from free of T2D to T2D, from free of T2D to death, and from T2D to death. No backflows were allowed, and only the first entry into a state was considered.

To obtain transition rates, we first calculated the overall age and sex-specific rates for each transition. Next, we calculated the prevalence of (i) current, former and never smokers and, of (ii) normal-weight smokers, overweight and obese ex-smokers, by 10-y age groups and sex , and separately for subjects with and without diabetes. Hazard ratios (HRs) comparing participants who were (i) current and former smokers with never smokers, and participants who were (ii) overweight and obese ex-smokers with normal-weight smokers, were calculated using Poisson regression ("Gompertz" distribution) in 2 models. Model 1 adjusted for age, education level, alcohol intake (continuous), physical activity (continuous), diet quality score (ordinal), coffee intake and cohort (I, II and III) and model 2 additionally adjusted for the following potential intermediates: systolic blood pressure, cholesterol levels, antihypertensive medication, statin use and comorbidities (prevalent cardiovascular disease (CVD), cancer and chronic obstructive pulmonary disease (COPD)). For the analysis on current, former and never smokers, we also added BMI in the second model. In our data, time since quitting smoking was not associated with diabetes risk, therefore we did not adjusted for it in our models. Finally, we calculated three sets of transition rates for each category using (i) the overall transition rates, (ii) the adjusted HRs for T2D and mortality, and (iii) the prevalence of smoking and smoking by BMI categories by presence of T2D. Similar calculations have been described previously[18].

The multistate life table started at age 50 years and closed at age 90 years. Multiple imputations were performed in case of missing covariates. Statistical analyses were conducted using IBM SPSS, version 21 (IBM Corp) and STATA, version 13 for Windows (StataCorp). We used Monte Carlo simulation (parametric bootstrapping) to calculate the confidence intervals of our life expectancy estimates with @RISK software (Palisade) 10 000 runs[19].

RESULTS

Baseline characteristics

The final study population consisted of 10 738 individuals: 4 615 men and 6 123 women as shown in **Table 3.1.1**. In total, we observed 884 incident diabetes events and 3 484 overall deaths during 14 years of follow-up. The mean age (standard deviation) of the population was 62 (SD=8.2) (**Table 3.1.1**). For the analysis on overweight or obese former smokers and normal-weight current smokers, there were 363 incidence T2D cases and 934 deaths.

Table 3.1.1. Baseline characteristics of 10 738 participants

Characteristics	Men (n= 4 615)	Women (n=6 123)
Age, mean (SD), y	61.6 (8.0)	62.3 (8.8)
Type 2 diabetes, Yes (%)	662 (14.3)	651 (10.6)
Education, No. (%)		
Primary	430 (9.3)	926 (15.1)
Lower	1310 (28.4)	3072 (50.2)
Intermediate	1697 (36.8)	1407 (23.0)
Higher/university	1178 (25.5)	718 (11.7)
Smoking Categories, Yes. (%)	709 (15.4)	2581 (42.2)
Never smoker		
Former smoker	2709 (58.7)	2328 (38.0)
Current smoker	1197 (25.9)	1213 (19.8)
Smoking/BMI categories, Yes. (%)	385 (8.3)	441 (7.2)
Current Normal weight		
Former Overweight	1949 (42.2)	1335 (21.8)
Former Obese	848 (18.4)	1089 (17.8)
Physical activity (METhours/week)	65.0 (45.4)	82.1 (49.7)
Diet quality score, mean (SD)	53.4 (9.9)	57.0 (10.2)
Coffee intake g/day, mean (SD)	529.4 (293.1)	456.3 (261.3)
Systolic blood pressure (mm/Hg), mean (SD)	142.4 (39.1)	140.3 (39.2)
Serum total cholesterol (mmol/l), mean (SD)	5.5 (0.9)	5.9 (1.0)
Anti-hypertensive medication use, Yes (%)	1516 (32.8)	2027 (33.1)
Body mass index, BMI, mean (SD), kg/m^2	27.1 (3.7)	27.5 (4.6)
Alcohol, mean (SD), g/day	13.5 (18.4)	7.8 (12.3)
Prevalent Cancer, Yes (%)	622 (13.5)	696 (11.4)
Prevalent COPD, Yes (%)	281 (6.1)	284 (4.6)
Prevalent CVD, Yes (%)	787 (17.0)	449 (7.3)

Smoking status, diabetes incidence, mortality and life expectancy

Diabetes incidence and mortality

The association between smoking categories and the risk of incident type 2 diabetes and mortality is shown in **Table 3.1.2.** In multivariable adjusted model, current smoking, but not smoking cessation, was associated with an increased risk of T2D in men 1.52 (95% CI, 1.06, 2.18) and women 1.54 (95% CI, 1.2, 1.96) compared to never smoking. Among those free of T2D, compared to never smokers, current but not former smokers, had an increased risk of mortality among men 1.98 (95% CI, 1.54, 2.55) and women 2.17(95% CI, 1.82, 2.58). Among those with T2D, the HRs for mortality were 2.81 (95% CI, 1.74, 4.54), and 1.42 (95% CI, 1.0, 2.03) for current smoker men and women, respectively, relative to never smokers **(Table 3.1.2)**.

Table 3.1.2. Hazard ratios for incident type 2 diabetes (T2D) and death among categories of smoking.*

Transition	Categories	Men			Women		
		Cases, Number/ Person-Years	Model 1 HR (95% CI)[a]	Model 2 HR (95% CI)[b]	Cases, Number/ Person-Years	Model 1 HR (95% CI)[a]	Model 2 HR (95% CI)[b]
Incident T2D	Never	383/29655	1.00	1.00	501/43000	1.00	1.00
	Former		1.13 (0.81-1.58)	1.04 (0.74-1.45)		1.07 (0.88-1.31)	1.03 (0.84-1.26)
	Current		1.48 (1.03-2.1)	1.52 (1.06-2.18)		1.37 (1.08-1.74)	1.54 (1.2-1.96)
No T2D to mortality	Never	958/31355	1.00	1.00	965/45488	1.00	1.00
	Former		1.23 (0.97-1.55)	1.17 (0.93-1.49)		1.08 (0.93-1.26)	1.06 (0.92-1.23)
	Current		2.11 (1.64-2.72)	1.98 (1.54-2.55)		2.15 (1.81-2.56)	2.17 (1.82-2.58)
T2D to mortality	Never	370/6445	1.00	1.00	307/7495	1.00	1.00
	Former		1.55 (0.99-2.43)	1.56 (0.99-2.47)		1.0 (0.78-1.29)	1.00 (0.77-1.29)
	Current		2.97 (1.85-4.77)	2.81 (1.74-4.54)		1.35 (0.95-1.91)	1.42 (1.00-2.03)

Abbreviations: HR, Hazard Ratio; CI, Confidence Interval; T2D, Type 2 Diabetes, BMI, Body Mass Index.

*Age 50 and over at start of follow-up

[a] Adjusted for age, cohort, alcohol intake, education, coffee, physical activity and diet quality score.

[b] Adjusted for age, cohort, alcohol intake, education, coffee, physical activity, systolic blood pressure, serum total cholesterol, anti-hypertensive medication use, statin use, BMI, diet quality score, and comorbidities.

Total life expectancy and life expectancy with and without diabetes

Compared to never smoker men, the total life expectancy of 50-y-old men in the current smoking group was 6.3 years (95% CI, -10.2, -2.7) shorter and for women, the difference was 5.0 (95% CI, -7.2, -3.3) years shorter **(STable 1)**. For both men 5.4 (95% CI, -8.9, -2.9) and women 5.4 (95% CI, -7.2, -3.5), current smoking was associated with fewer years lived without diabetes, but not with years lived with diabetes. Moreover, compared to never smokers, former smoker men, but not women lived 1.5 (95% CI, -4.0, -0.2) fewer free of T2D.

Smoking status by BMI categories, diabetes incidence, mortality and life expectancy

Diabetes incidence and mortality

The association between smoking categories by weight status and the risk of diabetes and mortality among men and women is shown in **Table 3.1.3**. Obese ex-smoker men 1.89 (95% CI, 1.15, 3.12) and women 2.19 (95% CI, 1.3, 3.68) had an increased risk of developing T2D, compared to normal-weight current smokers. Among those free of T2D, overweight ex-smoker men 0.62 (95% CI, 0.48, 0.78) and women 0.4(95% CI, 0.29, 0.55), as well as obese ex-smoker men 0.5 (95% CI, 0.35, 0.71) and women 0.36 (95% CI, 0.25, 0.51) had a reduced risk of mortality compared to normal-weight current smokers

Table 3.1.3. Hazard ratios for incident type 2 diabetes (T2D) and death among categories of smoking by BMI.*

		Men			Women		
Transition	Categories	Cases, Number/ Person-Years	Model 1 HR (95% CI)[a]	Model 2 HR (95% CI)[b]	Cases, Number/ Person-Years	Model 1 HR (95% CI)[a]	Model 2 HR (95% CI)[b]
Incident T2D	Current Normal weight	193/16159	1.00	1.00	170/14484	1.00	1.00
	Former Overweight		1.14 (0.73-1.77)	1.14 (0.73-1.78)		1.68 (1.02-2.76)	1.54 (0.92-2.55)
	Former Obese		1.97 (1.2-3.2)	1.89 (1.15-3.12)		2.58 (1.56-4.26)	2.19 (1.3-3.68)
No T2D to mortality	Current Normal weight	444/14914	1.00	1.00	235/13297	1.00	1.00
	Former Overweight		0.62 (0.48-0.78)	0.62 (0.48-0.78)		0.41 (0.30-0.56)	0.40 (0.29-0.55)
	Former Obese		0.54 (0.38-0.75)	0.50 (0.35-0.71)		0.36 (0.26-0.51)	0.36 (0.25-0.51)
T2D to mortality	Current Normal weight	182/4092	1.00	1.00	73/2866	1.00	1.00
	Former Overweight		0.51 (0.34-0.76)	0.51 (0.34-0.78)		0.39 (0.19-0.79)	0.32 (0.15-0.7)
	Former Obese		0.43 (0.26-0.69)	0.44 (0.27-0.73)		0.41 (0.21-0.83)	0.34 (0.15-0.73)

Abbreviations: HR, Hazard Ratio; CI, Confidence Interval; T2D, Type 2 Diabetes.

*Age 50 and over at start of follow-up

[a] Adjusted for age, cohort, alcohol, education, coffee, physical activity and diet quality score.

[b] Adjusted for age, cohort, alcohol, education, coffee, physical activity, systolic blood pressure, cholesterol, anti-hypertensive medication, statin, diet quality score and comorbidities

(Table 3.1.3). Also among those with T2D, obese ex-smoker men 0.44 (95% CI, 0.27, 0.73) and women 0.34 (95% CI, 0.15, 0.73) and overweight ex-smoker men 0.51(95% CI, 0.34, 0.78) and women 0.32 (95% CI, 0.15, 0.7) had a reduced risk of mortality **(Table 3.1.3)**.

Total life expectancy and life expectancy with and without diabetes

Compared to normal-weight current smoker men, the total life expectancy of obese and overweight ex-smoker men, was 4.8 (95% CI, 3.4, 5.9) and 4.0 (95% CI, 3.2, 4.9) years longer **(Fig 1 and STable 2)**. The total life expectancy of 50-year-old obese and overweight ex-smoker women, were 8.3 (95% CI, 7.4, 9.2) and 7.6 (95% CI, 6.8, 8.4) years longer than their current smoker normal-weight counterparts. For overweight ex-smoking men and women, this increased life expectancy included more years with and more years without diabetes; whereas for obese ex-smoking men and women this increased life expectancy was explained by only more years with diabetes. Overweight ex-smoker men lived 2.4 (95% CI, 1.0, 4.2) years more free of T2D and 1.6 (95% CI, 0.3, 2.8) with T2D; whereas obese ex-smoker men lived 4.8 (95% CI, 1.1, 8.8) years more with T2D. For women, compared to normal weight current smokers, overweight an obese ex-smokers, lived 3.5 (95% CI, 0.4, 5.9) and 1.4 (95% CI, -0.4, 6.6) years more free of T2D and 4.1 (95% CI, 1.3, 7.0) and 6.8 (95% CI, 1.6, 11.8) years more with T2D **(Fig 2 and STable 2)**.

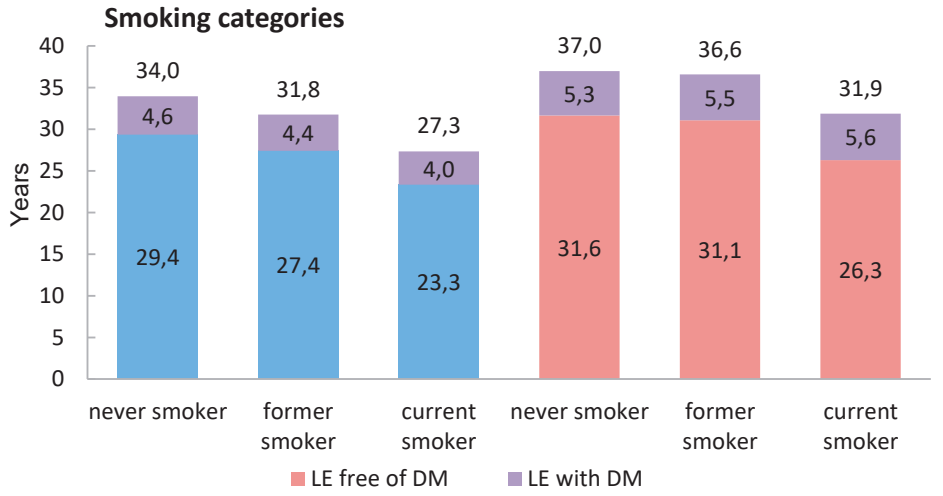


Figure 3.1.1. Life expectancy (LE) at 50 years among smoking categories, in men and women *
*All life expectancies have been calculated with hazard ratios adjusted for age, cohort, alcohol, coffee, education, physical activity and diet quality.

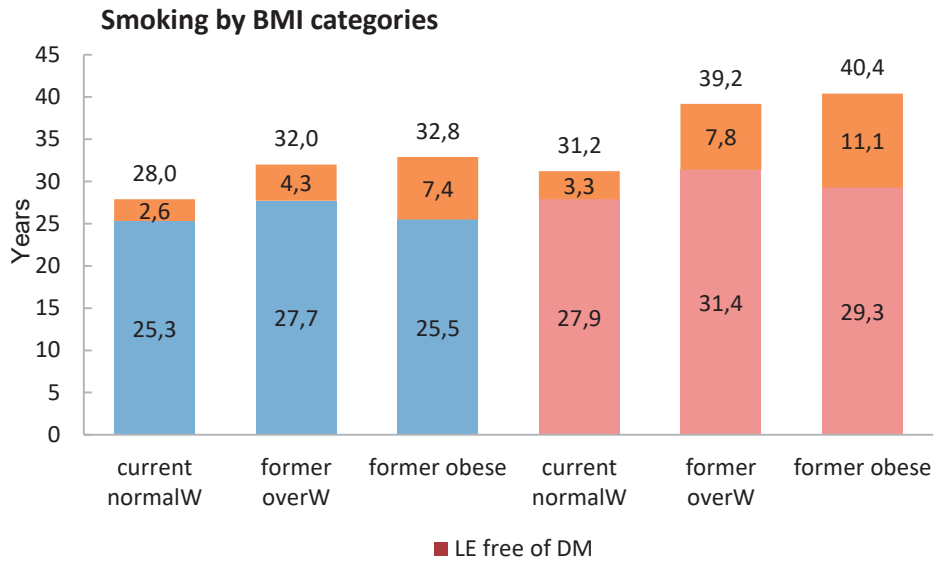


Figure 3.1.2. Life expectancy (LE) at 50 years among smoking categories by BMI, in men and women *.
*All life expectancies have been calculated with hazard ratios adjusted for age, cohort, alcohol, coffee, education, physical activity and diet quality.

Sensitivity analysis

The results did not change substantially after adjusting for potential intermediate risk factors, cancer and comorbidities **(STable 2, 3 and S Fig 3, 4)**, or excluding participants

with prevalent chronic diseases like cancer, CVD and COPD **(STable 5, 6 and S Fig 9, 10).** Furthermore, we repeated the analyses for 7 and 10 years of follow-up **(S Table 3, 4, 5, 6 and S Fig 5, 6, 7, 8)** and the results did not materially change.

DISCUSSION

Total life expectancy for men and women who were current smoker, at age 50 years was 6.3 and 5 years shorter, respectively, than for people who never smoked, with less years spending free of T2D, but with no difference in years lived with T2D. Similarly, men who were former smoker, but not women, lived overall 1.9 years less than never smokers, and spent fewer years free of T2D. However, former smokers who were overweight or obese presented a higher risk of developing T2D but also an extended number of years lived with diabetes, when compared with normal weight current smokers. Moreover, overweight but not obese ex-smokers spend more years free of T2D. On average, total life expectancy of an obese ex-smoker was 4.8 years higher in men and 8.3 years higher in women compared to current smoker normal weight, with most of these years spent with T2D.

In our study, total life expectancy in individuals aged 50 years and over for both men and women decreased drastically from never smokers to current smokers, with never and former smokers living longer than current smokers. Similar to our results, a long-term prospective cohort study from the Netherlands, based on 1 373 men participants showed earlier that at the age of 40 years, smoking cessation was associated with up to 5 years increase in total life expectancy[3]. We extended the previous evidence by calculating life expectancy in both men and women using multi state life tables and adjusting for a broader range of confounders and potential intermediate factors. Further, for the first time we show that smoking reduces the diabetes-free years with no impact on years lived with diabetes. Previous studies report an increase in number of disease-free life-years by 3 years associated with smoking cessation, but the disease free state did not include diabetes[3]. Therefore, our study supports the positive impact that smoking cessation has on cardiometabolic health and overall life expectancy.

Weight gain after smoking cessation is reported to be an important reason for not quitting[7]. Moreover, weight gain has adverse health effects as increased risk of diabetes and mortality[11]. Our results show that overweight/obese ex-smokers, especially women, compared to current normal-weight smokers are at increased risk of developing T2D, but they have lower risk of mortality. In line with our findings, Siahpush et al. show in their paper that compared with normal-weight smokers, the risk of mortality from T2D was 4% and 98% higher in overweight and obese ex-smokers, respectively[13]. Our study extended these results by calculating the association of obese and overweight

ex-smokers with incidence T2D and life expectancy, showing that normal-weight smokers lived shorter and spent more years with T2D than overweight or obese ex-smokers. The longer total life expectancy observed for obese ex-smokers was the result of the higher number of years lived without diabetes and the longer life expectancy with T2D. The increased life expectancy with T2D among participants, who were former smokers and overweight compared with the current smoker normal weight group, was statistically significant. The number of years lived with diabetes is a consequence of incident diabetes risk and mortality risk among those with diabetes. Higher incidence of diabetes would lead to an earlier occurrence of diabetes, whereas lower risk of mortality among those with diabetes would lead to greater number of years lived with diabetes.

The longer T2D–free life expectancy for overweight ex-smokers but not for obese ex-smokers, might be due to a harmful effect of a higher BMI on the incidence of diabetes combined with a protective effect of smoking cessation on mortality, among participants free of T2D. On the other hand, the longer life expectancy with T2D might be from the effect of BMI on mortality among participants with T2D: people with T2D, obese but former smokers lived longer and therefore experienced an increased burden of T2D. Another reason for the increase in years with T2D is that smoking cessation is associated with increased survival to advanced ages when the risks of diabetes are higher.

In our study, there is a difference in total life expectancy and in the number of years lived with diabetes among men and women. This gender difference might be influenced by the differential distribution of ever smokers (85% in man and 58% in woman), mainly due to the differences in former smokers, which could also explain some of the non-significant associations in women. Compared to men, women had an increased risk of diabetes when categorized by smoking status and BMI. These differences could be explained by the effects of smoking and obesity on T2D and mortality, expressed differently in men and women. Smoking has an anti-oestrogenic effect, which is related to hormonal imbalance that could lead to more adverse health effects in women [20]. Furthermore, genetic studies have found that levels of DNA adducts are higher[21] and DNA repair capacity lower[22] in women compared to men, suggesting that women may be more susceptible to the DNA damaging effects of smoking than men. Further, it should be taken in consideration the potential association and linkage to substance abuse, obesity and cardiovascular diseases, including hypertension, as a response to environmental mental stressor, which are attenuated by cigarette and alcohol use[23].

Several limitations of this study must be considered. Smoking status was assessed retrospectively by self-reported questionnaires. However, because T2D and mortality were assessed prospectively, the subjective measure of smoking would likely lead to non-differential misclassification with respect to the outcome, and therefore would likely dilute our estimates toward the null. Moreover, we did not have data on BMI before and after smoking cessation, therefore our results comparing normal weight current

smokers with overweight/obese ex-smokers should be interpreted with caution. Other longitudinal studies should investigate the process of weight gain and its occurrence in post smoking cessation. Further, the generalizability of these findings could be limited to middle-aged and older white European populations.

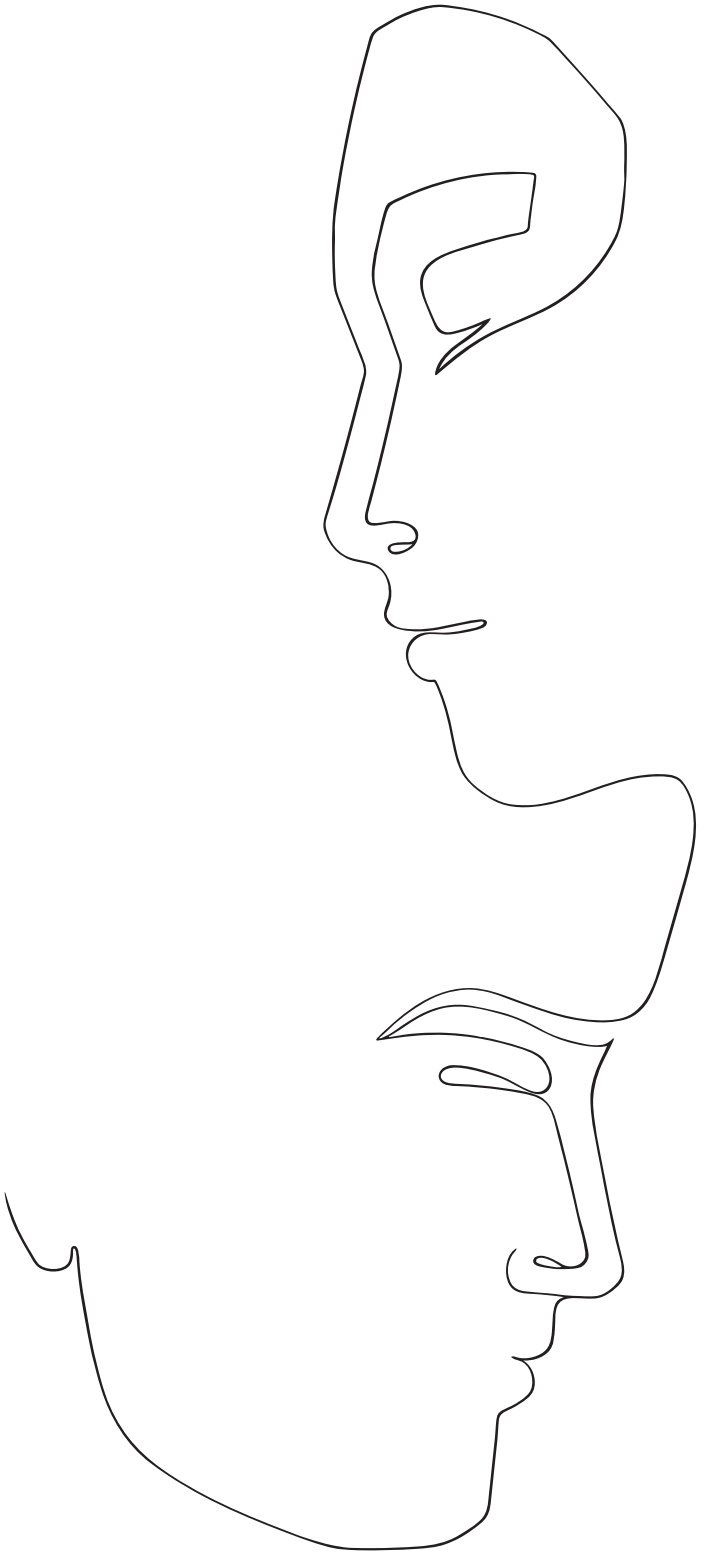
Conclusions

Our findings suggest that a potential risk of being overweight due to smoking cessation does not outweigh the benefits of smoking cessation in terms of reductions in mortality risks or spending more years diseased. In addition, healthcare providers and public health professionals should advise people who smoke that even if they gain weight because of quitting, it is still a healthier option than continuing to smoke. Nevertheless, due to the increased risk of diabetes, people who quit smoking and gain weight may be a group to target for proactive T2D prevention strategies.

REFERENCES

1. WHO report on the global tobacco epidemic, 2008: The MPOWER package. Popul Dev Rev 2008;**34**(3):581-81.
2. Pan A, Wang Y, Talaei M, et al. Relation of active, passive, and quitting smoking with incident type 2 diabetes: a systematic review and meta-analysis. Lancet Diabetes Endocrinol 2015;**3**(12):958-67.
3. Streppel MT, Boshuizen HC, Ocke MC, et al. Mortality and life expectancy in relation to long-term cigarette, cigar and pipe smoking: The Zutphen Study. Tob Control 2007;**16**(2):107-13.
4. Chang SA. Smoking and type 2 diabetes mellitus. Diabetes Metab J 2012;**36**(6):399-403.
5. Pan A, Wang YL, Talaei M, et al. Relation of active, passive, and quitting smoking with incident type 2 diabetes: a systematic review and meta-analysis. Lancet Diabetes Endo 2015;**3**(12):958-67.
6. Nagrebetsky A, Brettell R, Roberts N, et al. Smoking cessation in adults with diabetes: a systematic review and meta-analysis of data from randomised controlled trials. Bmj Open 2014;**4**(3).
7. Aubin HJ, Farley A, Lycett D, et al. Weight gain in smokers after quitting cigarettes: meta-analysis. Brit Med J 2012;**345**.
8. Lycett D, Munafo M, Johnstone E, et al. Associations between weight change over 8 years and baseline body mass index in a cohort of continuing and quitting smokers. Addiction 2011;**106**(1):188-96.
9. Pisinger C, Jorgensen T. Weight concerns and smoking in a general population: the Inter99 study. Prev Med 2007;**44**(4):283-9.
10. Pomerleau CS, Zucker AN, Stewart AJ. Characterizing concerns about post-cessation weight gain: results from a national survey of women smokers. Nicotine Tob Res 2001;**3**(1):51-60.
11. Dhana K, Nano J, Ligthart S, et al. Obesity and Life Expectancy with and without Diabetes in Adults Aged 55 Years and Older in the Netherlands: A Prospective Cohort Study. Plos Medicine 2016;**13**(7).
12. Global BMIMC, Di Angelantonio E, Bhupathiraju Sh N, et al. Body-mass index and all-cause mortality: individual-participant-data meta-analysis of 239 prospective studies in four continents. Lancet 2016;**388**(10046):776-86.
13. Siahpush M, Singh GK, Tibbits M, et al. It is better to be a fat ex-smoker than a thin smoker: findings from the 1997-2004 National Health Interview Survey-National Death Index linkage study. Tob Control 2014;**23**(5):395-402.
14. Koster A, Leitzmann MF, Schatzkin A, et al. The combined relations of adiposity and smoking on mortality. Am J Clin Nutr 2008;**88**(5):1206-12.
15. Freedman DM, Sigurdson AJ, Rajaraman P, et al. The mortality risk of smoking and obesity combined. Am J Prev Med 2006;**31**(5):355-62.
16. Consultation W. Obesity: Preventing and managing the global epidemic - Introduction. Who Tech Rep Ser 2000;**894**:1-253.
17. Leening MJG, Kavousi M, Heeringa J, et al. Methods of data collection and definitions of cardiac outcomes in the Rotterdam Study. Eur J Epidemiol 2012;**27**(3):173-85.
18. Franco OH, de Laet C, Peeters A, et al. Effects of physical activity on life expectancy with cardiovascular disease. Arch Intern Med 2005;**165**(20):2355-60.
19. Rindskopf D. An introduction to the bootstrap - Efron, B, Tibshirani, RJ. J Educ Behav Stat 1997;**22**(2):245-45.
20. Windham GC, Mitchell P, Anderson M, et al. Cigarette smoking and effects on hormone function in premenopausal women. Environ Health Perspect 2005;**113**(10):1285-90.

21. Ryberg D, Hewer A, Phillips DH, et al. Different Susceptibility to Smoking-Induced DNA-Damage among Male and Female Lung-Cancer Patients. Cancer Res 1994;**54**(22):5801-03.
22. Wei QY, Cheng L, Amos CI, et al. Repair of tobacco carcinogen-induced DNA adducts and lung cancer risk: a molecular epidemiologic study. J Natl Cancer I 2000;**92**(21):1764-72.
23. Nikpay M, Seda O, Tremblay J, et al. Genetic mapping of habitual substance use, obesity-related traits, responses to mental and physical stress, and heart rate and blood pressure measurements reveals shared genes that are overrepresented in the neural synapse. Hypertens Res 2012;**35**(6):585-91.



3.2

Trajectories of body mass index before the diagnosis of type 2 diabetes: The Rotterdam Study

ABSTRACT

Objective

Although obesity remains one of the most important risk factors for type 2 diabetes, the complexity of diabetes pathophysiology among individuals with different BMI has the potential to open new prevention strategies based on BMI trajectories. We investigated BMI trajectories and examined associated changes in other cardiometabolic risk factors before diabetes diagnosis.

Methods

We included 6223 participants from the Rotterdam Study, an observational prospective cohort study, followed over 20 years with clinical investigations every 4 years. Latent class trajectory analysis was used to identify BMI patterns before diagnosis of diabetes. Longitudinal changes of other cardiometabolic risk factors were studied using adjusted mixed-effects models.

Results

During a mean follow-up of 13.7 years, 565 participants developed diabetes among whom we identified 3 distinct trajectories of BMI including the "progressive overweight" group (n= 481, 85.1%), "progressive weight loss" group (n= 59, 10.4%), and "persistently obese" group (n=25, 4.4%). The majority, the "progressive overweight" group, was characterized a sharp increase of BMI 10-years before diabetes diagnosis. Moreover, they experienced a constant decrease of insulin levels and insulin resistance during the last 5 years prior to diabetes. The second group of "progressive weight loss" exhibited increased fluctuations of glucose levels during the follow-up and marked beta cell function loss, whereas insulin levels were constant with a slight increase in insulin resistance. The group of "persistently obese" were severely obese throughout the follow-up time before diabetes diagnosis. They were characterized by sharp increases of insulin levels and insulin resistance but modest decreases of beta cell function.

Conclusions

Our results suggest heterogeneity in BMI changes in a middle-aged and elderly white populations prior to diabetes diagnosis. Since the majority group of "progressive overweight" had incremental small gains of weight, strategies for weight loss are recommended to be applied for the whole population rather than focus in high-risk (obese) individuals. These findings highlight the value of tailored intervention according to BMI for diabetes prevention.

INTRODUCTION

Observational studies have extensively shown body mass index (BMI) to be associated with risk of type 2 diabetes [1]. Notably, the number of people with diabetes is expected to increase dramatically in the forthcoming years given the parallel increase in obesity rates worldwide [2 3]. However, patients with diabetes show great variability in terms of weight, weight gain and duration of obesity at the time of diagnosis [4-6]. Consequently, understanding diabetes complex pathophysiological pathways with regard to patterns of change in BMI might provide new insights into personalized prevention strategies to confront the new epidemiological challenges of obesity.

Former population studies investigating BMI changes in association with chronic diseases such as cardiovascular disease (CVD) and diabetes have showed heterogeneous signatures of disease development across BMI trajectories. Previously, we identified three distinct patterns of BMI prior to CVD development and the majority of participants who developed the disease were characterized with a stable BMI over time, highlighting a heterogeneous nature of CVD not entirely attributed to BMI [7]. Similarly, another study among 6705 British participants showed three BMI patterns accompanied with distinctive cardiometabolic risk profiles, with the majority of individuals showing modest weight gain prior to diabetes diagnosis [8]. This finding goes against the common assumption that people who experienced recent weight gain are more likely to be diagnosed with diabetes. Therefore, we aimed to explore this hypothesis in a population of middle-aged and elderly and identify BMI trajectories before diabetes development. Additionally, trajectories of cardiometabolic risk factors including glycaemic traits, lipids, blood pressure and waist circumference within each BMI pattern were further examined.

METHODS

Study population

The study was performed among participants of the prospective population-based Rotterdam Study (RS). In 1989, all residents aged 55 years or older in Ommoord, a suburb of Rotterdam, the Netherlands, 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 postal codes. The participants of the Rotterdam Study have been followed-up for more than 22 years for a variety of diseases and clinical data have been collected across five

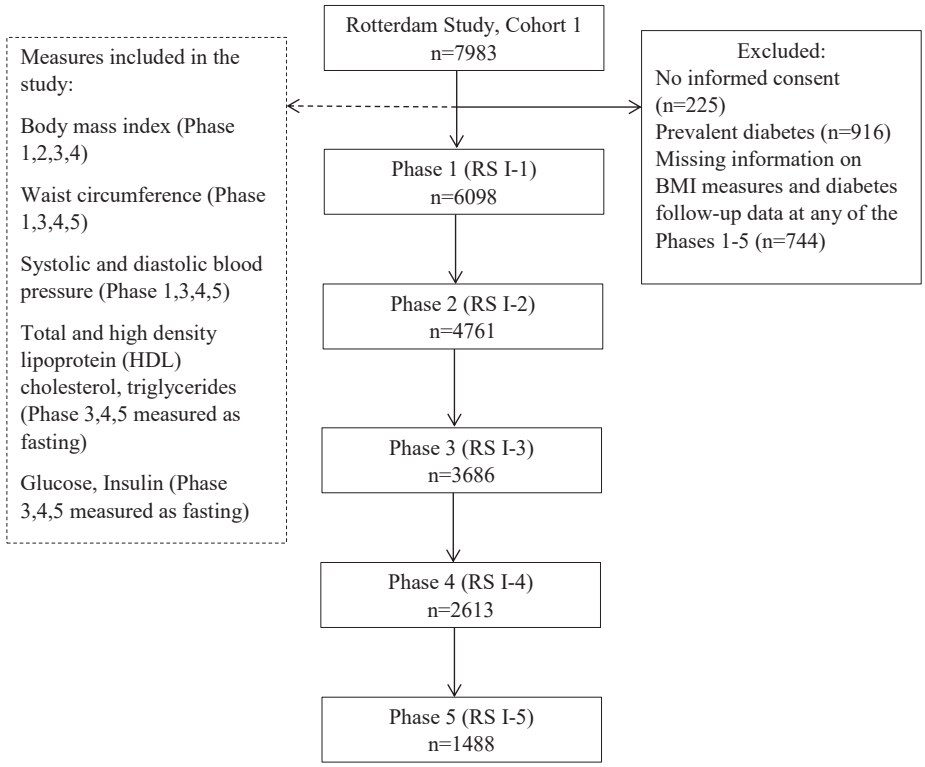


Figure 3.2.1. Flow diagram of the study participants for each phase.

subsequent phases every 3-4 years. A more detailed description of the Rotterdam Study can be found elsewhere [9]. 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.

For this study, we used the third visit of the first cohort (1997-1999). From 7983 participants at baseline, we excluded 225 without informed consent, 916 with prevalent diabetes, 743 without BMI measurement throughout phases 1-5 and 1 with missing information of diabetes follow-up. The final sample included 6098 individuals (**Figure 3.2.1**).

Assessment of cardio-metabolic risk factors

Information on cardio-metabolic risk factors were obtained through home interviews or measured at the study centre, as described previously [10 11]. Height and weight were measured in all five phases, whereas systolic and diastolic blood pressure and waist circumference were measured in phases 1, 3, 4 and 5, and fasting total cholesterol, high-density lipoprotein (HDL) cholesterol and fasting plasma glucose were measured

in phases 3, 4 and 5 (**Figure 3.2.1**). Height and weight were measured with the participants standing without shoes and heavy outer garments. BMI was calculated as weight divided by height squared (kg/m^2). Waist circumference was measured at the level midway between the lower rib margin and the iliac crest with participants in standing position without heavy outer garments and with emptied pockets, breathing out gently. Serum total cholesterol, HDL cholesterol, and glucose were measured using standard laboratory techniques. Blood pressure was measured at the right brachial artery with a random-zero sphygmomanometer with the participant in sitting position, and the mean of two consecutive measurements was used. Smoking status was classified as current smoking or others (former and never) in all phases. We assessed medication use for hypertension, hyperlipidaemia and diabetes through interview data.

Clinical outcome

The participants were followed from the date of baseline centre visit onwards. At baseline and during follow-up, cases of T2D were ascertained by use of general practitioners' records (including laboratory glucose measurements), hospital discharge letters, and serum glucose measurements from Rotterdam Study visits, which take place roughly every 4 years. According to the WHO guidelines, type 2 diabetes was defined as a fasting blood glucose > 7.0 mmol/L, or the use of blood glucose lowering medication [12]. 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 was 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 [13].

Statistical analysis

We used chi-square test for categorical variables and t-tests for continuous data when comparing the general characteristics between groups. Latent class trajectory analysis was performed to identify groups of participants with similar trajectories of BMI change during the follow-up until the occurrence of diabetes as previously described [7 14]. Next, within each identified BMI group, the trajectories of change in other cardio-metabolic risk factors during the time before diabetes diagnosis were developed [7].

The analysis is conducted by taking in account information from the population retrospectively from the date of diagnosis with diabetes. The model used for latent class trajectories are linear mixed-effects model with BMI as the dependent variable and time before diagnosis (time 0), age, sex, and phase of study as independent variables. The variable "time before diabetes diagnosis" describes the shape of the trajectories of BMI and was entered in the model as a covariate in a cubic specification. To assign the

number of classes in the analysis, the Bayesian information criterion (BIC) was used. The latent class trajectory model calculates a posterior probability of membership in each latent class for each participant, who is latter assigned to the class for which his/her posterior probability is the highest. To ensure that all obtained classes were of clinically meaningful size, we imposed the condition that each class should include at least 5 % of participants and the mean posterior probability of each class should be higher than 75 %.

Since the trajectories of change in BMI could differ between individuals who die during follow-up and among individuals who do not die or develop diabetes [15] during follow up we divided the rest of the population into two subgroups: (1) diabetes-free and alive until end of follow-up and (2) non-diabetes mortality. For each identified BMI group (among individuals diagnosed with diabetes) and the two other groups (diabetes-free, and non-diabetes mortality), we examined the trajectories of other cardio-metabolic risk factors including waist circumference, systolic and diastolic blood pressure, fasting total Cholesterol, LDL cholesterol, HDL cholesterol, fasting plasma glucose and fasting plasma insulin. The homeostasis model assessment was used to estimate insulin resistance (HOMA-IR) and beta cell function (HOMA-%B) [16]. The absolute 8-year risk of developing type 2 diabetes was calculated in all participants using the Framingham diabetes risk score [17] and Framingham cardiovascular disease (CVD) risk score was used to estimate absolute risk of developing CVD [18]. In our study, cardiovascular disease is composed of coronary heart disease (including fatal and non-fatal myocardial infarction and other CHD mortality) and stroke (fatal and non-fatal stroke) as previously described [10 19 20].

Because the aggregated effect of combined risk factors on diabetes might differ from each risk factor alone, we examined the trajectories of 8-years diabetes risk and 10-year diabetes risk in each group of BMI. The predicted 10-year CVD risk was calculated using the American College of Cardiology/American Heart Association (ACC/ AHA) Pooled Cohort Equation coefficients, which includes age, sex, total cholesterol, HDL cholesterol, systolic blood pressure, blood pressure lowering medication use, diabetes status, and smoking status in the prediction model [15]. These trajectories of cardio-metabolic risk factors were estimated using linear mixed-effects models controlling for follow-up time, age, sex, and study phase. Analyses of lipids were further adjusted for lipid-lowering treatment, analyses of blood pressure were further adjusted for antihypertensive treatment, and analyses of glucose were additionally adjusted for diabetes treatment. Quadratic and cubic terms for follow-up time were included in the BMI groups (latent classes) when significant ($p< 0.05$). For individuals not developing diabetes during follow-up (diabetes-free and non-diabetes mortality groups), year 0 is merely a time point in a normal life course, and we therefore fitted the trajectories by using linear models. Pair-wise differences in growth curves between BMI groups were tested using F-tests for each cardio-metabolic risk factor. Paired Chi square test (for categorical variables) was used to

Table 3.2.1. Characteristics of study participants in the first clinical visit

	Overall
n	6223
Age (years)	68.82 (8.85)
Gender (Women)	3681 (59.2)
Time before diagnosis/last visit (years)	13.75 (6.55)
Body Mass Index (kg/m2)	26.24 (3.70)
Waist circumference (cm)	90.15 (11.10)
Systolic blood pressure (mm Hg)	138.54 (22.00)
Diastolic blood pressure (mm Hg)	73.82 (11.44)
Total cholesterol (mmol/L)	5.83 (0.99)
LDL cholesterol (mmol/L)	3.76 (0.91)
Triglycerides (mmol/L)	1.49 (0.71)
HDL cholesterol (mmol/L)	1.41 (0.40)
Glucose (mmol/L)	5.68 (0.93)
Insulin (pmol/L)	78.68 (61.44)
HOMA-IR (units)	123.54 (119.42)
HOMA-%B (units)	1642.76 (1111.59)
Antihypertensive treatment (%)	894 (17.0)
Lipid lowering medication (%)	474 (13.6)
Current smoker (%)	1393 (23.1)

Data are n (%), mean(SD)

Abbreviations

HDL, high density lipoprotein; HOMA-IR: homeostatic model assessment –insulin resistance; HOMA-%B: homeostatic model assessment –beta cell function; LDL, low density lipoprotein. Fasting measurements of lipids and glycaemic indices were available in the third, fourth and fifth visits of the original Rotterdam Study cohort

compare participant characteristics between the groups. To account for multiple testing due to comparing three pairs of BMI groups, we used a Bonferroni-adjusted significance level of 0.05/3 = 0.0167 for the F-tests for each cardiometabolic risk factor. All other statistical tests used a significance level of 0.05, and all statistical tests were two sided. Analyses were conducted using R statistical software, version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria), with the package "lcmm" [10].

RESULTS

Baseline characteristics of the study population are presented in Table 3.2.1. Overall, 6223 participants with a mean age of 68.8 years, mostly women (n=3681, 59.2%), and overweight (mean BMI= 26.24) were included in the study. The mean (SD) follow-up

time was 13.7 (6.5) years during which 565 participants developed diabetes. Among individuals who did not develop diabetes, 1891 (30 %) remained alive until the end of follow-up and 3767 (60.5 %) died from non-diabetes causes. The baseline characteristics of these subgroups are presented in Table S1 in the Supplementary Material.

Patterns of BMI change over time

Among 565 participants who developed diabetes, we identified three distinct trajectories of change in BMI levels (**Figure 3.2.2**). The first group (n=481, 85.1%) representing the majority of individuals who developed diabetes, entered the study with a mean BMI of 28.0 kg/m^2 and experienced an increase in BMI within the overweight range. This group was named "progressive overweight". Thereafter, the second group (n= 59, 10.4%) who initially started with an average BMI of 26.6, continued to experience a decrease in BMI during all time of follow-up. We named this group the "progressive weight loss". The third group comprised 25 (4.4%) individuals who entered the study with an average BMI of 35.4 and maintained their obese status with fluctuating BMI values during the entire follow-up until the diagnosis of diabetes. Therefore, we named this group "persistently obese".

Among 1891 subjects who did not develop diabetes event and were alive until the end of follow-up, the "diabetes-free" group, the average BMI remained relatively stable (ranging from 25.9 to 27.3 during the follow-up). Among 3767 who died of other causes during the follow up, the "non-diabetes mortality" group, the average BMI at the start of the follow-up was in the overweight range (average BMI= 26.4) and reached the normal range just before. The analysis was performed in the total population but in order to plot the trajectories of change in BMI and other cardiometabolic risk factors, we assumed a hypothetical individual to be male with 65 years of age. Similar trajectories for a hypo-

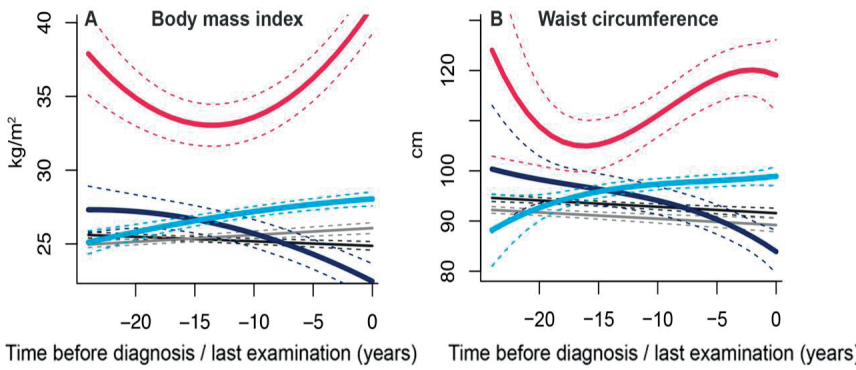


Figure 3.2.2. Trajectories of body mass index and waist circumference during 22 years of follow-up until diagnosis of type 2 diabetes, death or censoring from the study. The figures represent a hypothetical man of 65 years old. *Light blue* "progressive overweight" (including 85.1% of diabetes patients); *red* "persistently obese" (4.4% of diabetes patients); *dark blue* "progressive weight loss" (10.4%); *grey* "diabetes-free"; *black* "non-diabetes mortality".

thetical woman of 65 years of age are shown in Figures S1-S4B in the Supplementary Material.

Trajectories of waist circumference

Trajectories of waist circumferences differed significantly between the three groups (p <0.001 for all pairwise comparisons) (**Figure 3.2.2**). The trajectories for the "progressive overweight", "persistently obese" and "progressive weight loss" groups broadly resembled the trajectories of BMI in these groups. The mean waist circumference in the "diabetes-free" and "non-diabetes mortality" groups decreased slightly during follow-up.

Trajectories of glycaemic indexes (glucose, insulin and HOMA-IR measurements)

Trajectories between fating glucose levels differed between "progressive overweight" and "persistent obese" when compared to "progressive weight loss" group (**Figure 3.2.3**).

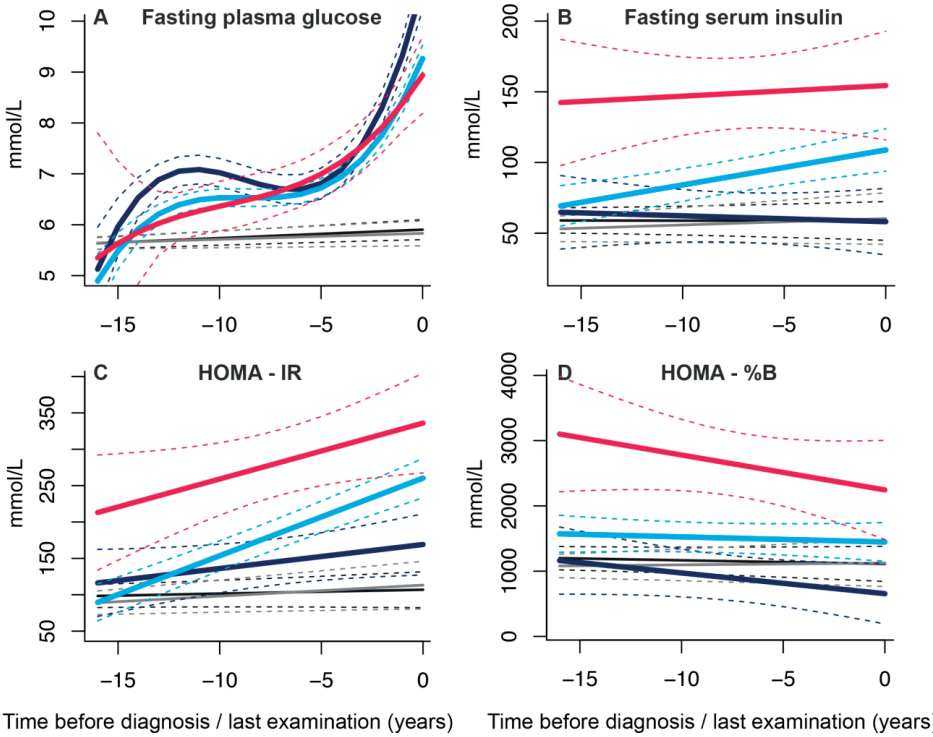


Figure 3.2.3. Trajectories of fasting plasma glucose, insulin, homeostatic model assessment –insulin resistance (HOMA-IR), homeostatic model assessment –beta cell function (HOMA-%B) during 14 years of follow-up until diagnosis of type 2 diabetes, death or censoring from the study. The figures represent a hypothetical man of 65 years old. *Light blue* "progressive overweight" (including 85.1% of diabetes patients); *red* "persistently obese" (4.4% of diabetes patients); *dark blue* "progressive weight loss" (10.4%); *grey* "diabetes-free"; *black* "non-diabetes mortality".

The mean glucose levels of the latter were fluctuating for the whole follow-up time. For the "progressive overweight" and "persistent obese" groups, we observed an increase in mean levels of fasting glucose from 4.9 mmol/L to 9.4 mmol/L during follow up.

All three groups showed significantly different trajectories for fasting insulin. The "progressive overweight" group experienced an increase in mean insulin levels (from 67 mmol/l to 109 mmol/L) during follow-up. A slight increase was observed for "persistent obese" who exhibited high insulin levels throughout the period, whereas modest decrease in insulin levels were observed for "progressive weight loss" group.

Trajectories of HOMA-IR differed between all three groups ($p < 0.01$ for all pairwise comparisons) demonstrating an incremental trend. The biggest increase change was observed for "progressive overweight" group (from 67 mmol/L to 258 mmol/L), followed by "persistent obese" group, which was characterized by the highest average HOMA-IR and lastly, "progressive weight loss" group. Contrary, a decreasing trend was observed for HOMA-%B for all the trajectories between the groups. The "persistent obese" group exhibited the highest average levels of HOMA-%B accompanied by a steep decrease during follow up. The "progressive overweight" group showed a stable trend with an average of 1500 mmol/L, whereas the "progressive weight loss" group experienced lowered HOMA-%B levels from 1200 mmol/L to 700 mmol/L (**Figure 3.2.3**).

Trajectories of lipid profile and blood pressure

We found no differences in fasting total cholesterol levels HDL, LDL and triglycerides between the three groups of individuals who developed diabetes during follow-up (**Figure 3.2.4**). For total cholesterol, we evidenced a mark increase in the "persistent obese" group starting from 4.5 mmol/L. The other two groups kept a lowering trend throughout

Table 3.2.2. Characteristics of study participants at the time of the diagnosis for the three groups with diabetes or at last visit for the groups without diabetes

	Individuals developing diabetes during follow-up (n = 565)			**Individuals free of diabetes during follow-up (n = 5658)**	
	Weight loss	**Progressive weight gainers**	**Persistently obese**	**Diabetes-free**	**Non-diabetes mortality**
	n = 59	n = 481	n = 25	n = 1891	n = 3767
Age at diagnosis/last contact (years)	67.2 (7.2)	66.4 (7.0)	64.5 (5.2)	62.2 (5.0)	72.4 (8.5)
Women (%)	30 (50.8)	282 (58.6)	20 (80.0)	1230 (65.0)	2119 (56.3)
Body Mass Index (kg/m2)	24.9 (2.9)	28.9 (3.3)	39.0 (3.8)	27.3 (4.1)	26.2 (3.9)
Waist circumference (cm)	90.5 (9.9)	98.3 (10.3)	117.7 (20.8)	91.3 (11.9)	92.6 (11.5)
Antihypertensive treatment (%)	23 (39.7)	200 (42.8)	20 (83.3)	883 (47.6)	1021 (29.3)
Lipid lowering medication (%)	11 (22.9)	62 (23.9)	5 (29.4)	420 (26.6)	255 (16.2)
Current smoker (%)	22 (43.1)	113 (32.7)	6 (30.0)	325 (18.4)	866 (32.2)

the study, with all groups having an average total cholesterol level within the reference range (< 5.5 mmol/L). On the other hand, decreasing levels of HDL were observed for both "progressive overweight" and "persistent obese" groups while the average levels of "progressive weight loss" group remained stable throughout the follow-up. For LDL and triglycerides levels, "persistent obese" group exhibited an increasing trend before

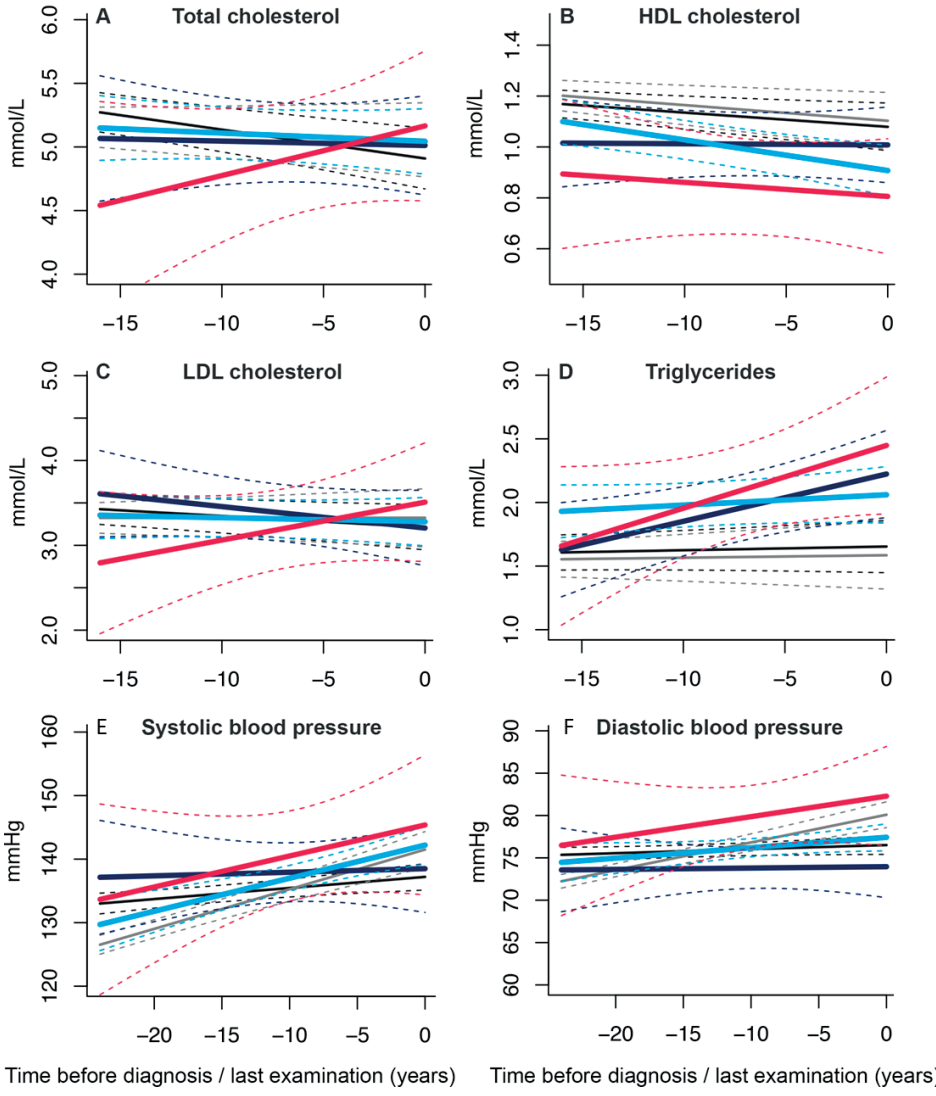


Figure 3.2.4. Trajectories of total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, triglycerides, systolic blood pressure and diastolic blood pressure. The figures represent a hypothetical man of 65 years old. *Light blue* "progressive overweight" (including 85.1% of diabetes patients); *red* "persistently obese" (4.4% of diabetes patients); *dark blue* "progressive weight loss" (10.4%); *grey* "diabetes-free"; *black* "non-diabetes mortality".

diabetes event. The average levels of LDL cholesterol demonstrated a modest decrease in the "progressive overweight" and "progressive weight loss" groups meanwhile, the trend was reversed in both groups for triglycerides levels during the follow-up.

Trajectories of systolic and diastolic blood pressure differed significantly between all BMI groups. Both "progressive overweight" and "persistent obese" groups showed increasing trend before diagnosis of diabetes in both systolic and diastolic blood pressure levels whereas "progressive weight loss" group was relatively stable during the follow-up (**Figure 3.2.4**).

Trajectories of estimated 8-year Type 2 Diabetes risk

Framingham 8-year diabetes risk followed nearly the same stable trend for "progressive weight loss", diabetes-free and non-diabetes mortality groups (Figure S4A). The "persistent obese" group demonstrated an increase of 8-year diabetes risk from 6% to 19% before diabetes diagnosis. A decreasing trend was shown for the "progressive overweight" group with a difference of nearly 4-5%.

DISCUSSION

We examined BMI trajectories in a middle-aged and elderly population based study followed for over 20 years using latent class trajectory analysis and identified three distinct groups of BMI changes: a "progressive overweight" group, a "persistent obese" group and a group of "progressive weight loss". Within the BMI groups that developed diabetes, trajectories of obesity, visceral fat as measured with waist circumference, glucose, insulin, HOMA-IR, HOMA-%B showed distinct patterns throughout the follow-up of the study. This study shed further insights into the timing and the extent of pathophysiological changes before diabetes diagnosis in a middle-aged and elderly European population highlighting the heterogeneous nature of diabetes diagnosis depending on the level of obesity.

The majority of individuals in our study diagnosed with diabetes were progressively gaining weight within the overweight range. Development of diabetes was not preceded by a recent weight gain, as commonly believed, but rather by a continuous, weight gain over the years. While there were relatively stable HOMA beta cell function, they exhibit progressively increasing trends of insulin levels and HOMA-insulin resistance starting from the beginning of the follow-up, whereas glucose levels worsened approximately 5 years before the diagnosis. In the same line, the "persistent obese group" showed accentuated parameters patterns of glucose metabolism as compared with "progressive overweight" group. When we measured the Framingham 8-year diabetes risk, we observed a decreasing trend throughout the period of follow-up in the "progressive overweight"

group, but the model was predicting well for the "persistently obese". This might indicate that prediction models do not perform well in the former group of individuals. The diagnosis of diabetes in the Rotterdam Study is done by active collection of information from general practitioners and screening at the research centre based on clinical values of glucose. Another interpretation of the result suggests that the diagnosis of diabetes might be bias towards enhanced screening efforts reserved to obese individuals rather than overweight. Similar findings are reported in an investigation of obesity trajectories prior to diabetes development in a UK cohort [8]. The "stable overweight" group was less often diagnosed with diabetes from the general practitioners than the "persistently obese" group. This indicate an inclination of physicians to more effectively screen obese individuals in comparison to overweight individuals.

We found that 10.4% our participants (second largest group) experienced progressive weight loss before diagnosis of diabetes, a pattern not observed in the UK study. Among the elderly, the relation between body weight, body composition and health behaviours is different than in younger adults [21 22]. Weight loss has been often been associated with a high risk of mortality [15 23 24] while its association with cardiovascular disease still remain inconclusive [24 25]. In this group, waist circumference trajectories followed the same decreasing trend as BMI while fluctuations of fasting glucose levels with a sharp increase 5 year before diabetes diagnosis were observed. However, these changes did not correspond to an increase of insulin levels, while HOMA-%B levels were the lowest among the three groups and decreased constantly. Despite the weight loss progression prior to diabetes diagnosis, the inability to respond adequately to high glucose levels together with the impaired beta-cell compensation from the pancreas in this category of individuals seems to be involved in the disease development regardless of obesity levels. Because of the low beta-cell function in this group before diagnosis, individuals might benefit from early prevention strategies focusing on prevention of further loss of beta cell function rather than tackling peripheral insulin sensitivity. This concept has shown familiarity before [26 27]. Notably, the predicted 8-year diabetes risk was nearly constant during follow-up for this category, similar to the diabetes-free group. These findings question the validity of diabetes prediction score in a population with heterogeneous disease development. One-size-fits-all model seems to be not a good metric.

Despite the differences in BMI trajectories, most of the other cardiometabolic risk factors including blood pressure and lipid profile developed without substantial changes in the three groups. Moreover, we were able to assess medication data for all BMI subgroups and we found that antihypertensive medication and lipid lowering drugs were bearing the highest proportionality of use among the persistently obese individuals followed by progressive overweight and progressive weight lost group. This data showed that most probably, overweight individuals and those losing weight over time are less likely to receive medication. Notably, the progressive overweight group and progressive weight

loss group constitute more than 95% of the middle aged and elderly population developing diabetes events. Therefore, treating in rightly manner these category of patients could have a big impact on decreasing the overall burden of diabetes and associated comorbidities in the total population.

Strength of our study include the prospective design with availability of repeated measurements for BMI and other cardiometabolic risk factors including medication use data over a long follow up time, which altogether allowed to perform latent class trajectories analysis. Previous literature has used BMI in pre-defined categories which might introduced some misclassification bias, whereas our analysis allows full exploration of heterogeneous patterns of BMI changes that might influence diabetes risk. Nevertheless, one of the drawbacks of this method is the assigned not-balanced sample size pertaining to the groups which make comparisons of the result difficult in the light of statistical power. Also, generalizability of the study may be limited due to the specific population analysed. The majority of individuals were middle aged and elderly with a mean age of 68.8 years old.

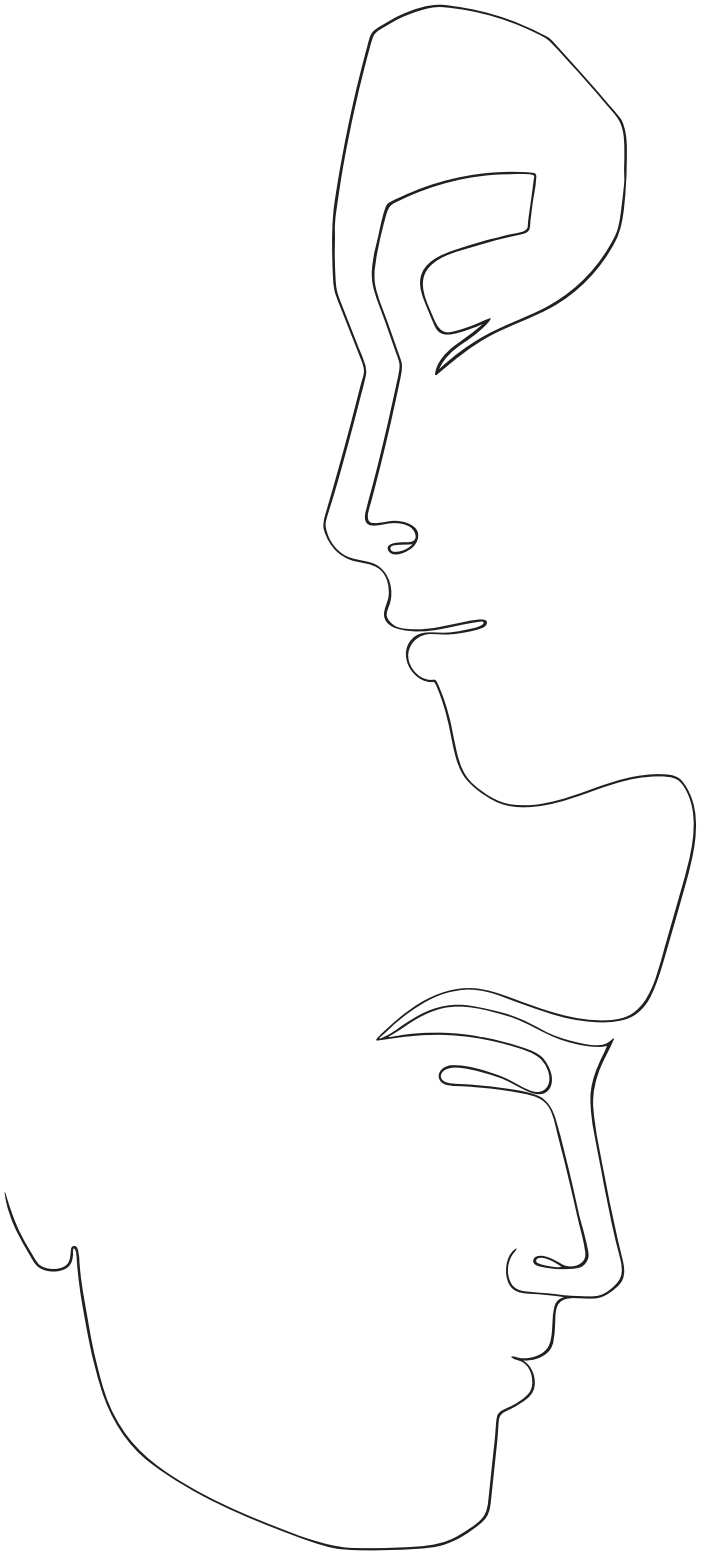
Conclusions

In conclusion, we identified three distinct patterns of BMI changes prior to diabetes diagnosis. These population growth curves contribute to our understanding of aetiology and pathophysiology of type 2 diabetes, as a heterogeneous disease with complex mechanism involved in its development. In general, the majority of individuals developing diabetes were characterized by weight gains within the overweight range before diabetes diagnosis suggesting strategies focusing in small weight reductions for the entire population rather than high risk groups in the total population. Future studies should establish whether there might be different treatment needs for diabetes prevention and management depending on disease subgroups.

REFERENCES

1. Dhana K, Nano J, Ligthart S, et al. Obesity and Life Expectancy with and without Diabetes in Adults Aged 55 Years and Older in the Netherlands: A Prospective Cohort Study. PLoS Med 2016;**13**(7):e1002086.
2. Collaboration NCDRF. Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19.2 million participants. Lancet 2016;**387**(10026):1377-96.
3. Collaboration NCDRF. Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4.4 million participants. Lancet 2016;**387**(10027):1513-30.
4. Brancati FL, Wang NY, Mead LA, et al. Body weight patterns from 20 to 49 years of age and subsequent risk for diabetes mellitus: the Johns Hopkins Precursors Study. Arch Intern Med 1999;**159**(9):957-63.
5. Ford ES, Williamson DF, Liu S. Weight change and diabetes incidence: findings from a national cohort of US adults. Am J Epidemiol 1997;**146**(3):214-22.
6. Hanson RL, Narayan KM, McCance DR, et al. Rate of weight gain, weight fluctuation, and incidence of NIDDM. Diabetes 1995;**44**(3):261-6.
7. Dhana K, van Rosmalen J, Vistisen D, et al. Trajectories of body mass index before the diagnosis of cardiovascular disease: a latent class trajectory analysis. Eur J Epidemiol 2016;**31**(6):583-92.
8. Vistisen D, Witte DR, Tabak AG, et al. Patterns of obesity development before the diagnosis of type 2 diabetes: the Whitehall II cohort study. PLoS Med 2014;**11**(2):e1001602.
9. Hofman A, Brusselle GG, Darwish Murad S, et al. The Rotterdam Study: 2016 objectives and design update. Eur J Epidemiol 2015;**30**(8):661-708.
10. Koller MT, Leening MJ, Wolbers M, et al. Development and validation of a coronary risk prediction model for older U.S. and European persons in the Cardiovascular Health Study and the Rotterdam Study. Ann Intern Med 2012;**157**(6):389-97.
11. Kavousi M, Elias-Smale S, Rutten JH, et al. Evaluation of newer risk markers for coronary heart disease risk classification: a cohort study. Ann Intern Med 2012;**156**(6):438-44.
12. consultation RoaWI. Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia, 2006.
13. Leening MJG, Kavousi M, Heeringa J, et al. Methods of data collection and definitions of cardiac outcomes in the Rotterdam Study. European Journal of Epidemiology 2012;**27**(3):173-85.
14. Proust-Lima C, Letenneur L, Jacqmin-Gadda H. A nonlinear latent class model for joint analysis of multivariate longitudinal data and a binary outcome. Stat Med 2007;**26**(10):2229-45.
15. Zajacova A, Ailshire J. Body mass trajectories and mortality among older adults: a joint growth mixture-discrete-time survival analysis. Gerontologist 2014;**54**(2):221-31.
16. Matthews DR, Hosker JP, Rudenski AS, et al. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;**28**(7):412-9.
17. Wilson PW, Meigs JB, Sullivan L, et al. Prediction of incident diabetes mellitus in middle-aged adults: the Framingham Offspring Study. Arch Intern Med 2007;**167**(10):1068-74.
18. D'Agostino RB, Sr., Vasan RS, Pencina MJ, et al. General cardiovascular risk profile for use in primary care: the Framingham Heart Study. Circulation 2008;**117**(6):743-53.
19. Goff DC, Jr., Lloyd-Jones DM, Bennett G, et al. 2013 ACC/AHA guideline on the assessment of cardiovascular risk: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. J Am Coll Cardiol 2014;**63**(25 Pt B):2935-59.

20. Bos MJ, Koudstaal PJ, Hofman A, et al. Modifiable etiological factors and the burden of stroke from the Rotterdam study: a population-based cohort study. PLoS Med 2014;**11**(4):e1001634.
21. Villareal DT, Apovian CM, Kushner RF, et al. Obesity in older adults: technical review and position statement of the American Society for Nutrition and NAASO, The Obesity Society. Obes Res 2005;**13**(11):1849-63.
22. Holder; HLGK. Unintentional weight loss in older adults. Am Fam Physician 2014;**89**(9):Online.
23. Flegal KM, Graubard BI, Williamson DF, et al. Excess deaths associated with underweight, overweight, and obesity. JAMA 2005;**293**(15):1861-7.
24. Dhana K, Ikram MA, Hofman A, et al. Anthropometric measures in cardiovascular disease prediction: comparison of laboratory-based versus non-laboratory-based model. Heart 2015;**101**(5):377-83.
25. Rimm EB, Stampfer MJ, Giovannucci E, et al. Body size and fat distribution as predictors of coronary heart disease among middle-aged and older US men. Am J Epidemiol 1995;**141**(12):1117-27.
26. Engberg S, Glumer C, Witte DR, et al. Differential relationship between physical activity and progression to diabetes by glucose tolerance status: the Inter99 Study. Diabetologia 2010;**53**(1):70-8.
27. Saito T, Watanabe M, Nishida J, et al. Lifestyle modification and prevention of type 2 diabetes in overweight Japanese with impaired fasting glucose levels: a randomized controlled trial. Arch Intern Med 2011;**171**(15):1352-60.



3.3

Lifestyle score and total life expectancy with and without diabetes

ABSTRACT

Objective

Several modifiable lifestyle factors are independently associated with increased risk for type 2 diabetes and mortality. However, the combined effect of combined lifestyle factors on type 2 diabetes and life expectancy remains unknown. Therefore, we aimed to assess and quantify the association of lifestyle score with diabetes risk and life expectancy among men and women.

Methods

This study was performed among 10,546 participants (mean age 63.0±10.1 year; 57.1% women) from a large prospective population-based cohort study among middle-aged and older adults. An overall lifestyle score was calculated including five lifestyle factors: smoking, alcohol consumption, diet quality, physical activity and body mass index. The lifestyle score was categorized as: unhealthier (reference), moderate and healthier. Multistate life tables were constructed to calculate the number of years lived overall as well as those lived with and without type 2 diabetes, adjusted for confounders.

Results

During an average follow-up of 11 years, 575 incident diabetes events and 1659 deaths occurred. In men, the healthier lifestyle category was associated with a lower risk of diabetes HR, 0.47 (95% CI 0.29-0.75), mortality among those without diabetes HR, 0.57 (95% CI 0.41-0.78) and mortality among participants with diabetes HR 0.50 (95% CI 0.29-0.86) compared to the unhealthier lifestyle category. A similar trend was observed in women, with HRs of respectively, 0.51 (95% CI 0.31-0.84), 0.63 (95% CI 0.43-0.93) and 0.96 (95% CI 0.44-2.09) for diabetes and mortality.

At the age of 45 years, men in the healthier lifestyle category lived overall 4.9 years longer compared to men in the unhealthier lifestyle category. Among women, this difference in total life-expectancy was 2.5 years. Men and women in the healthier lifestyle category lived 1.5 and 3.1 years shorter with diabetes. Life expectancy without diabetes was 6.4 years longer in men and 5.7 years longer in women with a healthy lifestyle.

Conclusion

A healthier lifestyle was associated with a decreased risk of developing diabetes by 50% among men and women. Overall, men and women who had a healthier lifestyle lived up to 5 years longer compared to participants who had an unhealthier lifestyle. More attention should be given in clinical practice and preventive care to adopting a healthy lifestyle since it could substantially reduce diabetes risk, premature mortality and prolong life expectancy.

INTRODUCTION

Lifestyle choises such as poor diet, physical inactivity, tobacco use, high adiposity and alcohol abuse, have been linked to an increased risk of multiple chronic diseases and premature death. According to a recent WHO report, altogether these lifestyle factors explain more than one third of the global burden of chronic diseases (WHO, 2015).

Smoking, physical inactivity, unhealthy diet, obesity and other lifestyle behaviors are associated with the development of diseases such as type 2 diabetes and mortality[1]. In 2010, approximately 25.6 million adults (prevalence of 11.3%) in the United States had diagnosed diabetes, including 10.9 million adults aged ≥ 65 years (prevalence 26.9%) (ref). Pharmacological management of diabetes has proven benefits, but these efforts are often costly, include side effects, and may not be as effective as lifestyle interventions[2]. Primary prevention of diabetes, therefore, would have major positive public health consequences.

Moreover, a few studies have investigated the combined impact of these lifestyle-related factors and mortality outcomes and total life expectancy. Research to quantify the overall impact of lifestyle-related factors on mortality outcomes will provide important information valuable for disease prevention. A recent prospective cohort study among 20,244 British men and women aged 45–79 y reported a 4-fold increase in risk of all-cause mortality for participants with no health behaviors compared to participants who had four health behaviors (nonsmoker, plasma vitamin C levels indicative of ≥5 daily servings of fruits and vegetables, moderate alcohol intake, and physically active)[3]

Furthermore, although a few strong examples exist, the independent and combined effect of lifestyle on overall health, have not been studied extensively. People that engage in multiple unfavorable lifestyle behaviors have a higher risk for mortality and incidence of chronic diseases than people who have no unfavorable lifestyle behaviors or only one and the sum of these single components might be more important than the single components itself. Another prospective cohort study of 78,865 participants of the Nurse's Health Study (NHS) and 44,354the Health Professionals Follow-up found that adherence to 5 low-risk lifestyle-related factors (never smoking, a healthy weight, regular physical activity, a healthy diet, and moderate alcohol consumption) could prolong life expectancy at age 50 years by 14.0 and 12.2 years for female and male US adults compared with individuals who adopted zero low-risk lifestyle factors.

However , to our knowledge, no study up to date has investigated the benefits of a healthier lifestyle in diabetes and their combined net effect on total life expectancy in Europe. Therefore, the aim of this study was to estimate the impact of lifestyle factors on total life expectancy and the number of years lived with and without diabetes in the Dutch population.

METHODS

Study Population

The Rotterdam Study is a population-based cohort study of individuals aged 45 years and over, living in the Ommoord district of Rotterdam, the Netherlands. The rationale and design of the study is described elsewhere[4]. In brief, all inhabitants of the Ommoord district aged 55 years and older were invited to participate (n = 10,215). A second extension of the cohort was initiated in 2006, in which 3,932 participants aged 45 years and older were included. The RS has been approved by the medical ethics committee according to the Population Study Act Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. A written informed consent was obtained from all participants.

For this study we used data from the third visit of the first cohort (RSI-3), first visit of the second (RSII-1) and third cohort (RSIII-1). From the 14,926 participants, we excluded: (i) 3,576 participants who didn't have information on lifestyle score; (ii) 798 were excluded because no information on T2D was available; or (iii) did not give informed consent for T2D follow-up (n=6). After exclusion, 10,546 participants (6,024 women) were available for the analyses on lifestyle score categories (healthier, moderate and unhealthier) and risk of T2D and mortality (**Figure 3.3.1**).

Assessment of Lifestyle score

Dietary quality

Dietary intake was assessed with a Food Frequency Questionnaire (FFQ)[5]. For RS-I and RS-II, a previously validated, two-step dietary assessment was used that comprised a simple self-administered questionnaire followed by a structured interview with a trained dietitian based on the completed questionnaire[6]. For RS-III, a validated FFQ based on 389 items was used[7]. Follow-up data from RS-I-3 did not include measurement of dietary intake, therefore data from RS-I-1 were used as a proxy. Participants' dietary quality was defined as adherence to the Dutch dietary guidelines, as previously applied to the Rotterdam Study[5]. For all participants, we examined adherence (yes/no) to fourteen items of the guidelines: vegetables, fruit, whole-grains, legumes, nuts, dairy, fish, tea, whole-grains, fats and oils, red and processed meat, sugar-containing beverages, alcohol, and salt. Total adherence was calculated as sum-score of the adherence to the individual items (0–14). For the analyses, we divided the dietary quality score into tertiles (low (0–6), medium (6–8) and high adherence (8–14)).

Physical activity

Physical activity was measured using two different questionnaires. For RS-I and RS-II, a validated adapted version of the Zutphen Physical Activity Questionnaire (ZPAC)[8] was used and for RS-III the validated LASA Physical Activity Questionnaire (LAPAQ)[9]. Both

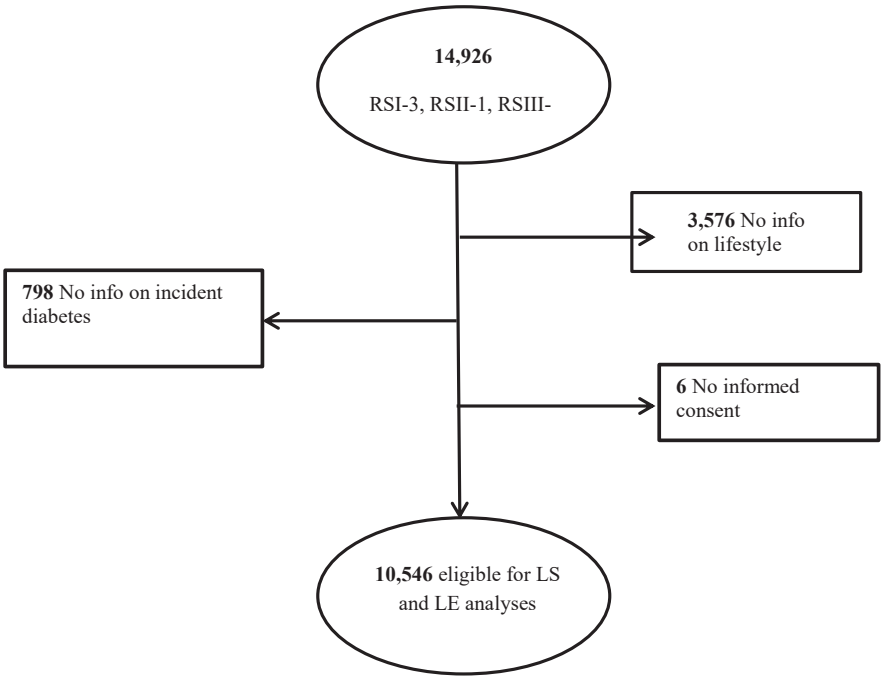


Figure 3.3.1. Flowchart of study participants

questionnaires included items regarding walking, cycling, gardening, sports, hobbies and housekeeping activities. Participants' physical activities were weighted by their intensity with the use of metabolic equivalent of task (MET). Questionnaire-specific tertiles of MET hours per week (low (<57MET/h for RS-I-1 and RS-II-1; <27MET/h for RS-III-1), moderate (57–93MET/h for RS-I-1 and RS-II-1; 27–65MET/h for RS-III-1) and high physical activity (>93MET/h for RS-I-1 and RS-II-1; >65MET/h for RS-III-1) were calculated.

Alcohol intake

Alcohol intake was measured using the previously described FFQ. Data were collected as the number of glasses consumed per week in a wide-range of alcoholic beverages. Alcohol consumption was divided into three sex-specific categories: (1) low alcohol intake (<2 glasses per day for men and <1glass per day for women), (2) moderate alcohol intake (2 to <4 glasses per day for men, 1to <3 glasses per day for women) and (3) harmful alcohol intake (≥4 glasses per day for men and ≥3 glasses per day for women). Harmful alcohol intake was defined according to the Dutch diagnostic classification system for mental disorders (DSM-IV-TR).

Smoking status

Smoking status was determined by self-reported computerized questionnaires during the home interview. Smoking status was categorized into current smoking, former smoking and never smoking and included the use of cigarettes, cigars, and/or pipes. Current smokers were participants who answered yes to the question: "are you currently smoking?" Former smokers were participants who answered no to this question but who positively answered the question: "are you a former smoker?"

Lifestyle score

An overall lifestyle score was calculated by combining dietary quality, physical activity, alcohol intake and smoking into one score. All four individual lifestyle variables were divided into three categories. The unhealthiest category was coded as 0, the moderate as 1, and the healthiest category as 2. Scores for all these individual lifestyle variables were summed up for each participant, resulting in a combined lifestyle score ranging from 0 to 8. According to the ranges the participants were categorized in three categories. Participants who had a score from 0 to 2 were categorized as the unhealthier lifestyle score category, from 3 to 5 as the moderate lifestyle category and from 6 to 8 as the healthier lifestyle category. The lifestyle score was calculated for participants whose data were available in at least two lifestyle variables.

Ascertainment of Type 2 Diabetes

The participants were followed from the date of baseline centre visit onwards. Cases of T2D were ascertained at baseline and during follow-up through: (i) active follow-up using general practitioners' records, (ii) glucose hospital discharge letters and (iii) glucose measurements from RS visits that take place approximately every 4 years[10]. T2D was defined according to recent WHO guidelines, as a fasting blood glucose ≥ 7.0 mmol/L, a non-fasting blood glucose ≥ 11.1 mmol/L (when fasting samples were absent), or the use of blood glucose lowering medication. Information regarding the use of blood glucose lowering medication was derived from both structured home interviews and linkage to pharmacy records[10]. At baseline, more than 95% of the population was covered by the pharmacies in the study area. Two study physicians independently adjudicated all potential events of T2D. In case of disagreement, consensus was sought with an endocrinologist. Follow-up data was complete until January 1st 2012.

Ascertainment of Deaths

Mortality data were obtained by notification from the municipal administration. Data on all-cause mortality and living status were updated until August 1st 2016. Participants were followed from the first day they entered the study till the day of death, the day of lost to follow-up or the last date of contact, whichever came first.

Assessment of Potential Confounders

Information on current health status, medical history, medication use and potential intermediate variables was obtained at baseline (RSI-3, RSII-1 and RSIII-1). Education was defined as low (primary education), intermediate (secondary general or vocational education), or high (higher vocational education or university). Blood pressure was measured in sitting position at the right upper arm with a random-zero-sphygmomanometer. The mean of two consecutive measurements was taken. Antihypertensive medication use was defined as use of diuretics, β-blockers, angiotensin-converting enzyme inhibitors, and calcium channel blockers. All biochemical parameters were assessed in fasting serum. Serum total cholesterol was measured in fasting serum on the COBAS 8000 Modular Analyser (Roche Diagnostics GmbH). Comorbidity was considered present when cancer or chronic obstructive pulmonary disease was prevalent at baseline. Pathology data of the cancers was obtained from linkage to the national cancer registry and the Dutch pathology database (PALGA). Chronic obstructive pulmonary disease was defined as a type of obstructive lung disease characterized by airflow limitation that is not fully reversible[11]. Height and weight at baseline were measured in our research center. BMI at baseline was calculated as weight in kg/height in m^2. We defined three BMI categories based on World Health Organization guidelines[12]: normal weight, BMI of 18.5 to 24.9 kg/m^2; overweight, BMI of 25 to 29.9 kg/m^2; and obese, BMI greater than or equal to 30 kg/m^2.

Data Analysis

We created multistate life tables to calculate the differences in life expectancy and years lived with and without T2D in: healthier, moderate and unhealthier lifestyle score. We considered three different health states: free of T2D, T2D, and death. Participants could experience the following transitions: from free of T2D to T2D, from free of T2D to death, and from T2D to death. No backflows were allowed, and only the first entry into a state was considered.

To obtain transition rates, we first calculated the overall age-specific rates for each transition. Next, we calculated the prevalence of healthier, moderate and unhealthier lifestyle score, by 10-y age groups, and separately for subjects with and without diabetes. Hazard ratios (HRs) comparing participants who were classified as having a healthier, moderate or unhealthier lifestyle score, were calculated using Poisson regression ("Gompertz" distribution) in 2 models. Model 1 adjusted for age, education level, BMI and cohort effect and model 2 additionally adjusted for the following potential intermediates: systolic blood pressure, cholesterol levels, antihypertensive medication, statin use and comorbidities (prevalent cardiovascular disease (CVD), cancer and chronic obstructive pulmonary disease (COPD)). Finally, we calculated three sets of transition rates for each category using (i) the overall transition rates, (ii) the adjusted HRs for T2D and mortality,

and (iii) the prevalence of lifestyle score categories by presence of T2D. Similar calculations have been described previously[13].

The multistate life table started at age 45 years and closed at age 95 years. Multiple imputations were performed in case of missing covariates. Statistical analyses were conducted using IBM SPSS, version 21 (IBM Corp) and STATA, version 13 for Windows (StataCorp). We used Monte Carlo simulation (parametric bootstrapping) to calculate the confidence intervals of the life expectancy estimates with @RISK software (Palisade) runs[14].

Sensitivity Analysis

To exclude any potential bias caused by prevalent comorbidities at baseline, we repeated the analysis among those who were without COPD, cancer and CVD (n = 7933). In addition, to check whether any of the lifestyle factors was driving the association of the overall score with diabetes risk and mortality, we excluded one by one each of the factors in separate models. Furthermore, to account for the variability in time of lifestyle habits, we evaluated the effects of lengths of follow-up on the relation between lifestyle score categories, T2D and mortality. Therefore, all analyses were repeated for different periods of follow-up: 7 and 10 years.

RESULTS

Baseline characteristics

The final study population consisted of 10,546 individuals: 4,522 men and 6,024 women. In total, we observed 575 incident diabetes events and 1659 overall deaths during a median follow up time of 11 years. The mean age (standard deviation) of the population was 64.4 (SD=9.8). Compared to women, men at baseline were on average higher educated, consumed higher alcohol amounts, and smoked more, but showed lower levels of physical activity **(Table 3.3.1)**.

Diabetes incidence and mortality

Table 3.3.2 shows the association between lifestyle score categories and risk of incident T2D and mortality. In multivariable adjusted model, the HRs for the association between a healthier lifestyle score and T2D for men and women accordingly were: 0.47 (0.29 to 0.75) and 0.51 (0.31-0.84). Among those free of T2D, compared to the unhealthier lifestyle score category, the healthier and moderate lifestyle categories, had a decreased risk of mortality among men 0.57 (0.41 to 0.78) and 0.72 (0.55 to 0.96) and women 0.63 (0.43 to 0.93) and 0.78 (0.53 to 1.14), respectively. Among those with T2D, the HRs for mortality in men were 0.50 (0.29 to 0.86), and 0.64 (0.42 to 0.96) for healthier and moderate lifestyle score , respectively, relative to the unhealthier lifestyle score category **(Table 3.3.2)**. For

Table 3.3.1. Baseline characteristics of 10546 participants

Characteristics	Men (n= 4522)	Women (n=6024)
Age, mean (SD), y	64.4 (9.4)	63.0 (10.1)
Diabetes mellitus*, Yes (%)	1017 (22.5)	1132 (18.8)
Education, Nr. (%)		
Primary	421 (9.2)	889 (14.8)
Lower	1272 (28.0)	3006 (49.9)
Intermediate	1671 (36.7)	1373 (22.8)
Higher/university	1158 (25.5)	711 (11.8)
Lifestyle Score (LS), Nr. (%)		
Unhealthier	1353 (29.9)	1040 (17.3)
Moderate	2567 (56.8)	3227 (53.6)
Healthier	602 (13.3)	1757 (29.2)
Body mass index, BMI, mean (SD), kg/m^2	27.2 (3.7)	27.5 (4.6)
Physical activity (METhours/week)	66.6 (47.4)	83.1 (50.1)
Smoking status, Nr. (%)		
Current	1218 (26.9)	1193 (19.8)
Former	2606 (57.6)	2278 (37.8)
Never	697 (15.4)	2552 (42.4)
Alcohol intake, Nr. (%)		
Harmful	172 (3.8)	210 (3.5)
Moderate	407 (9.0)	758 (12.6)
Low	3064 (67.8)	4100 (68.1)
Diet quality score, mean (SD)	7.2 (3.3)	6.8 (3.0)

this transition, the HRs for women with a healthier and moderate lifestyle score were in the same direction although not significant; 0.96 (0.44 to 2.09) and 0.94 (0.43 to 2.02) compared to their unhealthier counterparts.

Total life expectancy and life expectancy with and without diabetes

Compared to men in the unhealthier lifestyle category, the total life expectancy of 45-y-old men in the healthier lifestyle group was 4.9 years longer and for women, the difference was 2.5 years longer (Table 3). The difference in life expectancy free of diabetes for both men and women, in the healthier category was 6.4 years longer and 5.7 years longer. Moreover, compared to the unhealthier lifestyle group, the difference in life expectancy with diabetes for the healthier lifestyle category was 1.5 years shorter for men and 3.1 years shorter for women **(Table 3.3.3)**.

Table 3.3.2. Hazard ratios by transition among categories of lifestyle score.*

Transition	Categories	Men		Women	
		Cases, Number/ Person-Years	Model 1 HR (95% CI)[a]	Cases, Number/ Person-Years	Model 1 HR (95% CI)[a]
Incident T2D	Unhealthier LS	256/ 19240	1.00	319/ 29218	1.00
	Moderate LS		0.69 (0.47-1.00)		0.64 (0.39-1.04)
	Healthier LS		0.47 (0.29-0.75)		0.51 (0.31-0.84)
No T2D to mortality	Unhealthier LS	611/ 20482	1.00	631/ 30911	1.00
	Moderate LS		0.72 (0.55-0.96)		0.78 (0.53-1.14)
	Healthier LS		0.57 (0.41-0.78)		0.63 (0.43-0.93)
T2D to mortality	Unhealthier LS	236/ 4042	1.00	181/ 4805	1.00
	Moderate LS		0.64 (0.42-0.96)		0.94 (0.43-2.02)
	Healthier LS		0.50 (0.29-0.86)		0.96 (0.44-2.09)

Abbreviations: HR, Hazard Ratio; CI, Confidence Interval; T2D, Type 2 Diabetes.
*Age 45 and over at start of follow-up; The Unhealthier LS is the reference category.
[a] Adjusted for age, cohort and education.

Table 3.3.3. Effect of Lifestyle score on life expectancy at age 45*.

	Total LE	Difference in Total LE†	LE free of T2D	Difference in LE free of T2D†	LE with T2D	Difference in LE with T2D†
Men						
Unhealthier LS	29,7	Ref.	24,1	Ref.	5,6	Ref.
Moderate LS	32,5	2,9	27,5	3,4	5,0	-0,5
Healthier LS	34,5	4,9	30,5	6,4	4,1	-1,5
Women						
Unhealthier LS	35,6	Ref.	28,0	Ref.	7,7	Ref.
Moderate LS	37,1	1,4	31,6	3,6	5,5	-2,2
Healthier LS	38,2	2,5	33,6	5,7	4,5	-3,1

Abbreviations: LE, Life Expectancy; T2D, Type 2 Diabetes; Ref, Reference.
* All life expectancies have been calculated with hazard ratios adjusted for age, cohort and education.
† Differences are calculated using Unhealthier LS as reference: moderate vs. unhealthier and healthier vs. unhealthier.

Sensitivity analysis

The results did not change substantially after adjusting for potential intermediate risk factors, cancer and comorbidities **(Supplemental tables)**, or excluding participants with prevalent chronic diseases like cancer, CVD and COPD **(Supplemental tables).** Additionally, when excluded one by one the lifestyle factors from the overall score, we observed that smoking and BMI were the main drivers of the associations. Furthermore, we repeated the analyses for 7 and 10 years of follow-up **(Supplemental tables)** and the results did not materially change.

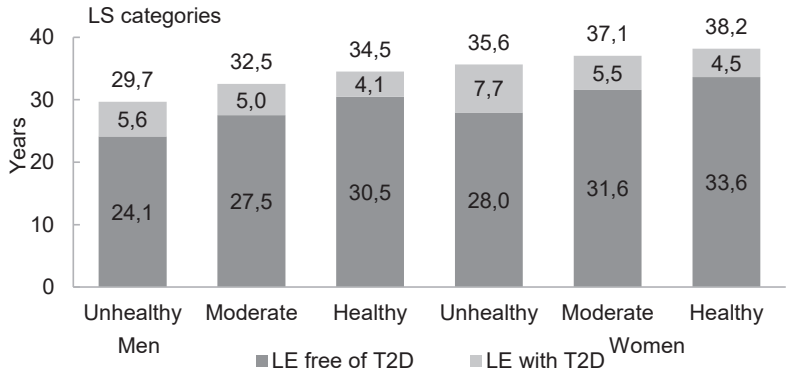


Figure 3.3.2. Effect of lifestyle score categories on LE with and without T2D at age 45 years *
*All life expectancies have been calculated with hazard ratios adjusted for age, cohort and education.

DISCUSSION

Overall we found that compared to unhealthy lifestyle, moderate and healthy lifestyle in middle aged and elderly were associated with longer total life expectancy and years lived without type 2 diabetes in both men and women. A healthier lifestyle decreased the risk of developing diabetes by 50% among men and women. Moreover, men and women who had a healthier lifestyle lived up to 5 years longer compared to participants who had an unhealthier lifestyle.

The increased life expectancy without diabetes in men and women with a healthier lifestyle might be due to the lower risk of diabetes and mortality associated with the healthier and moderate lifestyle categories. A lower risk of diabetes in men and women with a healthier lifestyle might reflect a later diagnosis of diabetes across the lifespan and therefore lead to a higher life expectancy without diabetes. This is also reflected in the total life expectancy which is also longer in men and women with a healthier lifestyle compared to an unhealthy lifestyle. Furthermore, being free from diabetes reduces the mortality risk and therefore leads to an increase in number of years lived and consequently the numbers of years lived without diabetes.

Lifestyle factors were quantified by combining five lifestyle factors, which were later categorized in three categories: 'unhealthy', 'moderate' and 'healthy'. The distribution of the lifestyle score was slightly different between men and women. The majority of both males and females was as expected in the moderate lifestyle category. But in women only a small percentage was represented in the unhealthy group, whereas the remaining part in men was more equally divided between the unhealthy and healthy categories. This could partially explain the differences in life expectancy estimates we see among genders. In addition, these findings show that a healthy lifestyle reduced

the risk of diabetes in men and women. This could indicate that not only single lifestyle factors but also the combination of several lifestyle factors is associated with a reduced risk of diabetes and mortality. It was not the case in transition 3 of our analysese where we assessed the mortality risk among for women who had diabetes (HR 0.96 (95%CI 0.44-2.09)).This could be due to the small number of mortality cases in this group and consenquently a lack of statistical power. However, the possibility that the associations of lifestyle factors on diabetes and mortality risk work differently in males and females should be considered. Previous evidence suggests sex differences in the association between lifestyle behaviors and important health outcomes[15 16]. In line with this, we we noted a difference in the number of years lived with diabetes among men and women. Compared to women, the healthier lifestyle in men was slightly lower risk of diabetes, indicating an earlier occurrence of diabetes during their life. Taken these results together, we could explain why women spend more years with diabetes than men. This is in accordance with previous research conducted in the US concluding that women spend more years living with diabetes than men[17 18], possibly due to larger differences in probabilities of death between males and females observed for patients with diabetes relative to those without diabetes [18 19].

Similar method on assessing the association of lifestyle factors on disease risk and life expectancy was used in a few recent studies[20 21]. Both studies, one in the general Chinese population and the other in the US population calculculated the cumulative effects of the main lifestyle factors and tranlated it into a score. Participants could score on all five lifestyle factor either a zero or one (representing the unhealthy or healthy category) resulting in composite score ranging from zero to five. Pan et al in the Chinese population. found a gain in life expectancy at age 50 years of 8.1 years in women and 6.6 years in men when adhering to 4-5 of the healthy lifestyle factors compared to adhering to none. In the study of Li et al. in the US population even higher number of years gained were found, 14.1 years in women and 12.1 years men. Although, our findings are in line with this evidence, our life expectancy estimates in the healthier category compared to unhealthy category were lower. This could be explained by the fact that in the studies of Li and Pan the two outer ranges are compared with each other, adhering to (almost) all lifestyle factors compared to adhering to none. In our study the unhealthy and healthy categories are a broader and less extreme in values. Another reason for this discrepancy could the differences in population sizes; in which our study population was much lower in numbers. However, our study is unique regarding the approach used for estimating life expectancy with and without diabetes. While Pan et al. and Li et al. obtained the mortality rates by modelling from the national register data , we calculated the mortality rates in the same population used for the analyses and estimated life expectancy with and without diabetes from direct observation of a well-defined population.

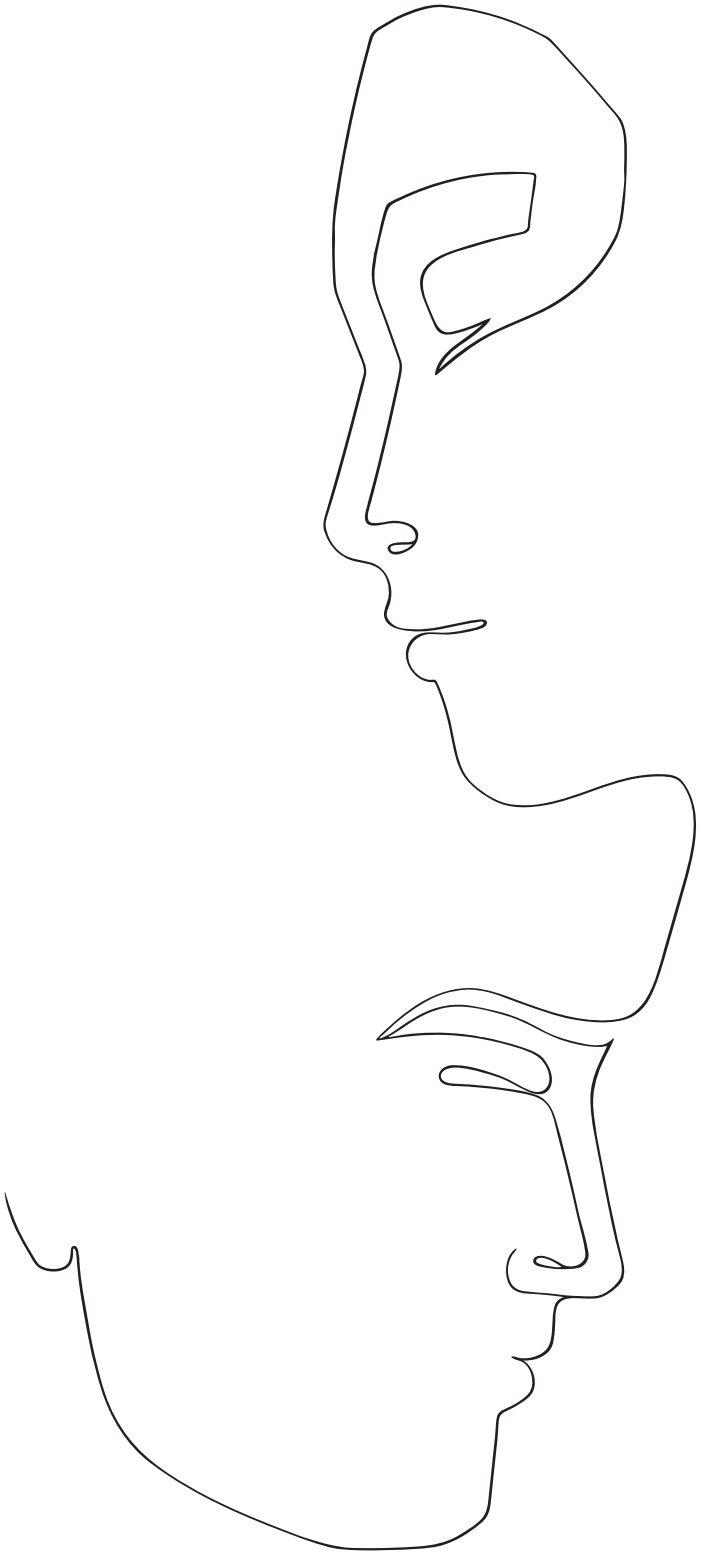
Strengths of our study include the prospective design with a long-term follow-up, the large number of participants and the adjustment for a broad range of potential confounders. The diagnosis of incident diabetes was done by standardized blood glucose measurements at the repeated study centre visits and electronic linkage with pharmacy dispensing records in the study area. Nevertheless, several limitations of this study must be acknowledged. First, diet and lifestyle factors were self-reported; thus, measurement errors are inevitable. This study included multiple waves of the Rotterdam Study, different questionnaires were used to measure physical activity rates and dietary quality. Yet, because we adjusted for cohort and calculated questionnaire-specific categories, the effect may be limited. Additionally, in the first cohort we used dietary intake at baseline as proxy for dietary intake at the third measurements, assuming that dietary patterns remained stable over time[22]. The generalizability of these findings could be limited to middle-aged and older white European populations, our results need confirmation in other populations. Additionally, this study was performed in a Dutch population which is on average a highly physically active population. Hence, confirmation of these findings in other study populations are needed.

A healthier lifestyle was associated with a decreased risk of developing diabetes by 50% among men and women. Overall, men and women who had a healthier lifestyle lived up to 5 years longer compared to participants who had an unhealthier lifestyle. More attention should be given in clinical practice and preventive care to adopting a healthy lifestyle since it could substantially reduce diabetes risk, premature mortality and prolong life expectancy.

REFERENCES

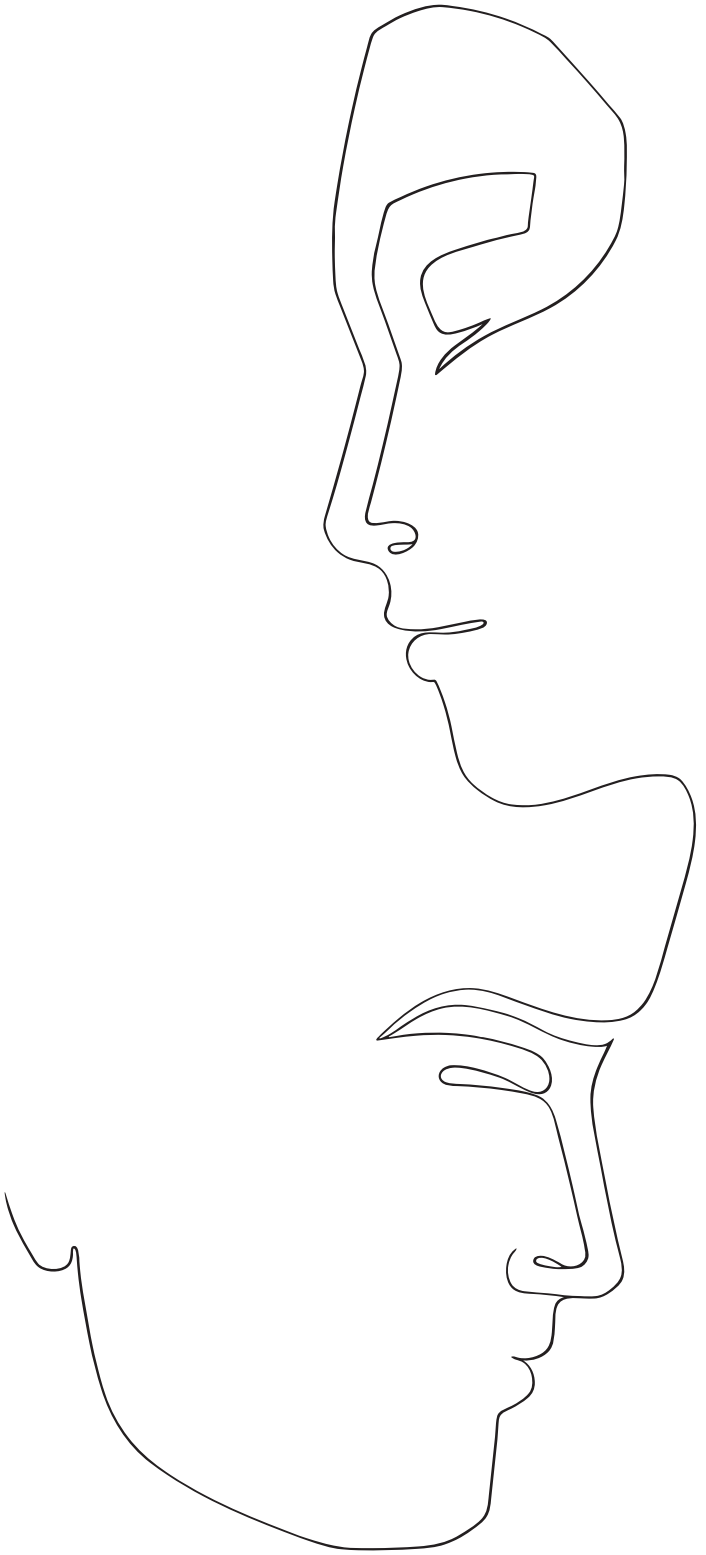
1. Lopez AD, Mathers CD, Ezzati M, et al. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. Lancet 2006;**367**(9524):1747-57.
2. Knowler WC, Barrett-Connor E, Fowler SE, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N Engl J Med 2002;**346**(6):393-403.
3. Khaw KT, Wareham N, Bingham S, et al. Combined impact of health behaviours and mortality in men and women: The EPIC-Norfolk prospective population study. Plos Med 2008;**5**(1):39-47.
4. Hofman A, Brusselle GGO, Murad SD, et al. The Rotterdam Study: 2016 objectives and design update. Eur J Epidemiol 2015;**30**(8):661-708.
5. Voortman T, Kiefte-de Jong JC, Ikram MA, et al. Adherence to the 2015 Dutch dietary guidelines and risk of non-communicable diseases and mortality in the Rotterdam Study. Eur J Epidemiol 2017;**32**(11):993-1005.
6. Klipstein-Grobusch K, den Breeijen JH, Goldbohm RA, et al. Dietary assessment in the elderly: validation of a semiquantitative food frequency questionnaire. Eur J Clin Nutr 1998;**52**(8):588-96.
7. Goldbohm RA, Vandenbrandt PA, Brants HAM, et al. Validation of a Dietary Questionnaire Used in a Large-Scale Prospective Cohort Study on Diet and Cancer. European Journal of Clinical Nutrition 1994;**48**(4):253-65.
8. Caspersen CJ, Bloemberg BPM, Saris WHM, et al. The Prevalence of Selected Physical Activities and Their Relation with Coronary Heart-Disease Risk-Factors in Elderly Men - the Zutphen Study, 1985. Am J Epidemiol 1991;**133**(11):1078-92.
9. Stel VS, Smit JH, Pluijm SMF, et al. Comparison of the LASA Physical Activity Questionnaire with a 7-day diary and pedometer. J Clin Epidemiol 2004;**57**(3):252-58.
10. Leening MJG, Kavousi M, Heeringa J, et al. Methods of data collection and definitions of cardiac outcomes in the Rotterdam Study. Eur J Epidemiol 2012;**27**(3):173-85.
11. van Durme YMTA, Verhamme KMC, Stijnen T, et al. Prevalence, Incidence, and Lifetime Risk for the Development of COPD in the Elderly The Rotterdam Study. Chest 2009;**135**(2):368-77.
12. Consultation W. Obesity: Preventing and managing the global epidemic - Introduction. Who Tech Rep Ser 2000;**894**:1-253.
13. Franco OH, de Laet C, Peeters A, et al. Effects of physical activity on life expectancy with cardiovascular disease. Arch Intern Med 2005;**165**(20):2355-60.
14. Rindskopf D. An introduction to the bootstrap - Efron, B, Tibshirani, RJ. J Educ Behav Stat 1997;**22**(2):245-45.
15. Sodergren M, McNaughton SA, Salmon J, et al. Associations between fruit and vegetable intake, leisure-time physical activity, sitting time and self-rated health among older adults: cross-sectional data from the WELL study. Bmc Public Health 2012;**12**.
16. Sodergren M, Wang WC, Salmon J, et al. Predicting healthy lifestyle patterns among retirement age older adults in the WELL study: A latent class analysis of sex differences. Maturitas 2014;**77**(1):41-46.
17. Narayan KM, Boyle JP, Thompson TJ, et al. Effect of BMI on lifetime risk for diabetes in the U.S. Diabetes Care 2007;**30**(6):1562-6.
18. Dhana K, Nano J, Ligthart S, et al. Obesity and Life Expectancy with and without Diabetes in Adults Aged 55 Years and Older in the Netherlands: A Prospective Cohort Study. PLoS Med 2016;**13**(7):e1002086.
19. Record Owner NLM. Trends in death rates among U.S. adults with and without diabetes between 1997 and 2006: findings from the National Health Interview Survey.

20. Pan XF, Li Y, Franco OH, et al. Impact of combined lifestyle factors on all-cause and cause-specific mortality and life expectancy in Chinese: the Singapore Chinese Health Study. J Gerontol A Biol Sci Med Sci 2019.
21. Li Y, Schoufour J, Wang DD, et al. Healthy lifestyle and life expectancy free of cancer, cardiovascular disease, and type 2 diabetes: prospective cohort study. BMJ 2020;**368**:l6669.
22. Schoufour JD, de Jonge EAL, Jong JCKD, et al. Socio-economic indicators and diet quality in an older population. Maturitas 2018;**107**:71-77.



4

Diabetes and cardiovascular disease risk



4.1

Type 2 diabetes, life expectancy and the number of years lived with and without cardiovascular disease

ABSTRACT

Objective

Total life expectancy may have increased over time together with the number of years spent diseased, due to improvements in treatment and preventive medicine. Therefore, the objective of this study was to estimate the association of type 2 diabetes with total life expectancy and calculate the number of years lived with and without cardiovascular disease.

Methods

Using data from the THIN Study including 2,304,408 participants, we built life tables to calculate the associations of having diabetes with life expectancy and years lived with and without cardiovascular disease among participants 50 years and older. For the life table calculations, we used hazard ratios for 3 transitions (healthy to death, healthy to cardiovascular disease, and cardiovascular disease to death), stratifying by sex and the presence of diabetes at baseline and adjusting for confounders.

Results

Participants with diabetes had a higher cardiovascular disease and mortality risk, with HRs of 1,41 (95%CI 1,39-1,43) and 1,45 (95%CI 1,43-1,47), respectively. The total life expectancy of men and women with diabetes at age 50 years and older was respectively 3.7 and 3.5 years shorter when compared to their counterparts without diabetes. The years lived without cardiovascular disease in men and women with diabetes was 3.6 and 3,5 years shorter compared to non-diabetic participants, respectively. Moreover, there were no differences in the number of years lived with cardiovascular disease between diabetic and non-diabetic men and women.

Conclusion

Our results suggest that people with diabetes have a higher risk of developing CVD and CVD mortality. However, when quantifying these risks into life expectancies we did not observe significant differences in years spent with and without CVD among diabetics and non-diabetics, suggesting that management, care and treatment of CVD in patients with diabetes might have improved in recent years.

INTRODUCTION

Epidemic of diabetes is one of the main threats to human health in our century, affecting about 3–5% of Western populations[1]. The global number of people with diabetes has risen dramatically over the past two decades and is expected to be more than 500 million adults by 2030, with most having type 2 diabetes[2].

Type 2 diabetes (T2D) is a chronic disease associated with increased morbidity and mortality[3, 4], mainly from cardiovascular disease[5]. Cardiovascular disease (CVD) is responsible for ~70% of all mortality among patients with type 2 diabetes[6] and is also a major contributor to diabetes-related healthcare strategies[1]. Previous research has shown that people with T2D live up to 10 years in total, less than those without T2D and approximately 8 years less free of CVD[7, 8]. Improvements in diabetes treatment and care have played a crucial role in decreasing mortality rates among diabetics. However, as life expectancy is increasing, the importance of whether the additional life year gains are spent in good or in poor health is increasing as well. Also, there is limited evidence on how life expectancy has changed over time among these individuals. Due to the heterogeneous results from current evidence, more research on the development of type 2 diabetes and cardiovascular disease is needed. Even though the mortality among people with diagnosed diabetes is decreasing due to better diabetes care, it still remains high. The decrease in mortality means an increase in longevity but does not necessarily lead to an increase of the number of healthy years in a person's life. Over the long term, living with type 2 diabetes decreases quality of life and increases the use of health care services[7]. Many studies has covered a limited age span (focused mainly in elderly) or were based on low case numbers. Our hypothesis is that total life expectancy may have increased over time together with the number of years spent diseased, due to improvements in treatment and preventive medicine. Therefore, our aim was to estimate the association of T2D with total life expectancy and calculate the number of years lived with and without CVD, using data from a large cohort.

METHODS

Study population and study sample

We undertook a cohort study with prospectively collected data from The Health Improvement Network (THIN) database, which contains computerized primary care records from primary care physicians who use the Vision IT system and have agreed at the practice level to participate (covering 6.2% of the U.K. population)[9]. THIN captures diagnoses, prescriptions, and tests from primary care, and referrals to specialists, hospital admissions, and diagnoses made in secondary care, which are typically reported back to the

primary care physician. THIN data are representative of the U.K. population[10], and comparisons to external statistics and other independent studies have shown that both the clinical diagnostic and prescribing information are well recorded and accurate [9, 10]. Data collection began in January 1995, and we used all data to September 2015. For this study, THIN's independent Scientific Review Committee granted approval in August 2016 (scientific review committee reference number: 16THIN078).

Out of 2,387,055 participants that were 50 years and older we excluded those with type 1 diabetes (n=19,642) and participants who didn't meet quality measures (63,005), which left us with 2,304,408 study participants for our analyses (**Figure 4.1.1**).

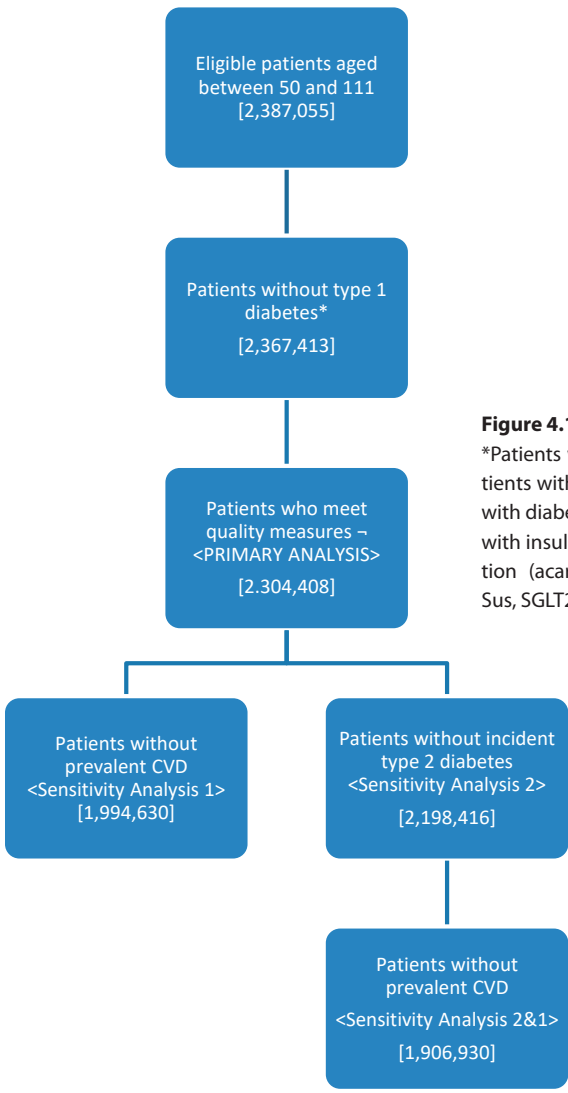


Figure 4.1.1. Flowchart of study participants
*Patients with type 1 diabetes are identified as patients with type 1 diabetes. Read Codes or patients with diabetes code (unspecified as type 1 or type 2) with insulin prescription and no oral drug prescription (acarbose/DPP4i, glitazones, glinides, GLP1, Sus, SGLT2) and age at diagnosis of diabetes < 40

Assessment of exposure

A clinical diagnosis of T2D by the general practitioner was the outcome of interest. In the UK, the Quality Outcome Framework (QOF) in general practices ensures high-quality data on important comorbidities such as cardiovascular disease, hypertension and T2D[11, 12]. Within the database, diagnostic codes for T2D were identified based on Read codes, a hierarchical coding system to record signs, symptoms, procedures and diagnosis in primary care[12, 13].

Assessment of outcomes

The primary outcome of our study was incident CVD. The endpoints were the first record of one of the following presentations of CVD: coronary heart and heart failure. Any events occurring after the first CVD presentation were ignored. A second outcome of the current study was mortality in the total study population. The definition of the primary outcome in THIN database has already been validated[14].

Assessment of Covariates

Covariates that are independent predictors of T2D other than the exposure of interest were selected on the basis of biological plausibility and previous literature[15]. Participant's age, sex, self-reported smoking status, and social deprivation were included in models. Data recorded at study entry were used to classify participants as never smokers, ex-smokers, or current smokers. Social deprivation was included as quintiles of the index of multiple deprivation[16], a score calculated for each participant's neighbourhood on the basis of indices such as income, education, and employment. BMI was defined using World Health Organization criteria as follows: underweight (BMI of<18.5kg/m^2), normal weight (BMI of 18 kg/m^2 to <25 kg/m^2), overweight (BMI of 25 kg/m^2 to <30 kg/m^2), and obese (BMI of ≥30 kg/m^2). Other covariates used were glycated haemoglobin A1c, systolic blood pressure, lipid-lowering medication, estimated glomerular filtration rate, presence of hypertension, Townsend social deprivation index, and comorbidities.

Data Analysis

We created life tables to calculate the differences in life expectancy and years lived with and without CVD by presence of diabetes. We used a period multistate life table. This type of life table combines information from people of different ages and from different birth cohorts[17, 18]. We considered 3 different health states: free of CVD, CVD, and death. Participants could experience the following transitions: from free of CVD to CVD or death and from CVD to death. Participants were not allowed to move back from a disease state (eg, from having CVD to not having CVD), and only the first entry into a state was considered[19]. To evaluate the differences in risk of mortality and CVD among

participants by presence of diabetes at baseline, we first calculated the overall sex- and age-specific rates for each transition. Hazard ratios (HRs) comparing diabetic with non-diabetic participants were calculated using Poisson regression ("Gompertz" distribution) in models adjusted for confounders[18].[8]. Model 1 adjusted for age; model 2 adjusted for for age, alcohol, Townsend, smoking and BMI in addition to age; and model 3 adjusted for lipid lowering medication, cholesterol, hypertension, eGFR, and comorbidities in addition to the variables in model 2. Finally, we calculated 3 sets of transition rates for diabetic and nondiabetic subjects using the overall transition rates, the adjusted HRs for CVD and mortality, and the prevalence of diabetes by sex and presence of CVD. Similar calculations have been described previously[8, 17, 18]. For our statistical analyses, we used STATA version 6.0 for Windows (StataCorp, College Station). We built life tables stratified by sex and presence of diabetes. The multistate life table started at age 50 years and closed at age 110 years. We calculated confidence intervals for all life expectancies and their differences using Monte Carlo simulation (parametric bootstrapping)[20]. To calculate the confidence intervals, we used @RISK software (MathSoft Inc, Cambridge, Mass; 1999), with 10 000 runs.

RESULTS

In **Table 4.1.1** are shown the baseline characteristics of your study population. Overall, there were slightly more men with type 2 diabetes and CVD than women. Also, men were more likely to smoke and drink while women were more hypertensive and suffered more from diseases such as depression and dementia.(**Table 4.1.1**)

In total, we observed 181,804 incident CVD cases and 387,781 deaths. The hazard ratios (HRs) calculated in the overall population and those calculated separately for men and women were similar. Therefore, we decided to calculate the life tables with the HRs obtained using the whole population. Overall, participants with diabetes had a higher CVD and mortality risk, with HRs respectively, HR 1,41 (95%CI 1,39-1,43) and HR1,45 (95%CI 1,43-1,47) (**Table 4.1.2**). As expected, also life expectancy estimates when using sex specific HRs in the sensitivity analyses did not differ from those calculated using the overall HRs.

The total life expectancy of men and women at age 50 years and older was respectively 3.7 and 3.5 years shorter when compared to their counterparts without diabetes (**Table 4.1.3 and Figure 4.1.1**). Diabetic men free of CVD lived 3.6 years shorter and diabetic women without CVD lived 3.5 years shorter compared to non-diabetic participants. Moreover, there were no differences in the number of years lived with CVD between diabetic and non-diabetic men and women.

For the statistical analyses we build several models and in all of them the estimates where comparable. The most important models were Model 1 (main model) in which was adjusted for age, alcohol, Townsend (deprivation), smoking and BMI. Additionally, in Model 2 was adjusted for lipid lowering medication, cholesterol, hypertension, eGFR, and comorbidities. In a third model, adding Hba1c did not significantly attenuate the

Table 4.1.1. Baseline characteristics of study participants

	Men	Women
Age, mean (SD), y	62.31(18.06)	63.69(19.66)
Diabetes mellitus, Yes (%)	135,573(4.15)	110,813(3.22)
Cardiovascular Disease, Yes (%)	202,786(6.21)	168,342(4.89)
Smoking Categories, Yes. (%)		
Never smoker	1,523,737(46.67)	2,059,423(59.79)
Former smoker	590,379(18.08)	551,273(16.00)
Current smoker	780,469(23.90)	682,337(19.81)
Missing	370,410(11.34)	151,634(4.40)
Drinker Categories, Yes. (%)		
Never drinker	395,633(12.12)	696,625(20.22)
Drinker no excess	1,803,058(55.22)	1,945,667(56.48)
Excessive drinker	114,718(3.51)	60,239(1.75)
Missing	951,586(29.15)	742,136(21.54)
Townsend, Yes. (%)		
1	663,609(20.32)	695,705(20.20)
2	603,392(18.48)	641,050(18.61)
3	625,280(19.15)	662,942(19.25)
4	579,702(17.76)	616,621(17.91)
5	423,326(12.97)	431,405(12.52
Missing	369,686(11.32)	396,744(11.52)
Body mass index, BMI, mean (SD), kg/m2	26.29(4.84)	25.89(5.83)
Hypertension, Yes (%)	391,898(12.00)	473,561(43.75)
Cholesterol (mmol/l), mean(SD)	4.96(1.10)	5.21(1.10)
Lipid lowering medication in the last 60 days, Yes. (%)	379,906(11.64)	339,761(9.86)
eGFR	84.76(22.87)	83.82(24.35)
Hba1c (%), mean(SD)	6.71(1.58)	6.45(1.51)
Prevalent Cancer, Yes (%)	69,588(2.13)	101,199(2.94)
Depression, Yes (%)	308,668(9.45)	586,429(17.02)
Dementia, Yes (%)	16,000(0.49)	36,073(1.05)
Chronic liver disease, Yes (%)	15,396(0.47)	11,727(0.34)
Prevalent COPD, Yes (%)	52,592(1.61)	19,101(1.43)
Prevalent Atrial Fibrilation, Yes (%)	106,523(3.269)	93,898(2.73)

results. Moreover, we performed several sensitivity analyses in which we 1) excluded prevalent CVD cases for transition 2 (in the whole population and sex specifically), 2) excluded incident T2D cases and 3) excluded incident T2D cases overall and prevalent CVD for transition 2 altogether. The results did not significantly differ in any of the sensitivity analyses.

Table 4.1.2. Hazard ratios for CVD and mortality by presence of T2D.

Transition	Categories	Cases/ Number of people	Model 1 HR (95% CI)[a]	Model 2 HR (95% CI)[b]	Model 3 HR (95% CI)[c]
Incident CVD	No T2D	181,804/ 6,241,606	1,00	1,00	1,00
	Yes T2D		1,41 (1,39-1,43)	1,14 (1,11-1,17)	1,28 (1,26-1,31)
No CVD to mortality	No T2D	250,763/ 6,241,606	1,00	1,00	1,00
	Yes T2D		1,51 (1,49-1,53)	1,38 (1,35-1,41)	1,41 (1,39-1,43)
CVD to mortality	No T2D	137,018/ 510,266	1,00	1,00	1,00
	Yes T2D		1,45 (1,43-1,47)	1,40 (1,37-1,43)	1,45 (1,43-1,47)

Abbreviations: HR, Hazard Ratio; CI, Confidence Interval; T2D, Type 2 Diabetes; CVD, cardiovascular disease.
[a] Adjusted for age, alcohol, Townsend, smoking and BMI.
[b] Adjusted for age, alcohol, Townsend, smoking, BMI, lipid lowering medication, cholesterol, hypertension, eGFR, Hba1c and comorbidities.
[c] Adjusted for age, alcohol, Townsend, smoking, BMI, lipid lowering medication, cholesterol, hypertension, eGFR and comorbidities.

Table 4.1.3. Life expectancy in years at age 50 years and older, stratified by gender.

	Total LE	Difference in Total LE†	LE free of CVD	Difference in LE free of CVD †	LE with CVD	Difference in LE with CVD †
Men						
No T2D	32,4	Ref	26,9	Ref	5,4	Ref
Yes T2D	28,7	-3.7	23,3	-3.6	5,4	0.0
Women						
No T2D	35,1	Ref	31,0	Ref	4,1	Ref
Yes T2D	31,6	-3.5	27,5	-3.5	4,0	0.0

Abbreviations: LE, Life Expectancy; T2D, Type 2 Diabetes; CVD, cardiovascular disease; Ref, Reference.
* All life expectancies have been calculated with hazard ratios adjusted for age, alcohol, Townsend, smoking and BMI.

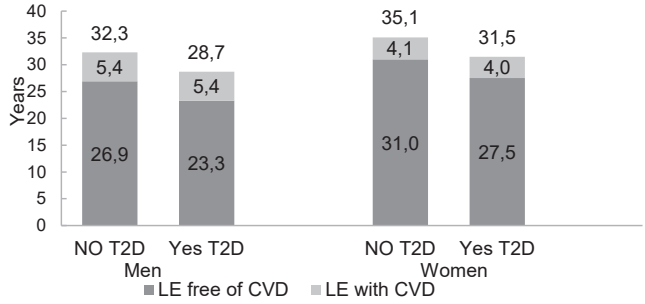


Figure 4.1.2. Effect of diabetes on life expectancy with and without cardiovascular disease at age 50 years and older .
All life expectancies have been calculated with hazard ratios adjusted for age, alcohol, Townsend, smoking and BMI.

DISCUSSION

In our study, participants with diabetes had a higher cardiovascular disease and mortality risk, with HRs of 1,41 (95%CI 1,39-1,43) and 1,45 (95%CI 1,43-1,47), respectively. The total life expectancy of men and women with diabetes at age 50 years and older was respectively 3.7 and 3.5 years shorter when compared to their counterparts without diabetes. The years lived without cardiovascular disease in men and women with diabetes was 3.6 and 3,5 years shorter compared to non-diabetes participants, respectively. Further, there were no differences in the number of years lived with cardiovascular disease between men and women with and without diabetes.

The shorter CVD-free life expectancies among diabetic subjects is due to their higher incidence of CVD combined with a higher risk of non-CVD mortality. We found no significant difference between the years spent with CVD between diabetic and nondiabetic subjects. This is not surprising, given that while those with diabetes are at a greater risk of developing CVD, once they have it, they are at a greater risk of dying.

The HRs that we used in our life tables fall within the range of the recently published associations of diabetes with CVD and mortality however,[21] previous studies mainly using cohort data from decades ago have reported higher HRs in comparison to what we observed [22-28]. This discrepancy could be explained due to the historical differences in diagnoses, treatment and prevention and lifestyle of type 2 diabetes and CVD. Improvement in cardiometabolic health prevention has led to an earlier diagnosis of diabetes now than decades ago. In addition, factors leading to diabetes could have changed; for example patients in the Framingham study were more family history type as opposed to now that are heavily driven by BMI. The sensitivity analysis illustrates the effects of smaller or larger associations of diabetes, none of which attenuated our results.

Historical comparisons between cohorts showed that the HRs for CVD among diabetics compared to age, sex and BMI controls where higher in the earlier subcohort (1997-2007); HR 1.79 (95% CI 1.55-1.86) than in the second subcohort (2007-2017); HR 1.51 (95% CI 1.40-1.63). When looking at the HRs for mortality in diabetics with and without CVD in the 2 subcohorts we found similar HRs between the cohorts. Surprisingly, diabetics without CVD had lower mortality risks compared to diabetics with CVD. These discrepancies could be due to the shorter follow up time in the second subcohort and the different medical follow-up patients with and without CVD receive.

The above estimates and differences in life expectancy could explain also the smaller differences we find in life expectancy estimates and number of years lived with and without CVD when comparing participants with and without diabetes. Franco et al. using data from the Framingham study found that men and women with diabetes 50 years and older lived on average 7.5 (95% confidence interval, 5.5-9.5) and 8.2 (95% confidence interval, 6.1-10.4) years less than their nondiabetic equivalents. The differences in life expectancy free of CVD were 7.8 and 8.4 years, respectively[28]. Using data from NHANES I, another study found that median LE was 8 years lower for diabetic subjects aged 55 to 64 years[29]. Similarly, using cross-sectional data from the National Health Interview Surveys (NHIS) and Markov models, Narayan et al estimated that the presence of diabetes among non-Hispanic, 50-year-old men would result in a loss of 8 years in LE[30]. However, our study includes recent patient data and also it is much larger and diverse in sample size.

To the best of our knowledge, this is the largest prospective study of the association between body size phenotypes with or without metabolic abnormalities and a range of incident CVD events with unprecedented precision and power.Moreover our mortality recordings and data on T2D and CVD are very accurate and validated.

Nevertheless, some limitations of this study must be considered. The current study has inhereted all the limitations of routinely collected data. Unlikely in a cohort study where every participant is screened, in routinely collected data many of the patients could be undiagnosed which could lead to missclassification of the exposure. Moreover, although we had validated data on all cause mortality, it is not the case for cause specific mortality data. Some of the acute deaths from CVD we may not have attributed it to cardiovascular disease.Further, some of the covariates had not been uniformly collected, such as smoking and alcohol data thus they should be viewed with caution. In addition, we did not have any data on physical activity levels among the participants. Lifestyle factors such as physical activity, smoking, alcohol and BMI play an important role especially in developing T2D and subsequently as well in cardiometabolic health in general[18, 31]. Li et al. showed that adherence to a healthy lifestyle at mid-life is associated with a longer life expectancy free of major chronic diseases[31].While two other studies investigating the effects of physical activity on diabetes, CVD and mortality risk conclude

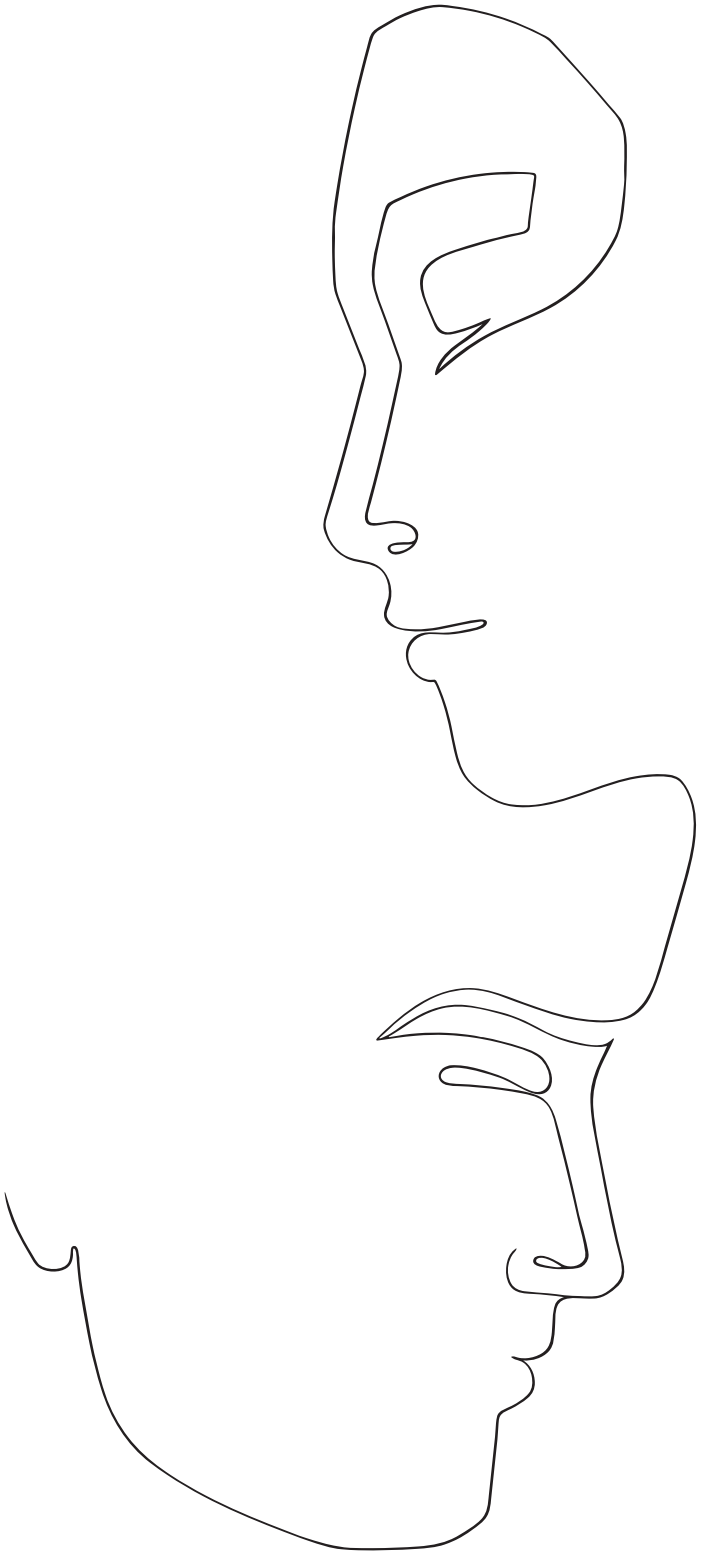
that avoiding a sedentary lifestyle during adulthood not only prevents cardiovascular disease independently of other risk factors but also substantially expands the total life expectancy and the cardiovascular disease–free life expectancy for men and women. This effect is already seen at moderate levels of physical activity, and the gains in cardiovascular disease–free life expectancy are twice as large at higher activity levels[18, 32]. Moreover, recent evidence suggests that adhering to the healthy range of lifestyle factors at 12 months after the first diagnoses of diabetes, patients could achieve remission to a non-diabetic state and off antidiabetic drugs[33].In the future, better designed randomized control studies or large national and international cohort studies should investigate the new trends of cardiometabolic diseases in order to determine which factors or what type of care could be appropriate in current times for these patients.

The total life expectancy of men and women with diabetes at age 50 years and older was respectively 3.7 and 3.5 years shorter when compared to their counterparts without diabetes. The years lived without cardiovascular disease in men and women with diabetes was 3.6 and 3,5 years shorter compared to non-diabetic participants, respectively. Our results suggest that people with diabetes have a higher risk of developing CVD and CVD mortality. However, when quantifying these risks into life expectancies we did not observe significant differences in years spent with and without CVD among diabetics and non-diabetics, suggesting that management, care and treatment of CVD in patients with diabetes might have improved in recent years.

REFERENCES

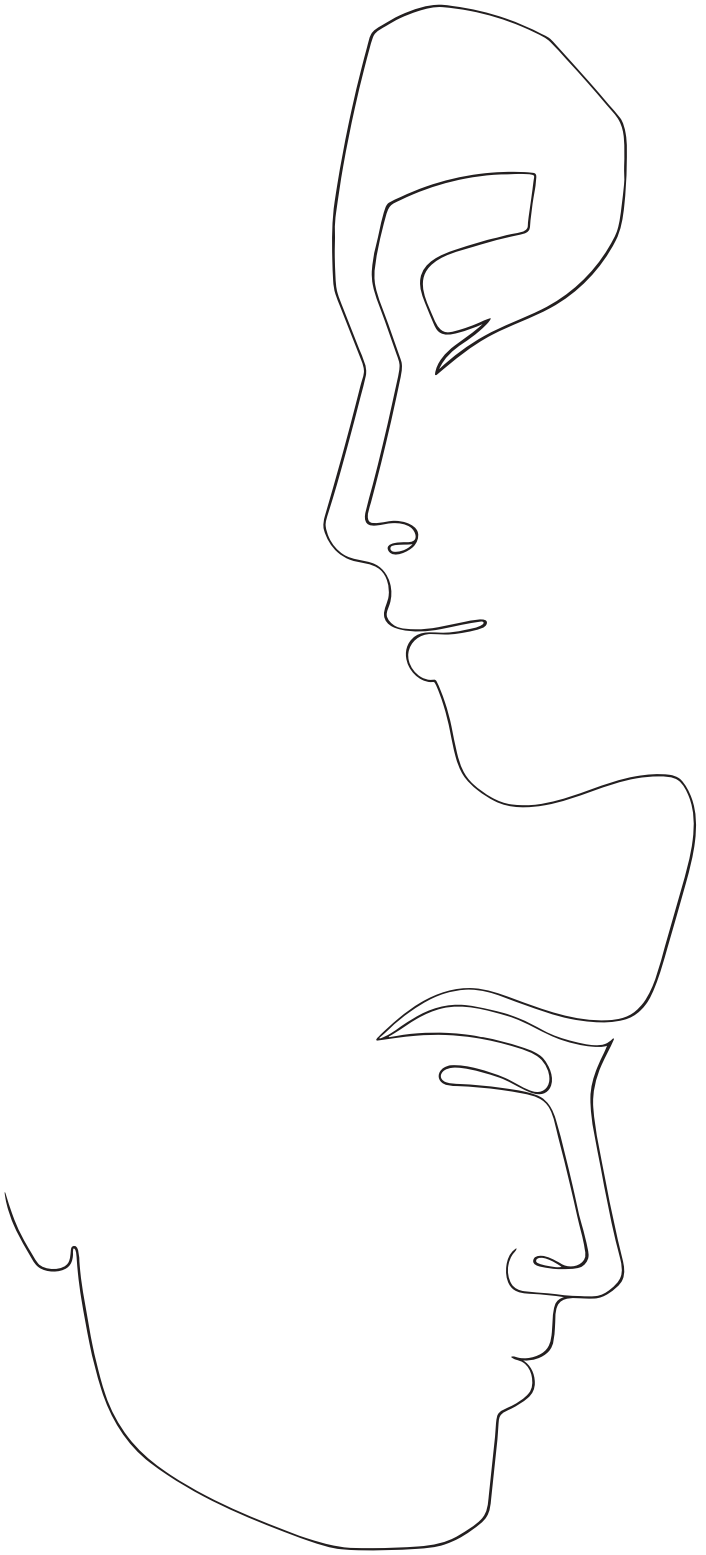
1. Brown, J.B., K.L. Pedula, and A.W. Bakst, *The progressive cost of complications in type 2 diabetes mellitus.* Arch Intern Med, 1999. **159**(16): p. 1873-80.
2. Whiting, D.R., et al., *IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030.* Diabetes Res Clin Pract, 2011. **94**(3): p. 311-21.
3. Inzucchi, S.E., et al., *Management of hyperglycemia in type 2 diabetes: a patient-centered approach: position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD).* Diabetes Care, 2012. **35**(6): p. 1364-79.
4. Tancredi, M., et al., *Excess Mortality among Persons with Type 2 Diabetes.* New England Journal of Medicine, 2015. **373**(18): p. 1720-1732.
5. Seshasai, S.R.K., et al., *Diabetes Mellitus, Fasting Glucose, and Risk of Cause-Specific Death.* New England Journal of Medicine, 2011. **364**(9): p. 829-841.
6. Laakso, M., *Hyperglycemia and cardiovascular disease in type 2 diabetes.* Diabetes, 1999. **48**(5): p. 937-42.
7. Loukine, L., et al., *Impact of diabetes mellitus on life expectancy and health-adjusted life expectancy in Canada.* Population Health Metrics, 2012. **10**.
8. Franco, O.H., et al., *Associations of diabetes mellitus with total life expectancy and life expectancy with and without cardiovascular disease.* Archives of Internal Medicine, 2007. **167**(11): p. 1145-1151.
9. Lewis, J.D., et al., *Validation studies of the health improvement network (THIN) database for pharmacoepidemiology research.* Pharmacoepidemiol Drug Saf, 2007. **16**(4): p. 393-401.
10. Blak, B.T., et al., *Generalisability of The Health Improvement Network (THIN) database: demographics, chronic disease prevalence and mortality rates.* Inform Prim Care, 2011. **19**(4): p. 251-5.
11. Kontopantelis, E., et al., *Recorded quality of primary care for patients with diabetes in England before and after the introduction of a financial incentive scheme: a longitudinal observational study.* Bmj Quality & Safety, 2013. **22**(1): p. 53-64.
12. O'Reilly, M.W., et al., *Serum testosterone, sex hormone-binding globulin and sex-specific risk of incident type 2 diabetes in a retrospective primary care cohort.* Clin Endocrinol (Oxf), 2019. **90**(1): p. 145-154.
13. Kumarendran, B., et al., *Polycystic ovary syndrome, androgen excess, and the risk of nonalcoholic fatty liver disease in women: A longitudinal study based on a United Kingdom primary care database.* Plos Medicine, 2018. **15**(3).
14. Hall, G.C., *Validation of death and suicide recording on the THIN UK primary care database.* Pharmacoepidemiol Drug Saf, 2009. **18**(2): p. 120-31.
15. Toulis, K.A., et al., *All-cause mortality in patients with diabetes under glucagon-like peptide-1 agonists: A population-based, open cohort study.* Diabetes Metab, 2017. **43**(3): p. 211-216.
16. Charlson, M.E., et al., *A new method of classifying prognostic comorbidity in longitudinal studies: development and validation.* J Chronic Dis, 1987. **40**(5): p. 373-83.
17. Asllanaj, E., et al., *Age at natural menopause and life expectancy with and without type 2 diabetes.* Menopause-the Journal of the North American Menopause Society, 2019. **26**(4): p. 387-394.
18. Franco, O.H., et al., *Effects of physical activity on life expectancy with cardiovascular disease.* Archives of Internal Medicine, 2005. **165**(20): p. 2355-2360.
19. Peeters, A., et al., *A cardiovascular life history - A life course analysis of the original Framingham Heart Study cohort.* European Heart Journal, 2002. **23**(6): p. 458-466.

20. Rindskopf, D., *An introduction to the bootstrap - Efron, B, Tibshirani, RJ.* Journal of Educational and Behavioral Statistics, 1997. **22**(2): p. 245-245.
21. Rawshani, A., et al., *Risk Factors, Mortality, and Cardiovascular Outcomes in Patients with Type 2 Diabetes.* New England Journal of Medicine, 2018. **379**(7): p. 633-644.
22. Huxley, R., F. Barzi, and M. Woodward, *Excess risk of fatal coronary heart disease associated with diabetes in men and women: meta-analysis of 37 prospective cohort studies.* BMJ, 2006. **332**(7533): p. 73-8.
23. Singer, D.E., A.W. Moulton, and D.M. Nathan, *Diabetic myocardial infarction. Interaction of diabetes with other preinfarction risk factors.* Diabetes, 1989. **38**(3): p. 350-7.
24. Smith, J.W., F.I. Marcus, and R. Serokman, *Prognosis of patients with diabetes mellitus after acute myocardial infarction.* Am J Cardiol, 1984. **54**(7): p. 718-21.
25. Waugh, N.R., et al., *Mortality in a cohort of diabetic patients. Causes and relative risks.* Diabetologia, 1989. **32**(2): p. 103-4.
26. Record Owner, N.L.M., *Risk Factors, Mortality, and Cardiovascular Outcomes in Patients with Type 2 Diabetes.*
27. Song, S.H. and P.M. Brown, *Coronary heart disease risk assessment in diabetes mellitus: comparison of UKPDS risk engine with Framingham risk assessment function and its clinical implications.* Diabet Med, 2004. **21**(3): p. 238-45.
28. Franco, O.H., et al., *Associations of diabetes mellitus with total life expectancy and life expectancy with and without cardiovascular disease.* Arch Intern Med, 2007. **167**(11): p. 1145-51.
29. Gu, K., C.C. Cowie, and M.I. Harris, *Mortality in adults with and without diabetes in a national cohort of the U.S. population, 1971-1993.* Diabetes Care, 1998. **21**(7): p. 1138-45.
30. Narayan, K.M., et al., *Lifetime risk for diabetes mellitus in the United States.* JAMA, 2003. **290**(14): p. 1884-90.
31. Li, Y.P., et al., *Healthy lifestyle and life expectancy free of cancer, cardiovascular disease, and type 2 diabetes: prospective cohort study.* Bmj-British Medical Journal, 2020. **368**.
32. Dhana, K., et al., *Physical activity types and life expectancy with and without cardiovascular disease: the Rotterdam Study.* Journal of Public Health, 2017. **39**(4): p. E209-E218.
33. Lean, M.E.J., et al., *Primary care-led weight management for remission of type 2 diabetes (DiRECT): an open-label, cluster-randomised trial.* Lancet, 2018. **391**(10120): p. 541-551.



5

Epigenetics and cardiometabolic health



5.1

A systematic review of sexually dimorphic DNA-methylation in cardiometabolic health

ABSTRACT

Sex is a major determinant of cardiometabolic risk**.** DNA methylation (DNAm), an important epigenetic mechanism that differs between sexes, has been associated with cardiometabolic diseases. Therefore, we aimed to systematically review studies in adults investigating sex-specific associations of DNAm with intermediate cardiometabolic traits and incident cardiovascular disease including stroke, myocardial infarction (MI) and coronary heart disease (CHD). Five bibliographic databases were searched from inception to 15 July 2019. We selected 35 articles (based on 30 unique studies) from 17,023 references identified; including a total of 14,020 participants from European, North American and Asian ancestries. Four studies reported sex differences between global DNAm and blood lipid levels and stroke risk. In 25 genome wide and candidate gene approach studies, DNAm at 31 gene sites were associated with sex differences in cardiometabolic diseases. The identified genes were *PLA2G7, BCL11A, KDM6A, LIPC, ABCG1, PLTP, CETP, ADD1, CNN1B, HOOK2, GFBP-7, PTPN1, GCK, PTX3, ABCG1, GALNT2, CDKN2B, APOE, CTH, GNASAS, INS, PON1, TCN2, CBS, AMT, KDMA6A, FTO, MAP3K13, CCDC8, MMP-2* and *ER-α*. Prioritized pathway connectivity analysis associated these genes with biological pathways such as vitamin B12 metabolism, statin pathway, plasma lipoprotein, plasma lipoprotein assembly, remodeling and clearance and cholesterol metabolism. Our findings suggest that DNAm might be a promising molecular strategy for understanding sex differences in the pathophysiology of cardiometabolic diseases and that future studies should investigate the effects of sex on epigenetic mechanisms in cardiometabolic risk. In addition, we emphasize the gap between the translational potential and the clinical utilization of cardiometabolic epigenetics.

INTRODUCTION

Cardiometabolic diseases include cardiovascular diseases (CVD), type 2 diabetes (T2D) and their associated risk factors including components of the metabolic syndrome and obesity[1]. Aging is associated with development of unfavorable cardiometabolic profile which in large contributes to increased incidence of major cardiovascular events and mortality[2]. Intermediate cardiometabolic risk factors are unequally distributed among sexes, and sex differences are also described in cardiometabolic diseases prevalence, clinical characteristics and prognosis[3, 4, 5]. Generally, before menopause women have better cardiometabolic risk profiles than same aged men; however, this sex advantage gradually disappears with advancing age, particularly after menopause[6]. Mechanisms underlying sex differences in CVD have been extensively studied in the past two decades and signaling pathways including epigenetic modifications of the genome emerged as possible pathways leading to sexual dimorphism in cardiometabolic diseases [7].

Epigenetic modifications comprise dynamic changes in the genome engaged in the modification of important cellular processes such as gene expression, chromosomal stability and genomic imprinting [8, 9]. DNA methylation (DNAm) is the best understood and most extensively studied epigenetic mechanism in regard to CVD risk [8, 9]. DNAm refers to the transfer of a methyl group into the C5 position of the cytosine to form 5-methylcytosine (5mC) and increases or decreases in genomic 5mC are referred as DNA hyper- and hypomethylation respectively [8, 9]. The global DNAm assessed at long-interspersed nuclear element (LINE-1) has been inversely associated with intermediate CVD risk factors and higher risk of metabolic status worsening[10]. Conversely, a higher degree of global DNA methylation measured at Alu repeats or by the LUMA method was associated with the presence of CVD[10]. Also, gene specific hyper- or DNA hypomethilation was associated with changes in gene expression and was shown to affect cardiometabolic risk including atherosclerosis, inflammation, blood pressure, serum lipid and glucose levels, subsequently leading to increased risk of developing T2D, stroke and myocardial infarction[11]. Also, sex-specific differences in DNAm have been found in brain, human pancreatic islets and blood[12, 13]. Men in general seem to have lower levels of methylation in their genome as compared to women[14, 15], indicating that similarly to sex chromosomes, methylation at the autosomes might be subject to sex differences. Despite this, only a relatively small number of studies in the ample field of cardiometabolic epigenetics stratify according to sex or focus in sex differences. Although a few studies[11, 16, 17] have summarized the existing literature on this complex topic, they did not focus on epigenetically induced sex differences in CVD, intermediate CVD risk factors or T2D, with the exception of a recent review that focused only on major CVD events[16].

Therefore, we aimed to systematically review the available evidence in human studies exploring the association between sex-specific DNAm and cardiometabolic diseases.

METHODS

Data Sources and Searches

This review was conducted using a predefined protocol (which was not registered at online platforms) and following a recently published guideline on how to perform systematic reviews[18] and in accordance with PRISMA guidelines[19]. A literature search was done using 5 electronic databases (Medline ALL via Ovid (1946-current), EMBASE (1974-current) via embase.com, Web of Science Core Collection (1900-current), Cochrane CENTRAL registry of trials (issue 7 2019) via Wiley and Google Scholar) until 15 July 2019 (date last searched) with the help of an experienced medical information specialist (WMB). All references were imported in an EndNote library and deduplicated with the algorithm developed by Bramer et al[20]. Additionally, we searched the reference lists of the included studies and relevant reviews. Two independent reviewers screened the titles and abstracts of all studies identified initially, according to the selection criteria. Full texts were retrieved from studies that satisfied all selection criteria. All disagreements were resolved through consensus or consultation with two other independent reviewers.

Study Selection

Observational (cross-sectional, case-control, prospective) studies conducted in adults and investigating the associations of global or gene-specific DNAm with cardiometabolic outcomes were selected. Studies were included if they investigated sex-stratified associations between DNAm and intermediate cardio-metabolic traits (blood lipids, glucose, blood pressure, inflammatory markers, atherosclerosis, T2D) and CVD (MI, CHD, stroke). Also, we included studies that reported a significant interaction term with sex but did not stratify by sex in their analysis. Furthermore, studies conducted only in men/women were not included in the current review.

Data Extraction

A predesigned electronic data abstraction form was used to extract relevant information. This included questions on study location, percentage of men and women included in the study, participants' age, cardio-metabolic outcome, tissue type, DNAm technique used and general and sex-specific findings. Two authors independently extracted the data and a consensus was reached in case of any inconsistency with involvement of an additional author.

Assessing the Risk of Bias

Two independent investigators used the Newcastle-Ottawa Scale[21] to assess the risk of bias of the included observational studies. The Newcastle-Ottawa Scale uses a star system (maximum of nine stars) to evaluate three domains: selection of participants; comparability of study groups; and the ascertainment of outcomes and exposures of interest. Studies that received a score of nine stars were judged to be of low risk of bias; a score of seven or eight stars was medium risk and those that scored six or less were considered at high risk of bias.

Pathway connectivity analysis

To identify biological pathways of the differentially methylated genes previously linked to CVD, we used the CPDB (ConsensusPathDB-human) tool from the Max Plank Institute for Molecular Genetics[22]. This tool integrates interaction networks in humans (in this study) and includes information on binary and complex protein-protein, genetic, metabolic, signaling, gene regulatory and drug-target interactions, as well as biochemical pathways[22]. Data that explains interactions was derived from 32 public resources.

RESULTS

Search Results and Study Characteristics

The search strategy identified 17,023 potentially relevant studies; after titles and abstracts were screened 16, 814 references were excluded (**Figure 5.1.1**). For the remaining 209 references, full-text articles were reviewed, 174 of which were excluded for various reasons outlined in **Figure 5.1.1**. A total of 35 articles based on 30 unique studies were included in this systematic review including a total of 14,020 non-overlapping participants, of whom approximately 53% were women. The studies included population with European (n=13), North American (n=3) and Asian (n=14) ancestries with age ranging from 32 to 75 years. Due to differences in the epigenetics marks and outcomes investigated, as well as different study designs of the included studies, we were not able to quantitatively pool the estimates from various studies. Therefore we report in this review a detailed descriptive summary of the available published literature. The characteristics of the included studies are described in **Table 5.1.1**. The median Newcastle-Ottawa quality score for the included studies was 7 of 9 possible points. The **Table 5.1.1** depicts the methodologic quality of all included studies.

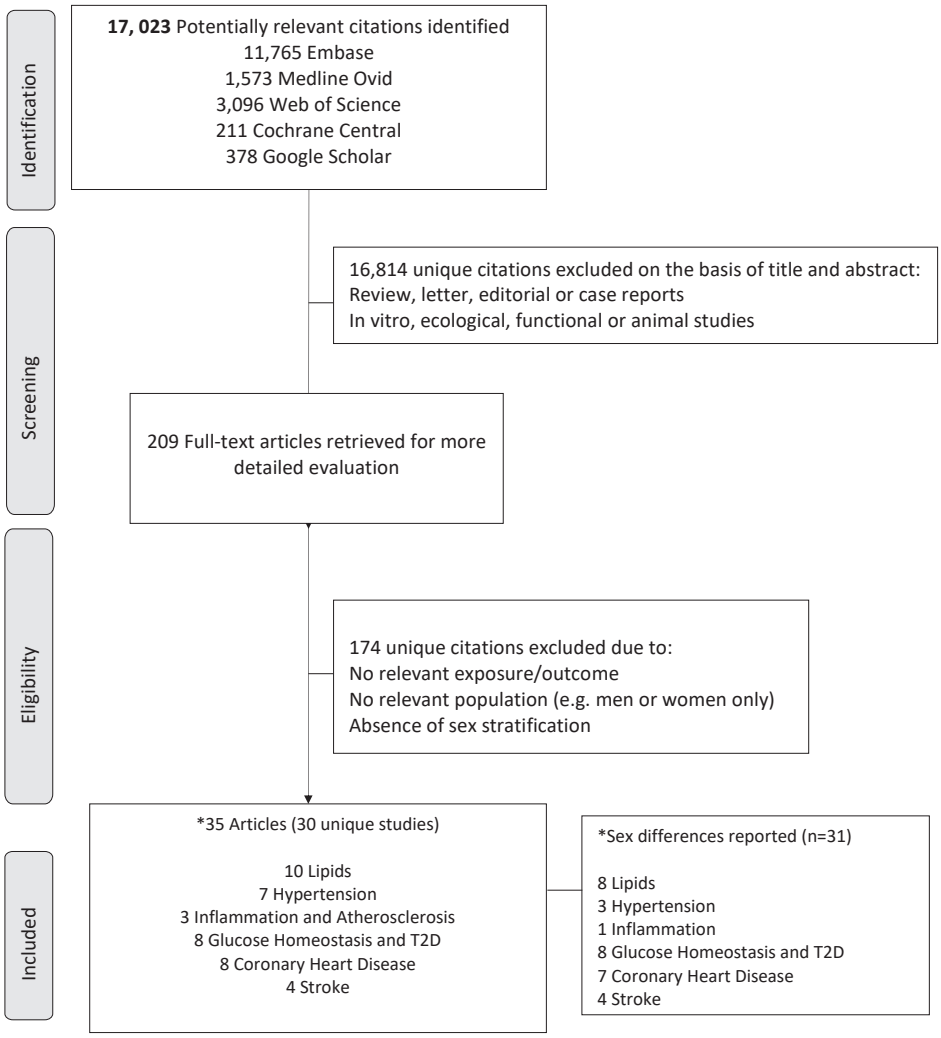


Figure 5.1.1. The flowchart of included studies
*In a specific study more outcomes might have been investigated, therefore, numbers refer to number of articles and not numbers of unique studies

The Role of Sex-Specific DNAm in Intermediate Cardio-metabolic Traits

Blood Lipids

Global DNA methylation is a frequent used marker for epigenetic screening since it captures the DNA methylation also at unknown genetic locations while the results of average DNA methylation correlate with the methylation of some trait-relevant genes[23, 24]. Ten articles[14, 25-33] investigated sex-specific associations between DNAm and blood lipid concentrations applying global (n=2), epi-genome wide (n=1) and candidate

gene (n=7) approaches. In total 2,443 non-overlapping participants (1,174 women and 1,269 men) from USA, Canada, Finland, UK and China were included in these studies.

Two cross-sectional studies[14, 25] investigating global DNAm and blood lipid levels reported sex differences. In the study conducted by Cash et al, LINE-1 methylation was significantly higher in men than in women, and among the entire sample, lower levels of *LINE-1* methylation was associated with higher levels of fasting low-density lipoprotein-cholesterol (LDL) and lower levels of fasting high-density lipoprotein-cholesterol (HDL) [14]. However, when stratifying by sex, the inverse association between global LINE-1 methylation, LDL and HDL remained significant only in men[14]. Malipatil et al, in their study reported that an increase of 10% in LINE-1 methylation was associated with decreased cholesterol/HDL ratio by 0.4 mmol/L in the overall sample of men and women. However, when stratifying by sex, the inverse association remained significant only in women[25]. In an EWAS performed by Garcia-Calzon et al, female samples displayed on average higher methylation in the X-chromosome, whereas males presented higher methylation in the autosomes. Further, women showed higher HDL levels, which were associated with higher *KDM6A* expression and epigenetic differences in human liver[31]. The results were not replicated. Further, the authors integrated DNA regulatory regions and other epigenetic factors for CpGs in the autosomal and X-chromosome based on sex ($q < 0.05$) for only four liver donors. Particularly, 42% of the autosomal CpG sites (13,817 CpGs) and 27% of the X-chromosome sites (2601 CpGs) differentially methylated by sex overlapped with histone marks related to active chromatin and enhancer regions (H3K4me1), whereas 14% of the autosomal sites (4760 CpGs) and 11% of the X-chromosome sites overlapped with histone marks related to heterochromatin (H3K27me3)[31].

In three candidate gene-studies *PTPN1*[32], *PLA2G7*[26] and *BCL11A*[27] DNAm was positively associated with serum lipids in women, but not in men. Another study reported that methylation at *ABCG1* was negatively associated with triglycerides in women only[28], whereas methylation at *LIPC* was negatively associated with triglycerides only in men[27]. This latter study also reported sex-specific associations for total cholesterol: whereas *ABCG1* was associated with triglycerides only in women, methylation at this CpG site was inversely associated with total cholesterol only in men[28]. For some CpG sites there were specific associations with HDL for males; methylation at *PLTP*[28], *CETP*[29], and *LIPC*[28] were negatively associated with HDL in men, but not in women. Moreover, a male-specific association was found between *GCK* CpG4 methylation at *GCK* and total cholesterol concentration[33]. However, a single study performed among 739 African Americans in the Genetic Epidemiology Network of Arteriopathy (GENOA) did not find overall or sex-specific significant associations between DNA methylation and lipid levels[30].

Blood pressure

Seven articles[25, 34-39] based on five unique studies reported associations between sex-specific DNAm and essential hypertension (EH), while one study investigated the cross-sectional association between DNAm and blood pressure[25]. Among these, five studies investigated candidate gene methylation[34-36, 38], while another study investigated epi-genome wide methylation[40] in regard to hypertension. Studies included 3,561 non-overlapping participants (2,029 women and 1,373 men) from China and UK.

In the study conducted by Malipatil et al, in the overall sample of men and women, a 10% increase in LINE-1 methylation was associated with a 2.5 mmHg lower baseline diastolic blood pressure. The stratified analysis by sex did not show any significant influence of sex on this association[25]. One candidate gene study reported higher methylation levels of the two CpG sites at *SCNN18* in women compared with men as controls (CpG1: t=-2.283, P=0.025; CpG2: t=-2.568, P=0.012) and incident EH cases (CpG1: t=-2.694, P=0.009; CpG2: t=-2.583, P=0.011)[35]. However, for these two CpG sites no significant difference was observed between males and females in the prevalent cases group (CpG1: t=0.409, P=0.068; CpG2: t=0.621, P=0.536)[35]. These results indicated a significant association between EH and *SCNN1B* methylation, which was affected by age, sex and antihypertensive therapy. Similarly, in one other study, higher *ADD1* DNAm levels were observed in females as compared to males (CpG1: P = 0.016; CpG2-5: P = 0.021)[34]. Further, the study showed that lower *ADD1* CpG1 methylation levels were significantly associated with EH in females (cases versus controls (%, SD): 10.00±1.41 versus 11.36±3.63, adjusted P =0.042) but not in males (adjusted P = 0.133). In contrast, lower levels of *ADD1* CpG2-5 methylation were associated with an increased risk of EH in males (cases versus controls: 22.48% versus 31.86%, adjusted P = 0.008) but not in females[34]. The prediction potential of EH for *ADD1* CpG1 and CpG2-5 methylation levels was assessed by the ROC curves. CpG2-5 methylation was reported as a significant predictor of EH in males (area under curve (AUC) = 0.855, P = 0.001), while CpG1 methylation showed a trend toward being an EH indicator in females (AUC= 0.699, P =0.054)[34]. In the same population, *AGTR1* CpG1 methylation was a significant predictor of EH in both sexes[36] and hypomethylation of CpG3 site at *IL-6* promotor was significantly associated with EH risk in both, men and women. Further, sex-specific DNAm levels were observed only at CpG1 and CpG2 sites of *IL-6* promoter (males were hypomethylated as compared to females)[38]. Another study by Han et al, investigated the interactions between alcohol consumption and DNA methylation of the *ADD1* gene promoter and its association with EH, involving 2040 cases and controls[36]. The researchers concluded that CpG1 methylation was associated with EH in females while CpG2-5 methylation was significantly associated with EH in males, suggesting that these interactions in the *ADD1* gene promoter might play a role in modifying EH susceptibility[36]. Finally, Bao et al reported that hypomethylation of the *IFNG* promotor region was associated with

a higher risk of EH. However, the authors did not observe any sex differences overall, except that in the control group DNAm levels were found to be higher in males when compared to females[39].

Inflammation and Atherosclerosis

Three articles[41-43], investigated the associations between epi-genome wide DNAm (n=2), candidate gene methylation and inflammatory markers. Also, we did not identify any study investigating the sex-specific role of DNAm in atherosclerosis. Studies in inflammatory markers included 2,771 non-overlapping participants (713 women and 317 men and one study did not report the number of men and women separately[41]) from Germany, China and USA. None of the epi-genome wide studies reported sex specific associations between global DNAm and inflammatory markers[41, 42]. Nevertheless, Guo et al, reported men-specific association between lower *PTX3* promoter methylation levels and higher neutrophil to lymphocyte ratio. Also, the level of *PTX3* promoter methylation in the coronary artery disease group (mean, SD: 62.69% ± 20.57%) was significantly lower than that of the group free of coronary artery disease (mean, SD: 72.45% ±11.84%), suggesting a role of this gene in developing coronary artery disease[43].

Glycemic Traits and T2D

Eight articles[12, 27, 32, 33, 44-47] reported sex-specific associations between DNAm and glycemic traits and T2D. Five studies were candidate gene studies and three were epi-genome wide studies. Among them, six studies focused on DNAm and T2D, one investigated the association between DNAm and metabolic syndrome and another investigated insulin metabolism. Studies included 2,239 non-overlapping participants (353 women and 554 men, with one study not specifying the number of men and women[46]) from Israel, Spain, Sweden, China and USA.

In a case-control study including 1,169 individuals, individual methylation levels at the *FTO* gene showed that CpG sites in the first intron were slightly (3.35%) hypomethylated in T2D cases relative to controls[46]. The odds of developing T2D increased by 6.1% for every 1% decrease in DNAm. Men were hypomethylated relatively to women and the effect of DNAm was stronger in males compared to females (P =0.034 for sex interaction, AUC = 0.675 among men and 0.609 among women)[46]. Also, in another case-control study association between *PTPN1* promoter methylation and the risk of T2D was observed in the overall population and in females[32] .

In the study by Burghardt et al., a significant increase in *CDH22* gene methylation in subjects with metabolic syndrome was identified in the overall sample[44]. However, when investigating males and females separately; differential methylation levels were observed within the *MAP3K13* gene in females and the *CCDC8* gene in males with metabolic syndrome. In the validation sample a significant difference in methylation was

again observed for the *CDH22* and *MAP3K13* genes, but not for *CCDC8* gene[44]. Another study investigated the impact of sex on the genome-wide DNAm pattern in human pancreatic islets from 53 males and 34 females, and relate the methylome to changes in expression and insulin secretion[12]. The study identified both chromosome-wide and site-specific sex differences in DNA methylation at the X chromosome of human pancreatic islets. However, the autosomal chromosomes showed differences in DNA methylation only on the level of individual CpG sites between sexes. Importantly, they found higher insulin secretion in pancreatic islets from females compared with males, as well as sex differences in gene expression[12]. Additionally, the authors did not find any difference in β-cell number between females and males. This could suggest that the DNA methylation differences seen between males and females might not be due to altered β-cell composition in the islets[12].

In a case-control study conducted by Rodriguez-Rodero et al[45], hypermethylation at CpG sites annotated to the HOOK2 gene was associated with the presence of T2D. Interestingly, when these results were analyzed by sex, female T2D samples were found hypermethylated at the cg04657146-region and the cg11738485-region of the *HOOK2* gene, whilst male samples were found hypomethylated in this latter region only[45]. Tang et al. reported a significant association only in males when investigating the overall *BCL11A* methylation in T2D patients[27]. While in another study among the same population, significantly elevated methylation levels of *GCK* CpG4 were observed in T2D patients than in the healthy controls. Also, this association was characteristic to males only[33]. Further, serum IGFBP-7 protein levels were similar among newly diagnosed and treated T2D patients and were not correlated with *IGFBP7* DNAm overall, but solely in males[47].

The Role of Sex-Specific DNAm in CVD

Coronary Heart Disease

Eight articles[48-55] investigated the associations between DNAm and CHD and MI. All studies applied a candidate-gene approach and included a total of 2,353 participants (1,010 women and 1,343 men) from China, Italy and the Netherlands.

One study reported that a higher DNAm at the imprinted loci of *INS* and *GNASAS* was associated with the incidence of MI in women (INS: +2.5%, $P = 0.002$; GNASAS: +4.2%, $P = 0.001$)[48]. Hypermethylation at one locus and at both loci was associated with odds ratios (ORs) of 2.8 and 8.6, respectively ($Ptrend = 3.0 \times 10{-}4$) while no association was observed among men. Similarly, one study revealed a female-specific significant association between methylation at *PLA2G7* promoter and risk of CHD[49]. Another study reported a female specific association of *CDKN2B* methylation with CHD [women with CHD (mean, SD: 7.21±2.40%) compared with women without CHD. In contrast, four studies reported men-specific associations between DNAm of various genes and

CHD[43, 50, 51]. Peng et al, reported significant associations of the methylated promoter of the *ABCG1* and *GALNT2* genes with an increased risk of CHD overall and among men only[50]. Also, CHD patients had significantly lower levels of *APOE* methylation than non-CHD controls. In addition, rs7412-T and rs7259620-A were protective factors for CHD in males (rs7412-T: OR=0.527, allele P=0.004; rs7259620-A: OR=0.743, allele P=0.029) [54]. Giannakopoulou et al reported a sex specific increased methylation in the *CTH* promoter gene in 34 patients who had coronary artery bypass surgery (CABG) (19.1%) compared to 16 control subjects (10.3%). Increased methylation levels were observed in male CABG patients compared to male control subjects while in females this was not observed[51]. Furthermore, Xu at al., showed that CHD cases had a significantly lower methylation level of the GCK gene (mean, SD: 49.77 ± 6.43%) compared with controls, while there was no difference of *GCK* methylation level between males and females and no significant interaction between gender and disease[52]. However, a significant difference of the CpG2 methylation level with CHD was observed in males only[52]. On the other hand, one study evaluated the association between the DNA methylation profiles of genes involved in One-Carbon Metabolism (OCM) and the homocysteine (Hcy) pathway, with the myocardial infraction risk due to the low B-vitamins intake[53], based in the rationale that B-vitamins and folates pathway may modulate DNA methylation[53]. The results from this study showed an inverse association between B-vitamins intake and the hypermethylation in three genes (*TCN2* promoter, *CBS* 50UTR, *AMT* gene-body) in male cases, as well as two genes (*PON1* gene-body, *CBS* 50UTR) in female cases[53].

Stroke

Four articles[15, 56-58] reported sex-specific associations between DNAm and stroke. Among these, three articles[56-58] used data from the same population for their analyses. Two studies applied global and two others candidate gene approaches. These studies applied a cross-sectional design, used blood samples and included a total of 1,045 non-overlapping participants (459 women and 586 men) from diverse ethnic backgrounds, such as Chinese-Taiwanese[56-58] and Spanish[15].

Two of the studies used a candidate gene approach and performed pyrosequencing to assess methylation of the targeted regions in the gene promoter[57, 58], while the two other studies investigated the global DNAm of the LINE-1 elements using pyrosequencing[56] or luminometric methylation assay (LUMA)[15]. Further, two studies found a significant decrease in LINE-1 methylation in men compared to women in cases of stroke[15, 56]. One of them also reported that cases of ischemic stroke presented a lower methylation level compared to controls. In addition, this hypomethylation of LINE-1 in men was associated with an increased stroke risk by 1.2-fold after adjusting for risk factors, while no significant association was observed in women[56]. On the other hand, among the two studies investigating the estrogen receptor alpha *ERα*[57] and

the matrix metalloproteinase-2 (*MMP-2*)[58] respectively, the methylation levels were lower in individuals with stroke compared to controls, in all the CpG sites analyzed in both studies. Further, when exploring the sex-specific associations, the two studies obtained contrary results. One study found a significant difference in methylation levels in all the investigated CpGs annotated to the gene *ERα* only between female cases and controls[57]. Whereas the other study reported a significant difference between the methylation levels in one out of eight CpGs annotated to *MMP-2* only between male cases and controls[58]. None of these studies investigated the difference between male and female cases.

Genes, pathways and cardiovascular disease

Studies included in this systematic review report that methylated CpG sites annotated to *KDM6A, PLA2G7, CETP, ABCG1, LIPC, BCL11A, ADD1, CNN1B, HOOK2, PLTP, GALNT2, PON1, TCN2, CBS, AMT, CTH, INS, GNAS-AS1, MMP2, CCDC8, MAP3K13, FTO, ESR1, CDKN2B, APOE, IGFBP7, PTPN1, GCK* and *PTX3* were differently methylated for men and women. An overview of these genes, function and their sex specific methylations is provided in **Table 5.1.1**. In addition, **Figure 5.1.2** shows the prioritized pathway connectivity between cardio-metabolic genes that were found to be differentially methylated in men and women. The most significant pathways, that in **Figure 5.1.2** are shown with darker red nodes with more representative enrichment include the Vitamin B12 Metabolism, Statin Pathway, Plasma lipoprotein, Plasma lipoprotein assembly, remodeling and clearance and Cholesterol metabolism. Hence, the most important genes connecting these pathways that merit further consideration were: *ABCG1, APOE, PLTP, LIPC, CETP, CTH* and *INS*. Overall, the majority of the genes reported in this review were previously known to be associated with CVD risk factors or CVD outcomes.

DISCUSSION

In this review, we systematically summarized the current evidence on sex differences in DNA methylation in relation to cardiometabolic diseases. We included 30 unique studies that had either stratified their analyses by sex and/or specified that their results did not differ among sexes by testing for statistical sex-interaction. Overall, our findings indicate that global DNAm might influence cardiometabolic risk in a sex-specific manner, and that DNAm at 31 gene sites could be differentially associated with various cardiometabolic traits in men and women.

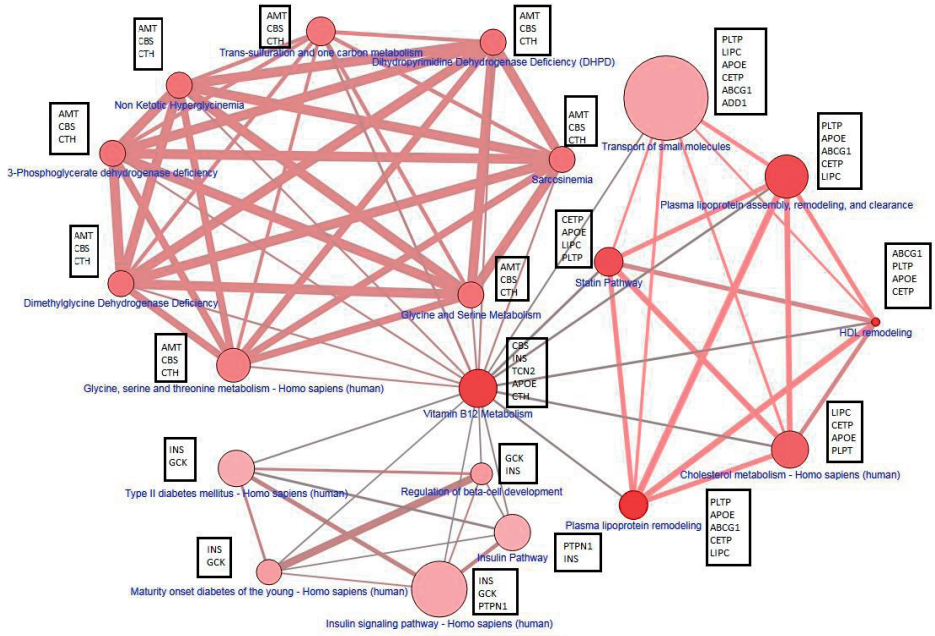


Figure 5.1.2. Pathway Connectivity between Nodes Harbouring Genes from cardio-metabolic genes that were found to be differentially methylated in men and women.
Circles represent nodes and their size reflect the number of genes ranging between 10 (small circle), 81 (medium-sized circles) up to 666 (largest circle) genes. Dark red and light red clusters have significant enrichment at $P<10^{-8}$ and $P<10^{-4}$ levels, respectively. Thicker connection lines represent larger number of shared genes across nodes, while coloured lines (dark and light red) represent higher number of input genes driving the connection between nodes. The nodes with more representative enrichment include the Vitamin B12 Metabolism, Statin Pathway, Plasma lipoprotein assembly, remodelling and clearance, Cholesterol metabolism, One carbon metabolism and Glycine, serine and threonine metabolism and HDL remodelling. The pathway diagram was constructed using the ConsensusPathDB (CPDB) web-based software.

Global DNA methylation

We identified four studies suggesting that altered LINE-1 DNAm may play a role in CVD risk in a sex-specific manner: (i) DNAm measured in LINE-1 repeats was inversely associated with different serum lipids in men and women[14, 25], (ii) decreased LINE-1 was associated with higher stroke risk in the overall sample and in men[56] and (iii) DNAm measured using genomic 5-methyl cytosine content and LUMA indicated hypomethylation in male as compared to female stroke cases[15].

In the current review, global DNA hypomethylation was associated with poorer outcomes. In particular, global DNA hypomethylation was associated with higher LDL and lower HDL levels in the overall sample and in Samoan men, but not in women[14] and increased stroke risk in Chinese men but not in women[53]. These findings are in line with observations in healthy Caucasian men where subjects with decreased LINE-1 methyla-

tion were more likely to develop ischemic heart disease and stroke (women were not included in this study)[59]. Global DNA hypomethylation has been previously reported in stroke patients as compared with healthy[59] and in a large study with participants from European ancestry, decreased global DNAm was observed in male as compared to female stroke cases[56]. In experimental studies, global DNA hypomethylation has been shown to precede the formation of atherosclerosis in Apoe-/- mice, and has been associated with hyperhomocysteinemia and aortic lipid deposition in mutant mice deficient in methylenetetrahydrofolate reductase[60]. While in humans global loss of DNAm has been previously associated with atherosclerosis in both, atherosclerotic lesions[61] and peripheral blood leukocytes[62] but also with blood lipids, inflammation and blood pressure[10] implying that LINE-1 hypomethylation could be associated with an unfavorable cardiovascular risk profile. Global DNAm is considered a robust measurement of the overall genomic methylation which is reported to be one of the earliest molecular changes in the transition of a cell from a normal to a diseased state[63]. Blood DNA hypomethylation might be easily measured and could be used to identify people at risk of cardiovascular events. Our findings emphasize the need of sex-specific approaches when further exploring the possible role of global DNAm as a biomarker and potential intervention target in cardiometabolic diseases.

Epigenome wide-association studies and candidate gene approach

We identified only six studies[12, 31, 41, 42, 44, 46] that investigated differentially methylated regions in the genome with cardiometabolic diseases in a hypothesis-free approach. Among these, three EWAS[12, 31, 44] reported sex-specific associations, and *KDMA6A, FTO, MAP3K13* and *CCDC8* were some of the important genes that were found to be methylated in a sex-specific manner with blood lipids and glycaemia traits. Among 25 candidate gene studies, 22 studies reported sex-specific associations between DNAm and cardiometabolic diseases at the following genes *PLA2G7, BCL11A, KDM6A, LIPC, ABCG1, PLTP, CETP, ADD1, CNN1B, HOOK2, GFBP-7, PTPN1, GCK, PTX3, ABCG1, GALNT2, CDKN2B, APOE, CTH, GNASAS, INS, PON1, TCN2, CBS, AMT, MMP-2* and *ER-α*. Based on the prioritized pathway connectivity analysis, although, limited, the evidence suggests an involvement of biological pathways related to vitamin B12 metabolism, statin pathway, plasma lipoprotein, plasma lipoprotein assembly, remodeling and clearance and cholesterol metabolism, with sex differences in cardiometabolic diseases (**Figure 5.1.2**). Some of the most relevant genes from the pathway analysis were *ABCG1, APOE, PLTP, LIPC, CETP, CTH* and *INS*. Overall, these genes have been associated to cardiometabolic outcomes, however little evidence links them to epigenetics and cardiometabolic diseases and even less to sex differences in cardiometabolic diseases.

ABCG1 gene is a transmembrane cholesterol transporter that effluxes cellular cholesterol from macrophages by delivering cholesterol to mature high-density lipoprotein

particles. Beyond a role in cellular lipid homeostasis, ABCG1 equally participates to glucose and lipid metabolism by controlling the secretion and activity of insulin and lipoprotein lipase. Moreover, there is a growing body of evidence suggesting that modulation of *ABCG1* expression might contribute to the development of diabetes and obesity[64], which are major risk factors of CVD. The *ABCG1, GALNT2* and *HMGCR* genes have been previously associated with pathogenesis and progression of CHD through manipulating the various lipid pathways[65, 66]. In the current review, hypermethylation of these three genes was associated with higher levels of total cholesterol and LDL , and increased CHD risk in men[50], while it was linked to higher levels of TG in women but not with risk of CHD[7]. The expression of *ABCG1* gene reduces cholesterol accumulation in macrophages by promoting the transfer of intracellular cholesterol into HDL pathway[67]. In contrast to this, hypermethylation at *PLA2G7* was associated with levels of total cholesterol, triglycerides and ApoB in females but not in males, and also only female CHD cases were hypermethylated as compared to controls[5]. *PLA2G7* is the coding gene for Lp-PLA2 whose abnormal activity can cause high risk of CHD and may serve as a diagnostic marker for CHD[68]. Therefore, the sex disparities in the *ABCG1* and *PLA2G7* methylation may have an effect in the molecular mechanisms underlying the sex-specific pathophysiology of CHD and may provide epigenetic clues to explain the inconsistency in the epidemiological studies. However, both studies were done in Han Chinese population, and sample size was rather small (only 85 CHD patients and 54 participants without CHD[50] and 36 CHD cases and 36 controls). Hence, further replication studies with larger sample size and in different ethnic populations are required to confirm these findings.

The *APOE* gene encodes apolipoprotein ε (ApoE), a protein that associates with lipids to form lipoproteins that package and traffic cholesterol and lipids through the bloodstream and has been linked with numerous physiological conditions, including healthy aging[69], cardiovascular disease[70], diabetes[71] and cognitive function[72]. One study using samples of 563 blood-bank donors, found that 1% of the inter-individual variation in plasma ApoE levels was attributable to variation of age and sex[73]. Researchers from the ApoEurope project, reported a sex-differential effect of age on mean levels of ApoE[74]. In men, the levels of ApoE levelled off after the age of 45 years, whereas they continued to increase in women[74].

PLTP (phospholipid transfer protein) is essential for the transfer of excess surface lipids from TG-rich lipoproteins to HDL particles. PLTP-mediated phospholipid transport among HDL particles is also known to be associated with HDL particle size and lipid composition. Sex disparities for HDL levels associated with *PLTP* have been previously reported[75]. In the PAGE study, the major allele of rs7679 was associated with higher HDL levels in women only. The locus with the most consistent evidence for sex disparities across the studies is *LPL*, or lipoprotein lipase. Different SNPs in this gene exhibited

sex disparities for HDL levels in two prior studies, with a larger effect in males[76, 77]. In two other studies, *LPL* exhibited sex differences for TG levels, also with a stronger effect in males[75, 78]. LPL is the rate-limiting enzyme for hydrolysis of triglycerides in lipoproteins and polymorphisms and mutations in LPL have been associated with lipid metabolism disorders. Hormone levels have been shown to affect regulation of LPL, including thyroid hormone, estrogen, and testosterone[79], which could possibly and partially explain the observed association in cardiometabolic diseases.

Although these pathways and the respective reported genes need further investigation, confirmation and translational research, the current evidence suggests they could be biologically relevant and could hold the key for future drug discovery, diagnosis and treatment of cardiometabolic diseases overall and in a sex specific manner. Determining the relationships between genes is essential for molecular biology and medicine. These relationships often cluster together into functional and disease pathways, and the characterization of these pathways is necessary to improve disease classification, patient stratification and, ideally, personalized treatment[80].

Epigenetic mechanisms in biological processes of sex differences

Sex differences in pathophysiology of cardiometabolic diseases could be attributed to several gender and sex-specific factors[81]. Lifestyle factors (smoking, diet, stress) can determine gender differences by modifying cardiometabolic risk directly, and they can also modify the epigenetic marks in a sex-specific manner leading to sex differences in cardiometabolic diseases[81](**Figure 5.1.2**). Sex differences may also be driven by biological dissimilarities rather than different environmental exposures among men and women[81]. In particular, the major mechanisms by which sex might influence cardiometabolic diseases epigenetics may include: (i) the genomic and non-genomic effects of steroid hormones and their receptors on DNAm enzymes, histone modifiers and miRNAs, (ii) genomic imprinting, leading to DNAm of either maternal or paternal alleles and (iii) increased expression of X-chromosomal escape genes in women targeting epigenetic modifications and the expression of non-pseudo-autosomal Y-chromosomal epigenetic modifiers in men[16] .

Sex hormones have been extensively studied in the past decade in regard to cardiometabolic diseases due to the better cardiometabolic profiles in women as compared to their male counterparts. In the current review, we found some implications for the interactions between sex hormones and methylation in modifying sex differences cardiometabolic diseases. Three[15, 57, 58] of the studies included in the current review, investigating epigenetic changes and stroke and reporting differences between men and women, highlight the importance of sex hormones and their receptors. Using a global DNA methylation approach, Soriano-Tarraga et al[15], found that global hypomethylation was independently associated with stroke subtypes only in

females. Moreover, there was an association between lower *ERα* methylation levels and large-artery and cardio-embolic stroke subtypes in women, while in men this association was not observed. It might be that women suffering from a major ischemic stroke may cause a more significant change in *ERα* methylation levels to reduce the brain injury[57]. In line with this, differential DNAm profiles mediated sex differences in the endogenous neuroprotective response to middle cerebral artery occlusion (MCAO) in mice were reported. In female mice, MACO induced selective demethylation of the ERα gene promoter, leading to the increase in ERα expression[82]. Also, sex differences were observed in *MMP-2* methylation, with expression of *MMP-2* being closely related to sex hormones[58]. Males with small-vessel ischemic stroke had lower methylation levels at all *MMP-2* CpG sites, while no association was observed in women[58]. Further exploration of the underlying mechanisms is needed. Sex- and stroke-subtype-specific effects must be taken into consideration when investigating potential strategies to alter the activity of *MMP-2* in patients with ischemic stroke. Steroid hormones can induce, among others, modification of histones. Androgen or estrogen receptors act by binding to hormone response elements in the DNA and attract various cofactors that have inherent histone acetyltransferase or methyl transferase activity. This is particularly known for the *CREB* binding protein and E1A binding protein p300[83]. The histone-modifying enzymes alter the epigenetic state of gene promoters to which the nuclear receptors bind, thereby changing gene expression.

It is known that DNAm contributes to X-chromosome inactivation in females[84], and findings by Garcia-Calzon et al, demonstrated that DNA methylation in the X-chromosome in human liver mirrors the methylome in other human tissues[31]. They reported higher average degree of X-chromosome methylation in females than in males with 37% of the significant sites on the X-chromosome having higher methylation in males[31]. Around 95% of the CpG sites in the X-chromosome that had differential DNAm in human liver between sexes also had different methylation levels between males and females in pancreatic islets and brain independently of the clinical characteristics of the population[12, 85]. Further, they identified four genes on the X-chromosome with large differences in DNA methylation between males and females and being more expressed in liver from females than males: *XIST*, *ARSE*, *RPS4X*, and *KDM6A*[31]. Also, higher *ARSE* and *RPS4X* mRNA expression has been found in pancreatic islets, and higher *XIST* and *RPS4X* mRNA expression was also found in brain from females compared with males[15, 85]. These differences in gene expression in several tissues may explain some metabolic differences between males and females. Interestingly, these four genes are known to escape X-chromosome inactivation[86-88]. In this study serum HDL levels were positively associated with *KDM6A* mRNA expression in human liver in addition to higher serum HDL levels and higher *KDM6A* expression in females. Also, silencing *KDM6A* in hepatocytes resulted in lower HDL levels and lower expression of key genes encoding proteins

that regulate HDL levels, supporting the direct contribution of *KDM6A* in the differences found in HDL levels between males and females[31].

Potential clinical implications and recommendations for future research

Although the clinical use of epigenetic marks in the field of cardiometabolic diseases is still in its infancy this is not the case with cancer research. Molecular risk stratification using (epi)genetic marks have been focused on identifying molecular features associated with clinical outcome and have applied them to patients' risk stratification and treatment guidance[89, 90]. Such results indicated that a gene expression score that incorporates prognostic genetic and epigenetic information could be used as a model for treatment response but also for risk stratification and early disease detection. In particular, sex-specific epigenetic marks against or as a supplement to existing risk scores (such as the Framingham Risk Score[91]) may be an added value when predicting the risk of cardiometabolic diseases. This is also supported by our findings suggesting that for cardiometabolic traits epigenetic markers may not be equally good predictors in men and women, emphasizing the role of sex in epigenetic patterns of cardiometabolic diseases. Further, as sex is one of the strongest predictors of treatment response, the epigenetic signatures may be used as markers to indicate the successfulness of pharmacological or dietary/lifestyle interventions in cardiometabolic diseases among sexes. Given the lack of sex-stratification in studies focusing on epigenetic mechanisms and the fact that the majority of the studies were focused to epigenetic changes in autosome chromosomes in regard of cardiometabolic diseases, our review underscores the emerging need for future studies to investigate the influence of sex on epigenetic mechanisms in cardiometabolic diseases. In complex phenotypes such as cardiometabolic diseases, the collection of high-quality blood samples and metabolically active tissues could provide the basis for the creation of large data sets that should accurately incorporate the many sources of variability (age, sex/gender, race/ethnicity). In particular, future prospective observational studies should aim to explore the role of sex when studying the associations between epigenetic marks and mechanistic pathways of cardiometabolic diseases by stratifying their analyses by sex and comparing male and female participants. Second, studying the associations between DNAm and intermediate CVD risk factors is valuable, however, from the clinical perspective, the value of DNAm as a biomarker of the risk factor is as good as the intermediate risk factor itself. Therefore, as we did not identify studies focusing on outcomes such as stroke or myocardial infarction, it might be of great value for future research to investigate the role of sex on the epigenetic determinants of stroke and myocardial infarction. Third, potential biological mechanisms underlying sex-specific associations should be further explored in an experimental setting. It is now known that sex differences in morphology and in response to stress exist also in cellular levels[92-94]. Therefore, when translating the observational findings into experimental

settings a clear distinction between male and female animal models or cell cultures is of high importance in order to obtain non-biased results on the sex-specific pathophysiology of cardiometabolic diseases.

Strengths and limitations

In this systematic review on sexually dimorphic DNAm, we critically appraised the literature following an a priori designed protocol with clearly defined inclusion and exclusion criteria using a comprehensive literature search in five databases. While previous systematic reviews on the topic are limited only to major CVD outcomes[16], our study took in consideration a broad range of cardiometabolic risk factors and diseases. However, the limitations of the findings from this study merit careful consideration. The included studies in this review were limited in sample size and the majority of studies included were cross-sectional assessments, making it difficult to conclude whether DNAm patterns are a cause or consequence of cardiometabolic changes. In addition, the results of some of the studies need cautious interpretation when it comes to the biological or functional relevance of their findings. Even though a study may report a significant difference in DNAm the biological relevance of small differences could be likely minimal and unknown. Studies investigating associations between metabolic syndrome and DNAm also need to be interpreted with caution given the heterogeneity of metabolic syndrome and that the subjects may or may not have dyslipidemia, elevated BP, and hyperglycemia. Therefore interpreting associations between changes in DNAm and subjects classified as having metabolic syndrome is Moreover, although individual studies attempted to adjust for established CVD risk factors, adjustment levels were inconsistent across the studies. Also, DNAm patterns reported in blood samples may not mirror the methylation patterns in the relevant targeted tissues. Further, we did not perform the search for non-coding microRNAs and histone modifications because the scope of our search was DNA methylation. Given the importance of microRNAs and histone modifications as epigenetic mechanisms, future systematic reviews and meta- analyses on microRNAs, histone modifications and sex differences in different types of cardiovascular tissues would be an added value on the topic. Moreover, we hand searched relevant reviews and references of studies included in the current review in order to minimize the possibility of missing important studies. Also, we cannot exclude the possibility of publication bias from underreporting negative findings. Lastly, a meaningful quantitative pooling of the existing data was unfeasible due to the heterogeneity in the input parameters, assumptions and study design.

Conclusions

Although a growing body of evidence suggests biological, genetic and epigenetic sex differences in cardiometabolic diseases, only a small number of studies in the field

stratify or present their results by sex. Nevertheless, the cumulative evidence from the studies that reported sex-based results, suggest that epigenetic changes in specific individual genes might be differently associated with cardiometabolic traits in males and females, encouraging further and larger-scale investigation. Robust, replicable results from carefully designed studies have the potential to uncover the molecular biological processes involved in disease onset and progression. In addition, future studies should help characterize gene regulatory effects of non-coding genetic variations, and, hopefully, give indications into disease-relevant biological pathways which could be addressed by preventive or therapeutic interventions. Clearly, a considerable amount of functional work is required in the future to expand our field of view beyond the classic biological mechanisms involved in sex differences of cardiometabolic diseases, and that could be important to design new drugs that target sex-specific mechanisms and permit more precise and efficient care.

REFERENCES

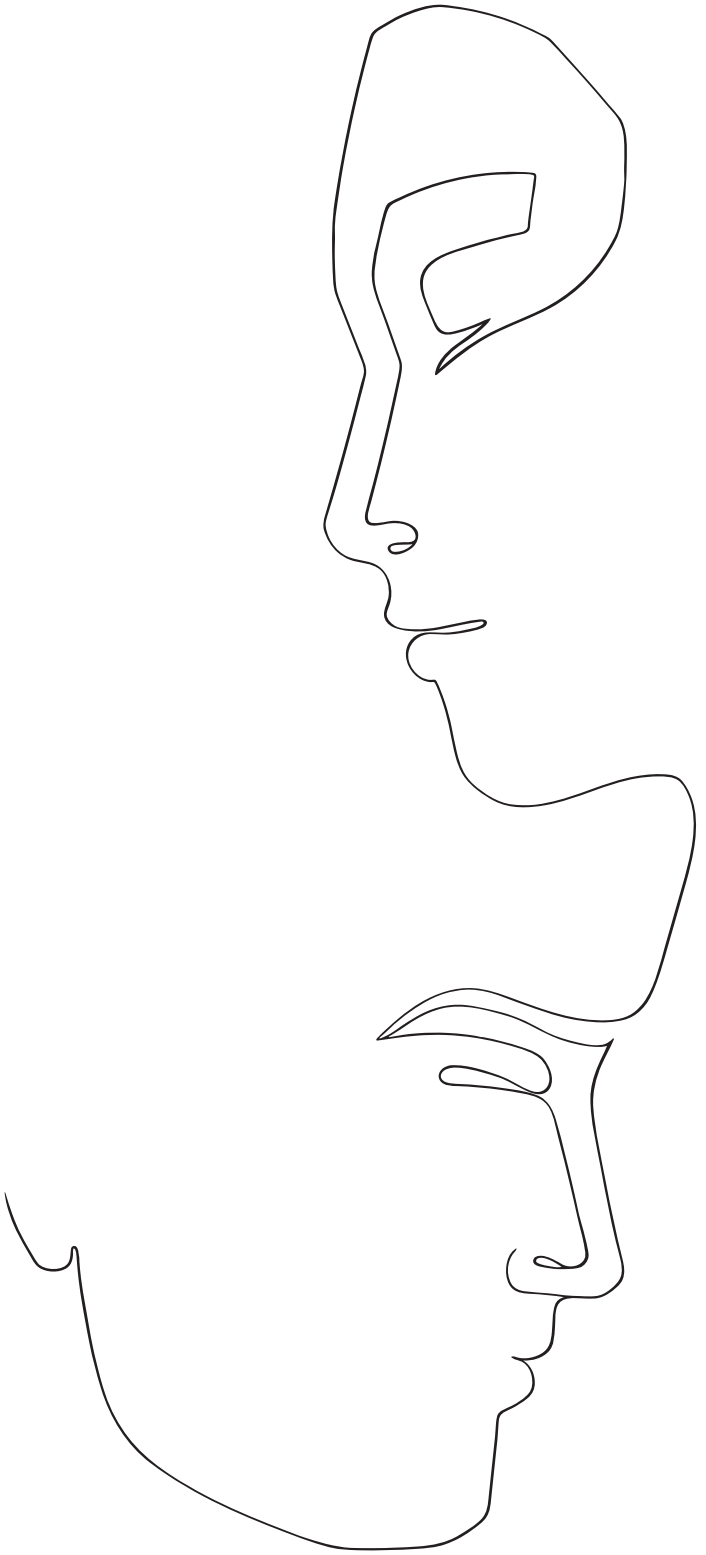
1. Manach, C., et al., Addressing the inter-individual variation in response to consumption of plant food bioactives: Towards a better understanding of their role in healthy aging and cardiometabolic risk reduction. Mol Nutr Food Res, 2017. 61(6).
2. Park, Y.W., et al., The metabolic syndrome: prevalence and associated risk factor findings in the US population from the Third National Health and Nutrition Examination Survey, 1988-1994. Arch Intern Med, 2003. 163(4): p. 427-36.
3. Bjornerem, A., et al., Endogenous sex hormones in relation to age, sex, lifestyle factors, and chronic diseases in a general population: the Tromso Study. J Clin Endocrinol Metab, 2004. 89(12): p. 6039-47.
4. Humphries, K.H., et al., Sex differences in cardiovascular disease - Impact on care and outcomes. Front Neuroendocrinol, 2017. 46: p. 46-70.
5. Appelman, Y., et al., Sex differences in cardiovascular risk factors and disease prevention. Atherosclerosis, 2015. 241(1): p. 211-8.
6. Mehta, L.S., et al., Acute Myocardial Infarction in Women: A Scientific Statement From the American Heart Association. Circulation, 2016. 133(9): p. 916-47.
7. Boyne, D.J., et al., Endogenous sex hormone exposure and repetitive element DNA methylation in healthy postmenopausal women. Cancer Causes Control, 2017. 28(12): p. 1369-1379.
8. Feinberg, A.P., Epigenetics at the epicenter of modern medicine. JAMA, 2008. 299(11): p. 1345-50.
9. Robertson, K.D., DNA methylation and human disease. Nat Rev Genet, 2005. 6(8): p. 597-610.
10. Muka, T., et al., The role of epigenetic modifications in cardiovascular disease: A systematic review. Int J Cardiol, 2016. 212: p. 174-83.
11. Braun, K.V., et al., The role of DNA methylation in dyslipidaemia: A systematic review. Prog Lipid Res, 2016. 64: p. 178-191.
12. Hall, E., et al., Sex differences in the genome-wide DNA methylation pattern and impact on gene expression, microRNA levels and insulin secretion in human pancreatic islets. Genome Biol, 2014. 15(12): p. 522.
13. Yousefi, P., et al., Sex differences in DNA methylation assessed by 450 K BeadChip in newborns. BMC Genomics, 2015. 16: p. 911.
14. Cash, H.L., et al., Cardiovascular disease risk factors and DNA methylation at the LINE-1 repeat region in peripheral blood from Samoan Islanders. Epigenetics, 2011. 6(10): p. 1257-1264.
15. Soriano-Tarraga, C., et al., Global DNA methylation of ischemic stroke subtypes. PLoS One, 2014. 9(4): p. e96543.
16. Hartman, R.J.G., S.E. Huisman, and H.M. den Ruijter, Sex differences in cardiovascular epigenetics-a systematic review. Biol Sex Differ, 2018. 9(1): p. 19.
17. Stoll, S., C. Wang, and H. Qiu, DNA methylation and histone modification in hypertension. Int J Mol Sci, 2018. 19(4).
18. Muka, T., et al., A 24-step guide on how to design, conduct, and successfully publish a systematic review and meta-analysis in medical research. Eur J Epidemiol, 2019.
19. Moher, D., et al., Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. Int J Surg, 2010. 8(5): p. 336-41.
20. Bremer, W.M., Reference checking for systematic reviews using Endnote. Journal of the Medical Library Association, 2018. 106(4): p. 542-546.
21. Lewis, J.E., et al., A randomized controlled trial of the effect of dietary soy and flaxseed muffins on quality of life and hot flashes during menopause. Menopause, 2006. 13(4): p. 631-642.

22. Kamburov, A., et al., The ConsensusPathDB interaction database: 2013 update. Nucleic Acids Res, 2013. 41(Database issue): p. D793-800.
23. Ohka, F., et al., The Global DNA Methylation Surrogate LINE-1 Methylation Is Correlated with MGMT Promoter Methylation and Is a Better Prognostic Factor for Glioma. Plos One, 2011. 6(8).
24. Knothe, C., et al., Disagreement between two common biomarkers of global DNA methylation. Clinical Epigenetics, 2016. 8.
25. Malipatil, N., et al., Assessment of global long interspersed nucleotide element-1 (LINE-1) DNA methylation in a longitudinal cohort of type 2 diabetes mellitus (T2DM) individuals. Int J Clin Pract, 2018: p. e13270.
26. Jiang, D., et al., Elevated PLA2G7 Gene Promoter Methylation as a Gender-Specific Marker of Aging Increases the Risk of Coronary Heart Disease in Females. PLoS One, 2013. 8(3).
27. Tang, L., et al., BCL11A gene DNA methylation contributes to the risk of type 2 diabetes in males. Exp Ther Med, 2014. 8(2): p. 459-463.
28. Guay, S.P., et al., Epipolymorphisms within lipoprotein genes contribute independently to plasma lipid levels in familial hypercholesterolemia. Epigenetics, 2014. 9(5): p. 718-729.
29. Guay, S.P., et al., DNA methylation variations at CETP and LPL gene promoter loci: New molecular biomarkers associated with blood lipid profile variability. Atherosclerosis, 2013. 228(2): p. 413-420.
30. Wright, M.L., et al., Joint Influence of SNPs and DNA Methylation on Lipids in African Americans From Hypertensive Sibships. Biol Res Nurs, 2018. 20(2): p. 161-167.
31. Garcia-Calzon, S., et al., Sex Differences in the Methylome and Transcriptome of the Human Liver and Circulating HDL-Cholesterol Levels. J Clin Endocrinol Metab, 2018. 103(12): p. 4395-4408.
32. Huang, Q., et al., Elevation of PTPN1 promoter methylation is a significant risk factor of type 2 diabetes in the Chinese population. Exp Ther Med, 2017. 14(4): p. 2976-2982.
33. Tang, L., et al., Elevated CpG island methylation of GCK gene predicts the risk of type 2 diabetes in Chinese males. Gene, 2014. 547(2): p. 329-33.
34. Zhang, L.-N., et al., Lower ADD1 gene promoter DNA methylation increases the risk of essential hypertension. PLoS One, 2013. 8(5): p. e63455.
35. Zhong, Q., et al., Association of SCNN1B promoter methylation with essential hypertension. Mol Med Rep, 2016. 14(6): p. 5422-5428.
36. Han, L., et al., The interactions between alcohol consumption and DNA methylation of the ADD1 gene promoter modulate essential hypertension susceptibility in a population-based, case-control study. Hypertens Res, 2015. 38(4): p. 284-90.
37. Fan, R., et al., Association of AGTR1 Promoter Methylation Levels with Essential Hypertension Risk: A Matched Case-Control Study. Cytogenet Genome Res, 2015. 147(2-3): p. 95-102.
38. Mao, S.Q., et al., Hypomethylation of interleukin-6 (IL-6) gene increases the risk of essential hypertension: a matched case-control study. J Hum Hypertens, 2017. 31(8): p. 530-536.
39. Bao, X.J., et al., Hypomethylation of the Interferon gamma Gene as a Potential Risk Factor for Essential Hypertension: A Case-Control Study. Tohoku J Exp Med, 2018. 244(4): p. 283-290.
40. Bostrom, A.E., et al., Longitudinal genome-wide methylation study of Roux-en-Y gastric bypass patients reveals novel CpG sites associated with essential hypertension. BMC Med Genomics, 2016. 9: p. 20.
41. Marzi, C., et al., Epigenetic Signatures at AQP3 and SOCS3 Engage in Low-Grade Inflammation across Different Tissues. PLoS One, 2016. 11(11): p. e0166015.
42. Sun, Y.V., et al., Gene-specific DNA methylation association with serum levels of C-reactive protein in African Americans. PLoS One, 2013. 8(8): p. e73480.

43. Guo, T.M., et al., Pentraxin 3 (PTX3) promoter methylation associated with PTX3 plasma levels and neutrophil to lymphocyte ratio in coronary artery disease. J Geriatr Cardiol, 2016. 13(8): p. 712-717.
44. Burghardt, K.J., et al., The Influence of Metabolic Syndrome and Sex on the DNA Methylome in Schizophrenia. Int J Genomics, 2018. 2018: p. 8076397.
45. Rodriguez-Rodero, S., et al., Altered intragenic DNA methylation of HOOK2 gene in adipose tissue from individuals with obesity and type 2 diabetes. PLoS One, 2017. 12(12): p. e0189153.
46. Toperoff, G., et al., Genome-wide survey reveals predisposing diabetes type 2-related DNA methylation variations in human peripheral blood. Hum Mol Genet, 2012. 21(2): p. 371-83.
47. Gu, H.F., et al., Evaluation of IGFBP-7 DNA methylation changes and serum protein variation in Swedish subjects with and without type 2 diabetes. Clin Epigenetics, 2013. 5(1): p. 20.
48. Talens, R.P., et al., Hypermethylation at loci sensitive to the prenatal environment is associated with increased incidence of myocardial infarction. Int J Epidemiol, 2012. 41(1): p. 106-15.
49. Jiang, D., et al., Elevated PLA2G7 gene promoter methylation as a gender-specific marker of aging increases the risk of coronary heart disease in females. PLoS One, 2013. 8(3): p. e59752.
50. Peng, P., et al., A preliminary study of the relationship between promoter methylation of the ABCG1, GALNT2 and HMGCR genes and coronary heart disease. PLoS One, 2014. 9(8): p. e102265.
51. Giannakopoulou, E., et al., Epigenetics-by-Sex Interaction for Coronary Artery Disease Risk Conferred by the Cystathionine gamma-Lyase Gene Promoter Methylation. OMICS, 2017. 21(12): p. 741-748.
52. Xu, L., et al., GCK gene-body hypomethylation is associated with the risk of coronary heart disease. Biomed Res Int, 2014. 2014: p. 151723.
53. Fiorito, G., et al., B-vitamins intake, DNA-methylation of One Carbon Metabolism and homocysteine pathway genes and myocardial infarction risk: the EPICOR study. Nutr Metab Cardiovasc Dis, 2014. 24(5): p. 483-8.
54. Ji, H., et al., APOE hypermethylation is significantly associated with coronary heart disease in males. Gene, 2019. 689: p. 84-89.
55. Chen, X., et al., Elevated methylation of cyclin dependent kinase inhibitor 2B contributes to the risk of coronary heart disease in women. Exp Ther Med, 2019. 17(1): p. 205-213.
56. Lin, R.T., et al., LINE-1 methylation is associated with an increased risk of ischemic stroke in men. Curr Neurovasc Res, 2014. 11(1): p. 4-9.
57. Lin, H.F., et al., Demethylation of Circulating Estrogen Receptor Alpha Gene in Cerebral Ischemic Stroke. PLoS One, 2015. 10(9): p. e0139608.
58. Lin, H.F., et al., Methylation in the matrix metalloproteinase-2 gene is associated with cerebral ischemic stroke. J Investig Med, 2017. 65(4): p. 794-799.
59. Baccarelli, A., et al., Ischemic heart disease and stroke in relation to blood DNA methylation. Epidemiology, 2010. 21(6): p. 819-28.
60. Chen, Z., et al., Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition. Hum Mol Genet, 2001. 10(5): p. 433-43.
61. Hiltunen, M.O., et al., DNA hypomethylation and methyltransferase expression in atherosclerotic lesions. Vasc Med, 2002. 7(1): p. 5-11.
62. Castro, R., et al., Increased homocysteine and S-adenosylhomocysteine concentrations and DNA hypomethylation in vascular disease. Clin Chem, 2003. 49(8): p. 1292-6.
63. Mikeska, T. and J.M. Craig, DNA methylation biomarkers: cancer and beyond. Genes (Basel), 2014. 5(3): p. 821-64.

64. Tavoosi, Z., et al., Cholesterol Transporters ABCA1 and ABCG1 Gene Expression in Peripheral Blood Mononuclear Cells in Patients with Metabolic Syndrome. Cholesterol, 2015. 2015: p. 682904.
65. Jeemon, P., et al., Implications of discoveries from genome-wide association studies in current cardiovascular practice. World J Cardiol, 2011. 3(7): p. 230-47.
66. Tietjen, I., et al., Segregation of LIPG, CETP, and GALNT2 mutations in Caucasian families with extremely high HDL cholesterol. PLoS One, 2012. 7(8): p. e37437.
67. Oram, J.F. and A.M. Vaughan, ATP-Binding cassette cholesterol transporters and cardiovascular disease. Circ Res, 2006. 99(10): p. 1031-43.
68. Grallert, H., et al., Eight genetic loci associated with variation in lipoprotein-associated phospholipase A2 mass and activity and coronary heart disease: meta-analysis of genome-wide association studies from five community-based studies. Eur Heart J, 2012. 33(2): p. 238-51.
69. Garatachea, N., et al., ApoE gene and exceptional longevity: Insights from three independent cohorts. Exp Gerontol, 2014. 53: p. 16-23.
70. Kathiresan, S., et al., Polymorphisms associated with cholesterol and risk of cardiovascular events. N Engl J Med, 2008. 358(12): p. 1240-9.
71. Yin, Y.W., et al., Influence of apolipoprotein E gene polymorphism on development of type 2 diabetes mellitus in Chinese Han population: a meta-analysis of 29 studies. Metabolism, 2014. 63(4): p. 532-41.
72. Rubino, E., et al., Apolipoprotein E polymorphisms in frontotemporal lobar degeneration: a meta-analysis. Alzheimers Dement, 2013. 9(6): p. 706-13.
73. Boerwinkle, E. and G. Utermann, Simultaneous effects of the apolipoprotein E polymorphism on apolipoprotein E, apolipoprotein B, and cholesterol metabolism. Am J Hum Genet, 1988. 42(1): p. 104-12.
74. Haddy, N., et al., The importance of plasma apolipoprotein E concentration in addition to its common polymorphism on inter-individual variation in lipid levels: results from Apo Europe. Eur J Hum Genet, 2002. 10(12): p. 841-50.
75. Asselbergs, F.W., et al., Large-scale gene-centric meta-analysis across 32 studies identifies multiple lipid loci. Am J Hum Genet, 2012. 91(5): p. 823-38.
76. Teslovich, T.M., et al., Biological, clinical and population relevance of 95 loci for blood lipids. Nature, 2010. 466(7307): p. 707-13.
77. Aulchenko, Y.S., et al., Loci influencing lipid levels and coronary heart disease risk in 16 European population cohorts. Nat Genet, 2009. 41(1): p. 47-55.
78. Taylor, K.C., et al., Investigation of gene-by-sex interactions for lipid traits in diverse populations from the population architecture using genomics and epidemiology study. BMC Genet, 2013. 14: p. 33.
79. Wang, H. and R.H. Eckel, Lipoprotein lipase: from gene to obesity. Am J Physiol Endocrinol Metab, 2009. 297(2): p. E271-88.
80. Barabasi, A.L., N. Gulbahce, and J. Loscalzo, Network medicine: a network-based approach to human disease. Nat Rev Genet, 2011. 12(1): p. 56-68.
81. Jaenisch, R. and A. Bird, Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nature Genetics, 2003. 33: p. 245-254.
82. Westberry, J.M., A.K. Prewitt, and M.E. Wilson, Epigenetic regulation of the estrogen receptor alpha promoter in the cerebral cortex following ischemia in male and female rats. Neuroscience, 2008. 152(4): p. 982-9.
83. Leader, J.E., et al., Epigenetic regulation of nuclear steroid receptors. Biochemical Pharmacology, 2006. 72(11): p. 1589-1596.

84. Carrel, L. and H.F. Willard, X-inactivation profile reveals extensive variability in X-linked gene expression in females. Nature, 2005. 434(7031): p. 400-4.
85. Xu, H., et al., Sex-biased methylome and transcriptome in human prefrontal cortex. Hum Mol Genet, 2014. 23(5): p. 1260-70.
86. Brown, C.J., et al., A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. Nature, 1991. 349(6304): p. 38-44.
87. Lan, F., et al., A histone H3 lysine 27 demethylase regulates animal posterior development. Nature, 2007. 449(7163): p. 689-94.
88. Lee, M.G., et al., Demethylation of H3K27 regulates polycomb recruitment and H2A ubiquitination. Science, 2007. 318(5849): p. 447-50.
89. Figueroa, M.E., et al., DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. Cancer Cell, 2010. 17(1): p. 13-27.
90. Marcucci, G., et al., Epigenetics meets genetics in acute myeloid leukemia: clinical impact of a novel seven-gene score. J Clin Oncol, 2014. 32(6): p. 548-56.
91. Wannamethee, S.G., et al., Metabolic syndrome vs Framingham Risk Score for prediction of coronary heart disease, stroke, and type 2 diabetes mellitus. Arch Intern Med, 2005. 165(22): p. 2644-50.
92. Addis, R., et al., Human umbilical endothelial cells (HUVECs) have a sex: characterisation of the phenotype of male and female cells. Biol Sex Differ, 2014. 5(1): p. 18.
93. Lorenz M, K.J., Kaufmann K, Kreye C, Mertens M, Kuebler WM, Baumann G, Gossing G, Marki A, Zakrzewicz A, Miéville C, Benn A, Horbelt D, Wratil PR, Stangl K, Stangl V, Does cellular sex matter? Dimorphic transcriptional differences between female and male endothelial cells. Atherosclerosis, 2015. 240(1): p. 61-72.
94. Du L, H.R., Bayir H, Watkins SC, Tyurin VA, Guo F, Kochanek PM, Jenkins LW, Ren J, Gibson G, Chu CT, Kagan VE, Clark RS, Starving neurons show sex difference in autophagy. J Biol Chem, 2009. 284(4): p. 2383-96.



5.2

Chromatin landscape and epigenetic biomarkers for clinical diagnosis and prognosis of type 2 diabetes mellitus

ABSTRACT

Type 2 diabetes and its accompanying complications constitute a major health burden worldwide, which can be partly attributed to the interplay between genetics and environments. Extensive research over the last decades has shown that our genome is not the only determinant of disease risk. Epigenetic marks induced by lifestyle and environmental factors are associated with altered gene expression patterns in important tissues, leading to altered susceptibility to disease later in life. Hence, the identification of epigenetic biomarkers unfolds the possibility for a novel personalized disease prevention strategy and at the same time holds the potential to be a promising prognostic tool for diabetes. So far, evidence on the predictive value of epigenetics in diabetes management is very limited. Unlike in cancer pathology, where examples of important epigenetic tools are now widely used in clinical practice as predictive/diagnostic biomarkers, for complex pathophysiological diseases such as diabetes, this still remains a challenge. These topics are discussed extensively in this chapter.

1. INTRODUCTION

Diabetes has become a major public health problem with type 2 diabetes (T2D) being the predominant condition that accounts for at least 90% of the cases [1]. According to the World Health Organization (WHO) reports in 2012, the estimated number of people living with T2D by 2030 would have been 366 million [2]. However, these numbers are expected to be higher since until 2017, 425 million people were reported having diabetes [3] overcoming all predictions [4].

T2D is characterized by insulin deficiency and insulin resistance [5], and its major complications comprise macrovascular, microvascular and neurologic changes which can lead to organ damage including heart, kidneys, eyes, feet and nerves [5]. According to the American Diabetes Association (ADA), diabetes diagnosis is defined as: fasting plasma glucose ≥ 126 mg/dL (≥ 7.0 mmol/L) where fasting is defined as no caloric intake for at least 8 hours or 2-h plasma glucose ≥ 200 mg/dL (≥ 11.1 mmol/L) during a 75-g oral glucose tolerance test (OGTT, the test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water) or A1C ≥ 6.5% (≥ 48 mmol/mol) or in a patient with classic symptoms of hyperglycaemia or hyperglycaemic crisis, a random plasma glucose ≥ 200 mg/dL (≥ 11.1 mmol/L) [6]. These definitions go in line with the current WHO diagnostic criteria, except for the glycosylated haemoglobin (HbA1c) test, which remains controversial[7].

Studies investigating the aetiology of T2D have been primarily focused into the genetic determinants of the disease. Recent evidence shows epigenetics could be a major player in the pathophysiology of the disease, through which environmental and lifestyle factors could affect T2D pathogenesis[8]. Lifestyle and other environmental factors could lead to changes in DNA methylation and histone modifications, which on the other hand, might affect the development of pancreatic β cells and the function of insulin secretion, contributing to the decline of insulin sensitivity resulting in the occurrence of T2D [8]. Animal and human studies investigating the genome-wide maps of epigenetic markers using islet tissue have provided a reliable resource for understanding the importance of the epigenetic mechanisms in T2D susceptibility [9].

In clinical practice, biomarkers are used routinely to identify individuals at risk and are of great importance in disease diagnosis. For T2D, fasting blood glucose, HbA1c and 2-hours oral glucose are commonly used, but they come with some drawbacks. Blood glucose levels do not reflect the impaired b-cell function or insulin resistance [10]; the optimal value for HbA1c for diagnosis of prediabetes state still remain controversial [11 12], whereas the 2 hours oral glucose tolerance test is a time consuming procedure. It is not known whether the deterioration in glucose tolerance and beta-cell function is linear or whether there is an accelerated loss of function at some point prior to the onset of diabetes [7]. Therefore, the early identification of high-risk individuals demands novel bio-

markers that adequately account for the inter-individual variance in the different pathological mechanisms underlying impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), each of which have distinct progression patterns towards diabetes [13]. Moreover, the different kind of complications from diabetes might leave different methylation signatures which could result in type-specific and predictive signatures with potential use as future prognostic biomarkers for T2D. Further, considering the rapidly increase in incidence and prevalence of T2D, it has become relevant to extend current knowledge and discover new biomarkers that could be identified and/or monitored during the diagnosis and progression of the disease.

In this chapter, the topics of epigenetic alterations, particularly DNA methylation and histone modifications and the importance of epigenetic biomarkers for risk prediction, diagnosis and prognosis of T2D will be discussed.

2. EPIGENETIC ALTERATIONS INVOLVED IN GLUCOSE HOMEOSTASIS AND INSULIN METABOLISM

The association between glucose homeostasis related traits and DNA methylation has been assessed through different approaches, such as global DNA methylation assessment, DNA methylation in candidate genes, and Epigenome-Wide Association Studies (EWAS).

Global DNA methylation refers to the overall level of 5-methylcitosine in the genome, expressed as percentage of total cytosine. Repetitive and transposable elements, such as LINE-1 and Alu, represent a large portion of the human genome and contain much of the CpG methylation found in normal human postnatal somatic tissues [14]. Given the existing correlation of methylation at such elements with the total genomic methylation content, they are considered surrogate markers for global genome methylation [14].

In a candidate gene methylation approach, the association is evaluated only for specific genes of interest that have been selected based on their possible role in the phenotype of interest. Therefore, the methylation level is assessed only in specific regions of the DNA.

EWAS, scan genome-wide epigenetic variants, such as DNA methylation, which might be associated with the phenotype of interest. EWAS are mainly performed using microarrays, which profile the methylation level of thousands of CpG islands in the genome, surveying multiple samples.

Information about the function of the genes mentioned in this chapter that have been studied in relation with T2D and glycaemic traits can be found in **Table 5.2.1**.

Table 5.2.1. Overview of genes studied in association of Epigenetics with T2D and glycemic traits (Database URL: www.genecards.org) [147]

Gene	Alias	Chr	Function
IGF1R	Insulin Like Growth Factor 1 Receptor	15	It encodes a receptor which binds insulin-like growth factor 1 (IGF1) with a high affinity, and IGF2 and insulin (INS) with a lower affinity. It is involved in cell growth and survival control, being crucial for tumor transformation and survival of malignant cell. Among its related pathways are Apoptotic Pathways in Synovial Fibroblasts and NFAT and Cardiac Hypertrophy. Associated diseased include Insulin-Like Growth Factor I Deficiency and Ring Chromosome 15 Syndrome.
IGFBP3	Insulin Like Growth Factor Binding Protein 3	7	It encodes a protein with an IGFBP domain and a thyroglobulin type-I domain. The protein forms a ternary complex with insulin-like growth factor acid-labile subunit (IGFALS) and either insulin-like growth factor (IGF) I or II. In this form, it circulates in the plasma, prolonging the half-life of IGFs and altering their interaction with cell surface receptors. Diseases associated with IGFBP3 include Insulin-Like Growth Factor I and Acid-Labile Subunit Deficiency.
IGFBP7	Insulin-like Growth Factor (IGF) Binding protein 7	4	It encodes a protein that binds IGFs to regulate their binding to its receptors. It also stimulates prostacyclin production, which is a potent inhibitor of platelet aggregation and a strong vasodilator that inhibits the growth of vascular smooth muscle cells. Associated diseases include Diabetic Angiopathy.
IGFBP1	Insulin-like growth factor binding protein 1	7	It encodes a protein that binds IGF-I and –II to regulate their binding to their receptors. It is mainly expressed in liver. Low levels of this protein may be associated with impaired glucose tolerance, vascular disease and hypertension in human patients. Associated diseases include Pancreatic Cancer, Childhood and Ovarian Disease.
IGF2	Insulin Like Growth Factor 2	11	It encodes a member of the insulin family of polypeptide growth factors, which are involved in development and growth. It plays a key role in regulating fetoplacental development. In adults, it may be involved in glucose metabolism in adipose tissue, skeletal muscle and liver. It undergoes glucose-mediated co-secretion with insulin, and acts as physiological amplifier of glucose-mediated insulin secretion. Associated diseases include Growth Restriction and Silver-Russell Syndrome.
C8orf31	Chromosome 8 Open Reading Frame 31 (Putative)	8	It is an RNA gene, and it is affiliated with the ncRNA class.
TXNIP	Thioredoxin Interacting Protein	1	It encodes a protein that may act as an oxidative stress mediator by inhibiting thioredoxin activity or by limiting its bioavailability. It also functions as a transcriptional repressor, possibly by acting as a bridge molecule between transcription factors and corepressor complexes, and over-expression will induce G0/G1 cell cycle arrest. It is required for the maturation of natural killer cells. Associated diseases include Hyperglycemia.

Table 5.2.1. Overview of genes studied in association of Epigenetics with T2D and glycemic traits (Database URL: www.genecards.org) [147] (*continued*)

Gene	Alias	Chr	Function
MMP-9	Matrix Metallopeptidase 9	20	It encodes an enzyme member of the Metallopeptidase (MMP) family, which are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Specifically, MMP-9 degrades type IV and V collagens. Associated diseases include Metaphyseal Anadysplasia 2 and Metaphyseal Anadysplasia.
EP300	Histone Acetyltransferase P300	22	It encodes a protein that functions as histone acetyltransferase, which regulates transcription via chromatin remodeling and is important in the processes of cell proliferation and differentiation. It mediates acetylation of histone H3 at Lys-122 (H3K122ac) and at Lys-27 (H3K27ac). It also functions as acetyltransferase for non-histone targets. Associated diseases include Rubinstein-Taybi Syndrome 2 and Colorectal Cancer.
SSTR5-AS1	SSTR5 Antisense RNA 1	16	It is a non-protein coding gene. It is an RNA Gene, and is affiliated with the non-coding RNA class
SSTR5	Somatostatin Receptor 5	16	It encodes a cyclic polypeptide which is an abundant neuropeptide and has a wide range of physiological effects on neurotransmission, secretion and cell proliferation. The activity of this receptor is mediated by G proteins which inhibit adenylyl cyclase, and different regions of this receptor molecule are required for the activation of different signaling pathways. Associated diseases include Pituitary Adenoma
LY86	Lymphocyte Antigen 86	6	It encodes a protein which is involved in the innate immune system. It may cooperate with CD180 and TLR4 to mediate the innate immune response to bacterial lipopolysaccharide (LPS) and cytokine production. Important for efficient CD180 cell surface expression. Associated diseases include Parametritis and Interstitial Emphysema.
TLR2	Toll-like Receptor 2	4	It encodes a cell-surface protein that cooperates with TLR1 or TLR6 to mediate the innate immune response after recognition of the pathogen-associated molecular patterns (PAMPs), such us bacterial lipoproteins or lipopeptides, and it is also thought to promote apoptosis in response to them. It is implicated in the pathogenesis of several autoimmune diseases.
SLC30A8	Solute Carrier Family 30 member 8	8	It encodes a zinc ion efflux transporter that is highly expressed only in the pancreas, particularly in islets of Langerhans. It provides zinc to insulin maturation or storage in the pancreatic beta-cells. Allelic variants of this gene can confer susceptibility to T2D.
GCK	Glucokinase (Hexokinase 4)	7	It encodes an enzyme that phosphorylates glucose to glucose-6-phosphate, the first step in most glucose metabolism pathways. It is expressed in pancreas and liver. In the pancreas, it plays a role in glucose-stimulated insulin secretion. In the liver, it is important in glucose uptake and conversion to glycogen. Mutations in this gene are associated with multiple types of diabetes and hyperinsulinemic hypoglycemia.

Table 5.2.1. Overview of genes studied in association of Epigenetics with T2D and glycemic traits (Database URL: www.genecards.org) [147] (*continued*)

Gene	Alias	Chr	Function
PRKCZ	Protein Kinase C Zeta type	1	It encodes an enzyme that plays a role as activator or downstream effector in diverse signaling cascades in different cell types. In adipocytes, upon insulin treatment may contribute to the activation of translocation of the glucose transporter SLC2A4/GLUT4 and subsequent glucose transport.
CTGF	Connective Tissue Growth Factor	6	It encodes a protein that is secreted by vascular endothelial cells, and plays a role in chondrocyte proliferation and differentiation, cell adhesion in many cell types, and it is related to platelet-derived growth factor. Polymorphisms in this gene can be related to a higher incidence of systemic sclerosis.
LEP	Leptin	7	It encodes a protein that is secreted by white adipocytes into the circulation and plays a major role in the regulation of energy homeostasis. Circulating leptin binds to its receptor in the brain, activating downstream signaling pathways that inhibit feeding and promote energy expenditure. It also has endocrine functions, participates in the regulation of immune and inflammatory responses, hematopoiesis, angiogenesis, reproduction, bone formation and wound healing. Mutations in this gene can cause severe obesity, morbid obesity with hypogonadism and T2D.
IRS-1	Insulin Receptor Substrate 1	2	It encodes a protein which, when is phosphorylated by insulin receptor tyrosine kinase, binds specifically to various cellular proteins, thus controlling diverse cellular processes. Mutations in this gene are associated with T2D and susceptibility to insulin resistance.
GIPR	Gastric Inhibitory Polypeptide Receptor	19	It encodes a G-protein coupled receptor for gastric inhibitory polypeptide (GIP), demonstrated to stimulate insulin release in the presence of elevated glucose. Defect in this gene thus may contribute to the pathogenesis of diabetes
CAMK1D	Calcium/Calmodulin Dependent Protein Kinase I Delta	10	It encodes an enzyme that is a component of the calcium-regulated calmodulin-dependent protein kinase cascade. It has been associated with the regulation of granulocyte function, activation of CREB-dependent gene transcription, aldosterone synthesis and secretion, differentiation and activation of neutrophil cells, and apoptosis of erythroleukemia cells.
CRY2	Cryptochrome Circadian Regulator 2	11	It encodes a transcriptional repressor which forms a core component of the circadian clock. It regulates various physiological processes, including metabolism, sleep, body temperature, blood pressure, endocrine, immune, cardiovascular and renal function. I also plays a key role in glucose and lipid metabolism modulation, in part, through the transcriptional regulation of genes involved in these pathways.
CALM2	Calmodulin 2 (Phosphorylase Kinase, Delta)	2	It encodes a calcium binding protein that plays a role in signaling pathways, cell cycle progression and proliferation. It mediates the control of a large number of enzymes, aquaporins and other proteins through calcium-binding and ion channels, such us the calcium-activated potassium channel KCNN2. Diseases associated with CALM2 include Long Qt Syndrome 15 and Long Qt Syndrome 1.

Table 5.2.1. Overview of genes studied in association of Epigenetics with T2D and glycemic traits (Database URL: www.genecards.org) [147] (*continued*)

Gene	Alias	Chr	Function
MCP1	Monocyte Chemoattractant Protein 1	17	It encodes a cytokine that displays a chemotactic activity, attracting monocytes and basophils but not neutrophils or eosinophils. It augments monocyte anti-tumor activity and has been implicated in diseases characterized by monocytic infiltrates, like psoriasis, rheumatoid arthritis or atherosclerosis.
TLR4	Toll-Like Receptor 4	9	It encodes a receptor that cooperates with LY96 and CD14 to mediate the innate immune response to bacterial lipopolysaccharide (LPS). It is also involved in LPS-sindependent inflammatory responses triggered by free fatty acids, such as palmitate, and Ni(2+). In complex with TLR6, promotes sterile inflammation in monocytes/macrophages in response to oxidized low-density lipoprotein (oxLDL) or amyloid-beta 42. Mutations in this gene have been associated with differences in LPS responsiveness.
FFAR3	Free Fatty Acid Receptor 3	19	It encodes a receptor that is activated by a major product of dietary fiber digestion, the short chain fatty acids (SCFAs), for the regulation of whole-body energy homeostasis, glucose homeostasis, intestinal immunity and indirectly LEP/Leptin production.
PP2Ac	Protein Phosphatase 2 Catalytic Subunit Alpha	5	It encodes the alpha isoform of the catalytic subunit of the Protein Phosphatase 2A, which can modulate the activity of some kinase enzymes. It is involved in signal transduction and in the negative control of cell growth and division. Diseases associated include Usher Syndrome, Type I.
PPARG	Peroxisome Proliferator-Activated Receptor Gamma	3	It encodes PPAR-gamma (PPARγ) a member of the peroxisome proliferator-activated receptor (PPAR) subfamily of nuclear receptors which form heterodimers to regulate transcription of various genes. PPARγ is a regulator of adipocyte differentiation and has been implicated in the pathology of numerous diseases including obesity, diabetes, atherosclerosis and cancer. Associated diseases include Familial Partial Lipodystrophy Type 3 and Intimal Medial Thickness of Internal Carotid Artery.
PPARGC1A	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha	4	It encodes a transcriptional coactivator that regulates the genes involved in energy metabolism, such us mitochondrial genes, and the muscle fiber type determination. It also plays a role in metabolic reprogramming in response to dietary availability through coordination of the expression of genes involved in glucose and fatty acid metabolism, and in the integration of the circadian rhythms and energy metabolism. It may be also involved in controlling blood pressure, regulating cellular cholesterol homoeostasis, and the development of obesity.
PDX-1	Pancreatic and duodenal Homeobox 1 / Insulin upstream factor 1	13	It encodes a transcriptional activator of several genes, including insulin, somatostatin, glucokinase, islet amyloid polypeptide and glucose transporter type 2. It is involved in the early development of the pancreas and plays a major role in glucose-dependent regulation of insulin gene expression. A defective gene can lead to early-onset insulin-dependent diabetes mellitus, as well as maturity onset diabetes of the young type 4.

Table 5.2.1. Overview of genes studied in association of Epigenetics with T2D and glycemic traits (Database URL: www.genecards.org) [147] (*continued*)

Gene	Alias	Chr	Function
INS	Insulin	11	It encodes a precursor form called proinsulin, which is cleaved to form the A and B chains, and then joined together to form insulin. It binds to the insulin receptor (INSR) to stimulate glucose uptake. It blood glucose concentration and increases cell permeability to monosaccharides, amino acids and fatty acids. It accelerates glycolysis, the pentose phosphate cycle, and glycogen synthesis in liver. Associated diseases include Hyperproinsulinemia and Insulin-Dependent Diabetes Mellitus 2.
GLP1R	Glucagon-Like Peptide 1 Receptor	6	It encodes a 7-transmembrane protein that functions as a receptor for glucagon-like peptide 1 (GLP-1) hormone, which stimulates glucose-induced insulin secretion, and plays an important role in the signaling cascades leading to insulin secretion. The protein is an important drug target for the treatment of type 2 diabetes and stroke. Polymorphisms in this gene are associated with diabetes, insulinoma and fasting hypoglycemia.
UBASH3A	Ubiquitin Associated And SH3 Domain Containing A	21	It encodes one members of the T-cell ubiquitin ligand family, which can negatively regulate T-cell signaling by facilitating the growth factor withdrawal-induced apoptosis in T cells. It can also interfere in the down-regulation and degradation of receptor-type tyrosine kinases and promotes accumulation of activated target receptors, such as T-cell receptors on the cell surface. Diseases associated include Dirofilariasis and Erysipeloid.
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	12	It encodes an enzyme that catalyzes an important energy-yielding step in carbohydrate metabolism, the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate in the presence of inorganic phosphate and nicotinamide adenine dinucleotide (NAD). It also has uracil DNA glycosylase activity in the nucleus. Related diseased include Fragile X Mental Retardation 1.
TFAM	Transcription Factor A, Mitochondrial	10	It encodes a protein involved in mitochondrial DNA replication and repair. Sequence polymorphisms in this gene are associated with Alzheimer's and Parkinson's diseases.
TRIM3	Tripartite Motif Containing 3	11	The protein encoded by this gene is a member of the tripartite motif (TRIM) family, also called the 'RING-B-box-coiled-coil' (RBCC) subgroup of RING finger proteins. This protein localizes to cytoplasmic filaments. It is similar to a rat protein which is a specific partner for the tail domain of myosin V, a class of myosins which are involved in the targeted transport of organelles. Among its related pathways are Cytokine Signaling in Immune system and Innate Immune System.
TCF7L2	Transcription Factor 7 Like 2	10	This gene encodes a high mobility group (HMG) box-containing transcription factor that plays a key role in the Wnt signaling pathway. The protein has been implicated in blood glucose homeostasis. Genetic variants of this gene are associated with increased risk of type 2 diabetes. Associated diseases include Noninsulin-Dependent Diabetes Mellitus and Colorectal Cancer.

Table 5.2.1. Overview of genes studied in association of Epigenetics with T2D and glycemic traits (Database URL: www.genecards.org) [147] (*continued*)

Gene	Alias	Chr	Function
PDK4	Pyruvate Dehydrogenase Kinase 4	7	It encodes a protein located in the matrix of the mitrochondria and plays a key role in the regulation of glucose and fatty acid metabolism and homeostasis via phosphorylation of the pyruvate dehydrogenase subunits PDHA1 and PDHA2. Expression of this gene is regulated by glucocorticoids, retinoic acid and insulin. Diseases associated include non-insulin-dependent diabetes mellitus.
HNF4A	Hepatocyte Nuclear Factor 4 Alpha	20	It encodes a nuclear transcription factor which binds DNA and controls the expression of several genes, including hepatocyte nuclear factor 1 alpha, which regulates the expression of several hepatic genes. It also may play a role in development of the liver, kidney and intestines. Mutations in this gene have been related to with monogenic autosomal dominant non-insulin-dependent diabetes mellitus type I.
KLF11	Kruppel Like Factor 11	2	It encodes a transcription factor that binds to SP1-like sequences in epsilon- and gamma-globin gene promoters. This binding inhibits cell growth and causes apoptosis. Defects in this gene are a cause of maturity-onset diabetes of the young type 7 (MODY7).
DUSP9	Dual Specificity Phosphatase 9	X	It encodes an enzyme that regulates mitogen-activated protein (MAP) kinases by dephosphorylating both the phosphoserine/threonine and phosphotyrosine residues. It shows selectivity for members of the ERK family of MAP kinases and is localized to the cytoplasm and nucleus. Aberrant expression of this gene is associated with type 2 diabetes and cancer progression in several cell types.
HHEX	Hematopoietically Expressed Homeobox	10	It encodes a member of the homeobox family of transcription factors, many of which are involved in developmental processes and it may play a role in hematopoietic differentiation. Related diseases include Gangliosidosis Gm2 and Sandhoff Disease. Among its related pathways are transcriptional misregulation in cancer and mesodermal commitment pathway.
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A	9	It encodes several transcript variants which differ in their first exons. Two of them encode structurally related isoforms known to function as inhibitors of CDK4 kinase. The remaining transcript contains an alternate open reading frame (ARF) that specifies a protein that functions as a stabilizer of the tumor suppressor protein p53. The three share a common functionality in cell cycle G1 control. This gene is frequently mutated or deleted in a wide variety of tumors, and is known to be an important tumor suppressor gene.
KCNQ1	Potassium Voltage-Gated Channel Subfamily Q Member 1	11	It encodes a voltage-gated potassium channel required for repolarization phase of the cardiac action potential, and plays an important role in a number of tissues, including heart, inner ear, stomach and colon. Mutations in this gene are associated with hereditary long QT syndrome 1 (also known as Romano-Ward syndrome), Jervell and Lange-Nielsen syndrome, and familial atrial fibrillation.

Table 5.2.1. Overview of genes studied in association of Epigenetics with T2D and glycemic traits (Database URL: www.genecards.org) [147] (*continued*)

Gene	Alias	Chr	Function
CIDEC	Cell Death Inducing DFFA Like Effector C	3	It encodes a member of the cell death-inducing DNA fragmentation factor-like effector family, which plays important roles in apoptosis. The protein binds to lipid droplets in adipocytes and regulates their enlargement, thereby restricting lipolysis and favoring storage. It also may mediate adipocyte apoptosis. This gene is regulated by insulin and its expression is positively correlated with insulin sensitivity. Mutations in this gene may contribute to insulin resistant diabetes. Diseases associated include Familial Partial Lipodystrophytype 5 and Adiposis Dolorosa.
ADCY5	Adenylate Cyclase 5	3	It encodes an enzyme that catalyzes the formation of the signaling molecule cAMP in response to G-protein signaling and regulates the increase of free cytosolic Ca (2+) in response to increased blood glucose levels and contributes to the regulation of Ca (2+)-dependent insulin secretion. Single nucleotide polymorphisms in this gene may be associated with low birth weight and type 2 diabetes. Diseases associated include familial dyskinesia with facial myokymia.
CDKN2B	Cyclin Dependent Kinase Inhibitor 2B	9	It encodes a cyclin-dependent kinase inhibitor, which forms a complex with CDK4 or CDK6, and prevents the activation of the CDK kinases, thus the encoded protein functions as a cell growth regulator that controls cell cycle G1 progression. Diseases associated include adult acute lymphocytic leukemia and scrotal carcinoma.
IDE	Insulin Degrading Enzyme	10	It encodes a zinc metallopeptidase that degrades intracellular insulin, and thereby terminates insulins activity, as well as participating in intercellular peptide signalling by degrading diverse peptides such as glucagon, amylin, bradykinin, and kallidin. The preferential affinity of this enzyme for insulin results in insulin-mediated inhibition of the degradation of other peptides such as beta-amyloid. Deficiencies in this protein's function are associated with Alzheimer's disease and type 2 diabetes mellitus but mutations in this gene have not been shown to be causitive for these diseases.
MTNR1B	Melatonin Receptor 1B	11	It encodes a high affinity form of a receptor for melatonin and it is likely to mediate the reproductive and circadian actions of melatonin. It is widely distributed, with high concentrations in the brain and in the retina. It is thought to participate in light-dependent functions in the retina and may be involved in the neurobiological effects of melatonin. Diseases associated include noninsulin-dependent diabetes mellitus and idiopathic scoliosis.
TSPAN8	Tetraspanin 8	12	It encodes a transmembrane glycoprotein that mediates signal transduction events that play a role in the regulation of cell development, activation, growth and motility. This gene is expressed in different carcinomas. Diseases associated include annular pancreas and autosomal recessive nonsyndromic deafness 3.

Table 5.2.1. Overview of genes studied in association of Epigenetics with T2D and glycemic traits (Database URL: www.genecards.org) [147] (*continued*)

Gene	Alias	Chr	Function
APN	Aminopeptidase N	15	It encodes an aminopeptidase of broad specificity which plays a role in the final digestion of peptides generated from hydrolysis of proteins by gastric and pancreatic proteases, as well as in the angiogenesis and promote cholesterol crystallization. It also participates in the processing of various peptides including peptide hormones, such as angiotensin III and IV, neuropeptides, and chemokines. It may be involved the cleavage of peptides bound to major histocompatibility complex class II molecules of antigen presenting cells. Defects in this gene appear to be a cause of various types of leukemia or lymphoma.
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A	9	It encodes a protein that acts as a tumor suppressor, capable of inducing cell cycle arrest in G1 and G2 phases. Its loss has been shown to be a significant event in a number of cancer types. Associated d diseases include Melanoma-Pancreatic Cancer Syndrome and Melanoma-Astrocytoma Syndrome.
CAV1	Caveolin 1	7	It encodes a scaffolding protein which is a main component of the caveolae plasma membranes found in most cell types. It is nvolved in the costimulatory signal essential for T-cell receptor (TCR)-mediated T-cell activation. It is also considered a tumor suppressor gene candidate and a negative regulator of the Ras-p42/44 MAP kinase cascade. Mutations in this gene have been associated with Berardinelli-Seip congenital lipodystrophy, congenital cataracts.
WFS1	Wolfram Syndrome 1 (Wolframin)	4	It encodes a transmembrane glycoprotein, which is located primarily in the endoplasmic reticulum and ubiquitously expressed with highest levels in brain, pancreas, heart, and insulinoma beta-cell lines. It participates in the regulation of cellular Ca(2+) homeostasis, at least partly, by modulating the filling state of the endoplasmic reticulum Ca(2+) store. Mutations in this gene are associated with Wolfram syndrome, also called DIDMOAD (Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy, and Deafness), an autosomal recessive disorder. The disease affects the brain and central nervous system.
MALT1	Mucosa Associated Lymphoid Tissue Lymphoma Translocation Protein 1	18	It encodes an aminopeptidase that catalyzes the removal of amino acids from the amino terminus of proteins and peptides. It may play a role in NF-kappaB activation in a BCL10-induced manner. Diseases associated with MALT1 include Immunodeficiency and Mucosa-Associated Lymphoid Tissue Lymphoma.
FTO	Alpha-Ketoglutarate Dependent Dioxygenase	16	It encodes an enzyme that repairs alkylated DNA and RNA by oxidative demethylation. It contributes to the regulation of the global metabolic rate, energy expenditure and energy homeostasis. In particular, it is involved in the regulation of thermogenesis and the control of adipocyte differentiation into brown or white fat cells. Diseases associated include growth retardation, developmental delay, facial dysmorphism and obesity.
ZNF668	Zinc Finger Protein 668	16	It encodes a protein that may be involved in transcriptional regulation. It is related to DNA binding transcription factor activity.

Table 5.2.1. Overview of genes studied in association of Epigenetics with T2D and glycemic traits (Database URL: www.genecards.org) [147] (*continued*)

Gene	Alias	Chr	Function
HSPA2	Heat Shock Protein Family A (Hsp70) Member 2	14	It encodes a chaperone protein implicated in a wide variety of cellular processes, including protection of the proteome from stress, the protein quality control system, by ensuring the correct folding of proteins, the re-folding of misfolded proteins and controlling the targeting of proteins for subsequent degradation, and the transport of newly synthesized polypeptides. It also plays a role in spermatogenesis. Diseases associated include inflammatory bowel disease and papillary cystadenocarcinoma. Heat Shock Protein Family A (Hsp70) Member 2
CD320	CD320 Molecule	19	It encodes a receptor for transcobalamin saturated with cobalamin (TCbl), that is expressed at the cell surface. It mediates the cellular uptake of transcobalamin bound cobalamin (vitamin B12), and is involved in B-cell proliferation and immunoglobulin secretion. Mutations in this gene are associated with methylmalonic aciduria.
HNF1A	SFT2 Domain Containing 3	2	It encodes a protein that may be involved in fusion of retrograde transport vesicles derived from an endocytic compartment with the Golgi complex.
TWIST1	Twist Family BHLH Transcription Factor 1	7	It encodes a transcription factor that plays an important role in embryonic development, regulating the transcription of genes involved in neural crest differentiation and brown fat metabolism. It also represses the expression of proinflammatory cytokines such as TNFA and IL1B, and the activity of the circadian transcriptional activator: NPAS2-ARNTL/BMAL1 heterodimer. it is involved in the osteoblast differentiation. Represses Mutations in this gene cause Saethre-Chotzen syndrome and Craniosynostosis 1.
MYO5A	Myosin VA	15	It encodes a class of actin-based motor proteins involved in cytoplasmic vesicle transport and anchorage, spindle-pole alignment and mRNA translocation, and it is abundant in melanocytes and nerve cells. Mutations in this gene cause Griscelli syndrome type-1 (GS1), Griscelli syndrome type-3 (GS3) and neuroectodermal melanolysosomal disease, or Elejalde disease.
MAPK1	Mitogen-Activated Protein Kinase 1	22	It encodes a member of the MAP kinase family. The MAPK cascade plays also a role in initiation and regulation of meiosis, mitosis, and postmitotic functions in differentiated cells by phosphorylating a number of transcription factors. The activation of this kinase requires its phosphorylation by upstream kinases. Associated diseases include Chromosome 22Q11.2 Deletion Syndrome, Distal and Pertussis.
MYO18B	Myosin XVIIIB	22	It encodes a protein which may be involved in intracellular trafficking of the muscle cell when in the cytoplasm, whereas entering the nucleus, may be involved in the regulation of muscle specific genes. May play a role in the control of tumor development and progression. Mutations in this gene are associated with lung cancer.

Table 5.2.1. Overview of genes studied in association of Epigenetics with T2D and glycemic traits (Database URL: www.genecards.org) [147] (*continued*)

Gene	Alias	Chr	Function
HOXC6	Homeobox C6	12	It encodes a member of the homeobox family of transcription factors that play an important role in morphogenesis in all multicellular organisms. This is a sequence-specific transcription factor which is part of a developmental regulatory system that provides cells with specific positional identities on the anterior-posterior axis.
PRKAB1	Protein Kinase, AMP-Activated, Beta 1 Non-Catalytic Subunit	12	It encodes a regulatory subunit of the AMP-activated protein kinase (AMPK), which is an energy sensor protein kinase that plays a key role in regulating cellular energy metabolism. In response to reduction of intracellular ATP levels, AMPK activates energy-producing pathways and inhibits energy-consuming processes: inhibits protein, carbohydrate and lipid biosynthesis, as well as cell growth and proliferation. Associated diseases include Body Mass Index Quantitative Trait Locus 11.
NF-KB	Nuclear Factor Kappa B	4	It encodes a transcription regulator that is activated by various intra- and extra-cellular stimuli such as cytokines, oxidant-free radicals, ultraviolet irradiation, and bacterial or viral products. It translocates into the nucleus and stimulates the expression of genes involved in a wide variety of biological functions. It is a homo- or heterodimeric complex formed by the Rel-like domain-containing proteins RELA/p65, RELB, NFKB1/p105, NFKB1/p50, REL and NFKB2/p52 and the heterodimeric p65-p50 complex. Associated diseases include Immunodeficiency.
RELA	RELA Proto-Oncogene, NF-KB Subunit		It encodes the NF-Kappa-B transcription factor P65. The most abundant form of NF-kappa-B is NFKB1 complexed with the product of this gene, RELA. Associated diseases include Ependymoma and Reticuloendotheliosis. Can modulate chromatin function through deacetylation of histones and can promote alterations in the methylation of histones and DNA, leading to transcriptional repression.
Sirt1	Sirtuin 1	10	It encodes a NAD-dependent protein deacetylase that links transcriptional regulation directly to intracellular energetics and participates in the coordination of several separated cellular functions such as cell cycle, response to DNA damage, metabolism, apoptosis and autophagy. Associated diseases include Aging and Ovarian Endodermal Sinus Tumor.
IL-1A	Interleukin 1 Alpha	2	It encodes cytokine member of the interleukin 1 cytokine family and it is involved in various immune responses, inflammatory processes, and hematopoiesis. It is produced by activated macrophages and released in response to cell injury, and thus induces apoptosis. Polymorphism may be associated with rheumatoid arthritis and Alzheimer's disease, Irritant Dermatitis and Cholesteatoma of middle ear.

Table 5.2.1. Overview of genes studied in association of Epigenetics with T2D and glycemic traits (Database URL: www.genecards.org) [147] (*continued*)

Gene	Alias	Chr	Function
PTEN	Phosphatase And Tensin Homolog	10	It encodes a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase which functions as a tumor suppressor by negatively regulating AKT/PKB signaling pathway, and that is commonly lost in cancer. The isoform alpha plays a role in mitochondrial energetic metabolism by promoting COX activity and ATP production. It may also be a negative regulator of insulin signaling and glucose metabolism in adipose tissue. Related diseases are dysplastic gangliocytoma of the cerebellum, and macrocephaly multiple lipomas and hemangiomata.
TNF-α	Tumor Necrosis Factor	6	It encodes a proinflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. It is mainly secreted by macrophages and can induce cell death of certain tumor cell lines. It is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. It is implicated in a variety of diseases, including autoimmune diseases, insulin resistance, and cancer. Diseases associated include asthma and malaria.
COX-2	Cyclooxygenase 2	1	It encodes cyclooxygenase isoform 2, also known as prostaglandin-endoperoxide synthase 2 (PTGS2). It is an inducible isozyme, which converts arachidonate to prostaglandin H2 (PGH2), during the prostanoid synthesis. It is constitutively expressed in some tissues in physiological conditions, such as the endothelium, kidney and brain, and in pathological conditions, such as in cancer. It is responsible for production of inflammatory prostaglandins. Up-regulation of PTGS2 is also associated with increased cell adhesion, phenotypic changes, resistance to apoptosis and tumor angiogenesis.
IL-8	C-X-C Motif Chemokine Ligand 8 ; interleukin-8	4	It encodes a protein that is secreted primarily by neutrophils, where it serves as a chemotactic factor by guiding the neutrophils to the site of infection. It may also be released from several cell types in response to an inflammatory stimulus. Is also attracts basophils and T-cells, but not monocytes. Associated diseases include Bronchiolitis and Extrinsic Allergic Alveolitis.
UNC13B	Unc-13 Homolog B	9	This gene is expressed in the kidney cortical epithelial cells and it is upregulated by hyperglycemia. It contains three C2 domains and a diacylglycerol-binding C1 domain. Hyperglycemia increases the levels of diacylglycerol, which has been shown to induce apoptosis, thus contributing to the renal cell complications of hyperglycemia. Associated diseases include Hyperglycemia and Hemophagocytic Lymphohistiocytosis
PAI-1	Serpin Peptidase Inhibitor, Clade E (Nexin, Plasminogen Activator Inhibitor Type 1), Member 1	7	It encodes a member of the serine proteinase inhibitor (serpin) superfamily, which is the principal inhibitor of tissue plasminogen activator and urokinase, and hence is an inhibitor of fibrinolysis. Defects in this gene are the cause of plasminogen activator inhibitor-1 deficiency (PAI-1 deficiency), and high concentrations of the gene product are associated with thrombophilia. Associated diseases include Plasminogen Activator Inhibitor-1 Deficiency and Complete Plasminogen Activator Inhibitor 1 Deficiency.

Table 5.2.1. Overview of genes studied in association of Epigenetics with T2D and glycemic traits (Database URL: www.genecards.org) [147] (*continued*)

Gene	Alias	Chr	Function
RAGE	Receptor For Advanced Glycation End-Products Variant 20	6	It encodes a receptor for glycosylation end products (AGE). AGE accumulate in vascular tissue in aging and at an accelerated rate in diabetes. Besides AGE, it also interacts with other molecules implicated in homeostasis, development, and inflammation, and certain diseases, such as diabetes and Alzheimer's disease. Acts as a mediator of both acute and chronic vascular inflammation in conditions such as atherosclerosis and in particular as a complication of diabetes. Associated diseases include Diabetic Angiopathy and Thymic Hyperplasia.

2.1. Glucose homeostasis

Epigenetic alterations can have great influence on islet cells and glucose homeostasis that can alter their pathophysiological processes and consequently result in T2D [15].

2.1.1. DNA methylation

Different studies have investigated the association between global DNA methylation and glucose levels, reporting inconsistent results [16-18]. Increased levels of plasma glucose were associated with higher methylation levels in LINE-1 when assessed in adipose tissue, blood or skeletal muscle [16 17 19]. However, one study showed no association or an inverse association between LINE -1 methylation or other markers of global DNA methylation and glucose levels assessed in B and NK lymphocytes human cells [18]. This stresses the relevance of using cell type-specific assays when investigating epigenetic signatures in clinical tissue samples especially those characterized by a high heterogeneity in cell types frequency and phenotype, such as blood.

Candidate gene studies have revealed lower methylation levels of *GIPR* gene and *PPARGC1A* gene in blood and skeletal muscle [20 21]. Both genes are believed to contribute to improve insulin sensitivity, mitochondrial biogenesis and browning of white adipose tissue. In another study, the authors reported that high glucose levels affect human pancreatic islet gene expression and several of these genes also exhibit epigenetic changes [22]. This might contribute to the impaired insulin secretion seen in T2D. Moreover, one study reported that increased levels of plasma glucose might be associated with higher methylation levels of LY86 gene in blood, which has been suggested to play a role in inflammation, obesity and insulin resistance [23]. Volkmar et al. have investigated DNA methylation in human pancreatic islets by exposing the pancreatic cells from nondiabetic donors to high glucose levels [9]. The study reported a non-significant association between DNA methylation and the 16 CpG sites tested, concluding that the methylated changes in the islets from T2D patients would not likely be a cause of hyperglycaemia [9].

Furthermore, studies conducted using placenta tissue and cord blood have yielded interesting results between methylation levels and fasting glucose. Lower DNA methylation levels of *ADIPOQ, LPL, IGF1R* and *IGFBP3* on the fetal side of the placenta were associated with higher maternal 2-h post oral glucose tolerance test levels during pregnancy, although the association did not remain significant with 2 h post-oral glucose tolerance test levels [24]. Also, the maternal gestational glucose levels were positively associated with placental DNA methylation, and negatively associated with cord blood DNA methylation of the *PPARGC1A* promoter in a CpG site-specific manner. The researchers concluded that epigenetic alteration of the *PPAGRC1A* promoter may be one of the potential mechanisms underlying the metabolic programming in offspring exposed to intrauterine hyperglycaemia [25]. Another study investigating whether epigenetic dysregulations of the insulin-like growth factor system in placenta were exposed to maternal impaired glucose tolerance, confirmed their hypothesis [26]. Also, in this study, maternal glucose 2 h post oral glucose tolerance test and fasting glucose at the second trimester of pregnancy were negatively correlated with *GF1R-L4* (7 CpGs) and *IGFBP3-L1* DNA methylation levels [26]. Both *GF1R-L4* and *IGFBP3-L1* are important genes in foetal metabolic programming and impaired glucose tolerance.

Limited evidence exists on EWAS and glucose metabolism. Also, the existing evidence so far is inconclusive and inconsistent with studies reporting no association [27] and another reporting a positive association between epigenome-wide DNA methylation levels and fasting glucose [28]. Using whole blood samples from a population-based prospective study, one study recently reported 6 CpG sites related to fasting glucose and 2-hour glucose, independent of age, sex, smoking, and estimated white blood cell proportions [26]. Moreover this study showed that effect strengths were reduced on average by around 30% after adjustment for BMI, suggesting an influence of BMI on the investigated phenotypes [26]. The findings provide evidence for the first time that DNA methylation may be associated with glucose metabolism, a relationship which can be measured in DNA isolated from whole blood.

2.1.2. Histone modifications

Histone modifications may also play a pivotal role in glucose metabolism, but this is an understudied research topic [29]. Studies have shown that *TXNIP* gene might be important in glucose metabolism, especially in diabetes-related phenotypes [30 31]. *TXNIP* is a key component of pancreatic β-cell biology, nutrient sensing, energy metabolism, and regulation of cellular redox [30 31]. Moreover, *TXNIP* expression is highly induced by glucose through activation of the carbohydrate response element-binding protein, which binds the *TXNIP* promoter, making it an attractive target for diabetes therapy. Previous studies have identified several critical transcription factors, enzymes important in histone activation and acetylation, like the ChREBP and p300, as the specific chro-

matin modification mediating this glucose-induced transcription of beta cell *TXNIP* [31]. Recently, another study published similar results confirming the findings [32]. They found that the glucose-induced *TXNIP* gene expression is greatly reduced by p300 silencing, and Ep300 cells are protected from high glucose-induced cell death and have elevated insulin secretion [32]. In the current study, elevated levels of EP300 and *TXNIP* gene expression in human diabetic islets were correlated with reduced glucose-stimulated *TXNIP* genes expression [32]. These data provide evidence that histone acetylation could be a key regulator of glucose-induced increase in *TXNIP* gene expression and thereby glucotoxicity-induced apoptosis.

2.2. Insulin metabolism

A common feature of T2D that affects the liver and the peripheral tissues is insulin resistance (IR). The most relevant tissues that develop insulin resistance are liver cells, skeletal muscle, and adipose tissue [33]. Impaired response to insulin fails to clear the blood stream from glucose, and additionally, stimulates the secretion of adipokines from the adipose tissue which may further negatively affect the whole body glucose homeostasis [33].

2.2.1. DNA Methylation

Several studies have investigated the association between global DNA methylation and insulin metabolism, focusing on fasting plasma insulin levels, insulin secretion [19], and insulin resistance as measured by homeostatic model assessment [34 35]. The studies on insulin secretion and insulin resistance did not report significant associations between insulin metabolism and global DNA methylation. However, one study reported an interaction of global DNA methylation with circulating folate concentrations in relation to insulin resistance [34]. The authors found that a lower degree of methylation and lower plasma folate concentrations were associated with higher insulin resistance [34]. Folate metabolism is linked to phenotypic changes through DNA methylation by the knowledge that folate, a coenzyme of one-carbon metabolism, is directly involved in methyl group transfer for DNA methylation, making them important epigenetic players. Another study assessed global DNA methylation as a percentage of 20-deoxycytidine plus 5-methyl-deoxy-cytidine (5mdC) in genomic DNA and reported a positive association between insulin levels and global DNA methylation assessed in lymphocyte B cells but no association in natural killer cells [36]. Zhao et al. assessed global DNA methylation in Alu elements in peripheral blood leukocytes, which was quantified by bisulphite pyrosequencing [35]. The study showed a positive association with insulin resistance and reported that a 10% increase in mean Alu methylation was associated with an increase of 4.55 units in homeostatic model assessment [35].

Many candidate gene studies have examined methylation sites in or near known candidate genes in relation to plasma insulin, insulin expression and insulin resistance [15].

Most of them reported a positive correlation between plasma insulin and methylation at PPARGC1A in the liver and at HTR2A and LY86 in blood cells. Lower levels of methylation at PPARGC1A were identified in skeletal muscle and lower levels of methylation were identified at the insulin promoter gene associated with increased levels of plasma insulin or mRNA insulin expression [15]. Moreover, inverse associations were found between insulin resistance and the degree of methylation of TFAM and GIPR3 genes in blood cells and PPARGC1A gene in skeletal muscle [15]. Furthermore, studies in pregnant women, reported a negative association of methylation levels of the maternal side of placenta of ADIPOQ gene with insulin resistance [37]. While another study reported a positive correlation between the methylation of IGFBP3 with fasting insulin levels and insulin resistance [26].

Further, a few EWAS have been performed in regard to insulin metabolism [38 39]. Hidalgo et al. reported a significant association between the methylation of a CpG site in *ABCG1*gene on chromosome 21 with insulin and homeostatic model assessment-IR, suggesting that methylation of the CpG site within *ABCG1* merits further evaluation as a novel disease risk marker [39].

The majority of the above mentioned genes are reported to have important functions in metabolic traits and have been associated to insulin metabolism through different biological mechanisms. The *HTR2C* gene is involved in energy expenditure and polymorphisms in this gene coding for many receptors are thought to influence insulin homeostasis [40]. While, *PPARGC1A* upregulates transcription of genes involved in mitochondrial oxidative metabolism and biogenesis as well as skeletal muscle glucose transport. Because mitochondrial defects have been associated with peripheral insulin resistance in healthy subjects it has been suggested that reduced *PPARGC1A* expression in skeletal muscle may be a primary feature of insulin resistance [41]. Furthermore, *PPARGC1A* is involved in biological functions with implications in insulin action including protection against oxidative stress, formation of muscle fiber types as well as regulation of microvascular flow [41 42]. Moreover, there is evidence linking the LY86, TFAM and GIPR3 genes to insulin resistance, mainly their respective encoded proteins that play crucial roles in the pathophysiological regulation of inflammation and insulin resistance [42].

2.2.2. ***Histone modifications***

The evidence pertaining the possible role of histone modifications in insulin metabolism is also very limited. One study investigated the effects of insulin on alterations in post-translational modifications of histone H3 in L6 myoblasts under a hyperglycaemic condition [43]. The authors demonstrated that insulin induced intracellular generated oxidative stress is involved in modulating multiple histone modifications under hyperglycaemic conditions [43]. Their results also revealed that phosphorylation of histone H3 at Ser 10 was independent of known histone kinases and suggest the role of serine/threonine

phosphatase in modulating insulin signalling, suggesting a possible role of phosphatase and its inhibitor in diabetes [43].

3. EPIGENETIC ALTERATIONS IN DIABETES

T2D is a complex disease, product of the interaction of genetic and environmental factors [44] (**Figure 5.2.1**). Epigenetic mechanisms could underlie the connection between environmental exposures and pathology of T2D [45]. For this reason, in recent years, it has been of great interest to study DNA methylation and histone modifications in relation to T2D.

3.1. DNA methylation

When comparing diabetic versus non-diabetic individuals, no difference in global DNA methylation has been reported in overall peripheral blood [46 47], lymphocytes or monocytes populations assessed separately [18], pancreatic islets [9], omental visceral adipose tissue and subcutaneous adipose tissue [17]. Also studies that used skeletal muscle and subcutaneous adipose tissue from monozygotic twins discordant for T2D did not report any differences in global DNA methylation [16]. However, significant differences in the degree of global DNA methylation have also been reported. Luttmer et al reported hypomethylation in blood samples from T2D patients [48], whereas Simar et al reported an increased degree of global DNA methylation specifically in B-cells and natural killer cells from T2D donors [18].

In a candidate gene approach, DNA methylation at several selected genes has been investigated in different tissues, comparing diabetic and non-diabetic donors.

The genes that have been reported to have higher methylation levels in T2D patients are: *IGFBP7* [49], *IGFBP1* [50], *TLR2* [51], *SLC30A8* [52], *GCK* [53], *PRKCZ* [54], *CTGF* [46] and leptin gene in peripheral blood; *PPARGC1A* [55], *PDX-1* [56], insulin promoter gene [57] and *GLP1R* in pancreatic islets [56] and *APN* in adipose tissue [58].

On the other hand, in diabetic donors, lower levels of methylation have been found at genes: *GIPR* [59], *CAMK1D, CRY2, CALM2* [60], *MCP1* [61], *TLR4, FFAR3* [51], *PP2Ac* [62] and *CTGF* [46] in the in peripheral blood samples; *UBASH3A* in B-cells [18] and *PDK4* in skeletal muscle tissue [63]. Additionally, differential methylation between type 2 diabetic patients and matched controls has been found at *TCF7L2* [64].

Further, no clear difference was observed for genes IRS-1 in the peripheral blood [65]; *GADPH, TFAM* and *TRIM3* in B-cells [18]; *GLP1R* in pancreatic islets [66] and *PPARGC1A* in the skeletal muscle [67].

When comparing monozygotic twins discordant for T2D, the genes that were hypermethylated in the diabetic subjects are: *HNF4A, KLF11, DUSP9, HHEX* and *PPARGC1A* in

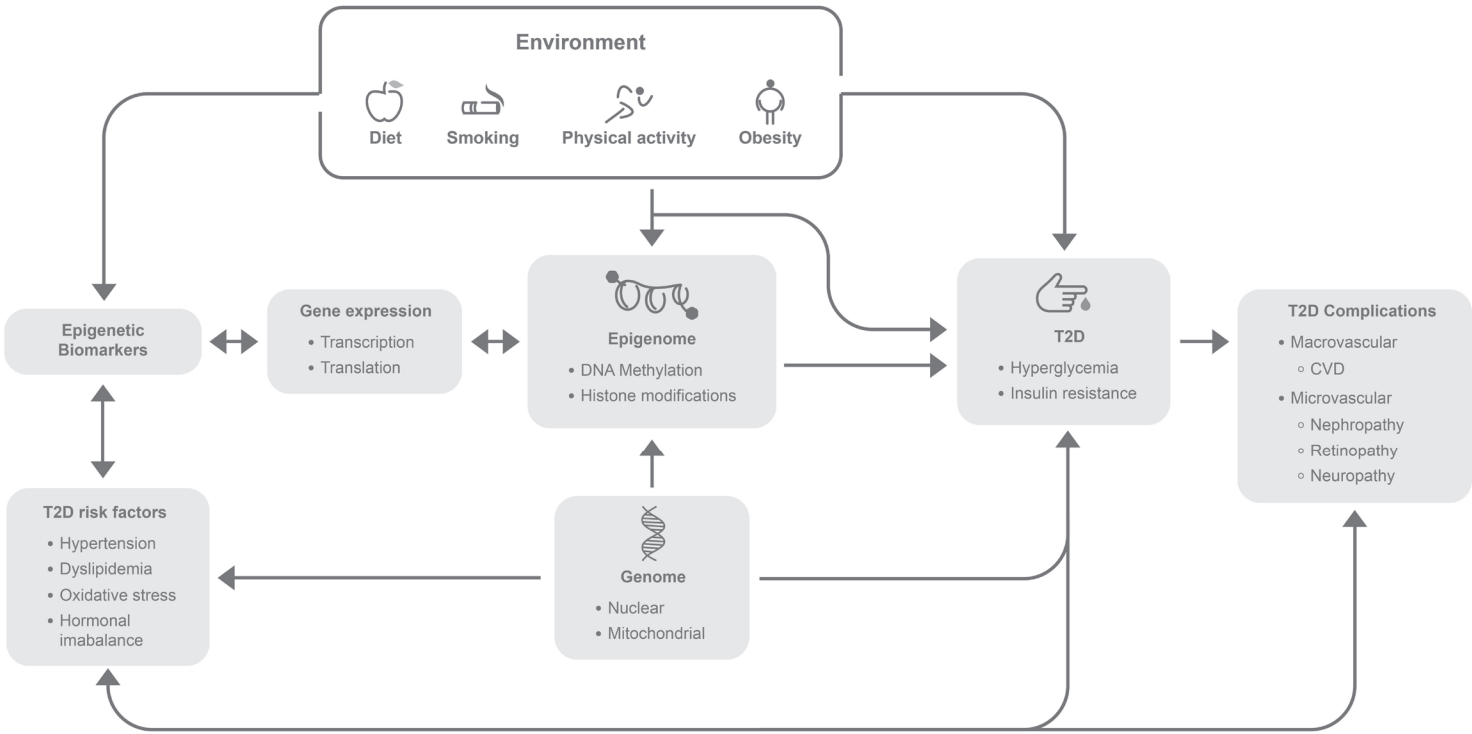


Figure 5.2.1. A conceptual model linking epigenomics to T2D and T2D complications.
Epigenomics represents a critical link between genomic coding and phenotype expression that is influenced by both underlying genetic and environmental factors. Epigenetic biomarkers which can be influenced by T2D risk factors and remodeled epigenetic patterns may contribute to the development of T2D and its complications.

muscle tissue and *CIDEC, HNF4A, ADCY5, CDKN2B, IDE, KCNQ1, MTNR1B* and *TSPAN8* in subcutaneous adipose tissue. Whereas the hypomethylated genes found in the diabetic twins are *CDKN2A, KCNQ1* and *SLC30A8* in muscle tissue and *CAV1, CDKN2A, DUSP9, IRS1* and *WFS1* in subcutaneous adipose tissue. However, after adjustment for multiple testing only two methylation sites, at *CDKN2A* and *HNF4A* genes, in subcutaneous adipose tissue, remained significant [16].

EWAS have raised and evolved as the technology for epigenetics research gradually develops. Progressively, scientists have had more access to methylation arrays with a higher number of methylation probes and new sequencing techniques that have allowed researches to perform genome-wide studies in shorter times. Thus, genome-wide DNA methylation profiling to find associations with T2D has allowed identifying new genes differentially methylated that might provide new insights in the pathogenesis of the disease and the search for biomarkers. To date, EWAS in association with T2D have been performed in peripheral blood, pancreatic islets, skeletal muscle and subcutaneous adipose tissue.

For the study of methylation in pancreatic islets, a Human Methylation 27 BeadChip array was used. It was observed that 276 CpGs affiliated to promotors of 254 genes were displaying differentially methylated sites in tissue from T2D donors compared with non-diabetic controls [9]. Further, a Human Methylation 450 K BeadChip array was utilized to carry out the same approach. As a result, 1649 CpGs, annotated to 853 genes, were reported to have sites of differential methylation in diabetic islets [68]. In skeletal muscle tissue, methylation levels were compared between monozygotic twins discordant for T2D using a Human Methylation 27 Bead Chip array. The test identified one CpG, annotated at IL8, to be differentially methylated [16]. A similar analysis was performed in subcutaneous adipose tissue, revealing that in the diabetic donor, CpGs annotated to *ZNF668*, *HSPA2, C8orf31, CD320, SFT2D3, TWIST1* and *MYO5A* genes had methylation levels significantly different from the non-diabetic twins [16]. DNA methylation studies in blood samples reports dissimilar results, depending on the methylation assessment and multiple testing correction methods. When Human Methylation 450 K Bead Chip array was used, one study reported 51 significant CpGs associated to T2D (correction for multiple tests of FDR < 5%) [69], while other authors observed 5 CpGs associated to incident T2D (correction for multiple testing of $P < 5 \times 10^{-7}$) [30]. A different study used Methylated DNA immunoprecipitation sequencing (MeDIP-seq). It identified as the strongest signal, a differential methylated region at the promoter of *MALT1* (FDR < 5%). A study using the affimetrix SNP6 microarray with posterior in-deep sequencing of the tip-ranking regions showed that 13 out of 93 CpG sites exhibited differences between T2D patients and controls [70]. The researchers found that, among those sites, a methylation site located in the first intron of *FTO* was significantly hypomethylated in blood samples from diabetic subjects [70].

3.2. Histone modifications

Histone changes in acetylation or methylation patterns might induce modifications in chromatin structure and, as a consequence, it may promote dysregulated gene transcription and disease progression [71 72].

A limited number of studies have explored the association of histone modifications with T2D. Histone methyltransferase Set7 is an enzyme involved in histones methylation. Using peripheral blood mononuclear cells from diabetic patients, it was found a Set7-dependent monomethylation of lysine 4 of histone 3 (H3K4m1) on *NK-kβ* p65 promoter, along with an upregulation of the enzyme [73]. Another study observed an increased level of histone H3 lysine 9 dimethylation (H3K9me2) around *IL-1A* promoter and *PTEN* coding regions in circulating monocytes from T2D patients relative to non-T2D controls [74]. A different study investigating histone acetylation found that histone 3 (H3) acetylation at *TNF-α* promoter and *COX-2* promoter was increased in peripheral blood monocytes from type 2 diabetics, compared to controls [75].

4. EPIGENETIC MECHANISMS AS BIOMARKERS FOR RISK PREDICTION

When T2D is not well managed, it can lead to health complications in different organs of the body, increasing the risk of disability and premature death [76]. Direct and indirect costs of T2D impose a large burden that impacts the economy of the country's [77]. These adverse consequences make the development of prevention strategies highly relevant, like the early identification of individuals at risk of developing T2D.

Recently, next to the traditional prevention methods, a novel approach to assess individuals risk is developed, by using statistical models that are able to predict future onset of the disease, based on epigenetic markers. The performance of a prediction model is evaluated by means of the area under the receiver operating characteristic (ROC) curve (AUC), being an AUC value of 1.0 a perfect discrimination of the outcome to be predicted. So far, the models using conventional components, such as anthropometric measurements, family history of diabetes, lifestyle factors and biomarkers like glucose, insulin and lipid levels, are able to predict T2D with an AUC that ranges from 0.7 to 0.9 [78]. However, a large number of predictor factors and measurements of different nature are required, and models do not perform similarly in different populations. As Genome-Wide Association Studies (GWAS) have been developed, genetic markers associated with T2D, glucose, insulin and insulin resistance, have been included as predictors in prediction models. Unfortunately, the associated genetic variants have not improved the performance of the overall models [79 80], which might not be surprising since studies until now, agree that genetic variants explain up to 10% of the heritable risk [81].

Based on findings from previous reports in the field of epigenetics, a limited number of studies have been conducted to explore the suitability of the reported epigenetic markers to predict the onset of T2D and, whether the findings in blood samples can be used as surrogate markers for epigenetic modifications in target tissues for this disease [82 83].

4.1. DNA methylation

Investigating global DNA methylation, a study examined the predictive value for *LINE-1* as a risk marker for T2D and other metabolic disorders. Worsening of metabolic status or T2D onset after 1 year of follow-up was assessed. A model based on classic risk factors (age, sex, body mass index and physical activity) showed an AUC of 0.646[82]. The addition of *LINE-1* methylation measures to the previous model, significantly improved the predictive performance to an AUC of 0.650 [82]. The study was performed among European Spanish women 40 to 65 years old. No other evidence has been reporting the added value of global DNA methylation as a predictive tool in diabetes.

Methylation sites in the DNA, previously reported to be associated with T2D and glucose homeostasis in candidate gene studies or EWAS, have been taken into consideration in the search for biomarkers. One longitudinal study found that the methylation marker cg06500161, annotated to gene *ABCG1*, was associated with a 9% increased risk for future T2D, whereas methylation at cg02650017 annotated to *PHOSPHO1*, was associated with a 15% decreased risk for future T2D [83]. Nevertheless, no further investigation of the predictive value of these CpGs was performed.

The role of DNA methylation in predicting onset of T2D is still in its infancy of investigations. An ongoing longitudinal effort is combining clinical investigation, omics profiling (metabolomics, lipidomics, transcriptomics and epigenomics) with exercise and dietary interventions to provide novel diagnostic and predictive biomarkers to effectively detect the progression towards diabetes in high risk individuals, and also to predict responsiveness to lifestyle interventions known to be effective in the prevention of diabetes [84]. The study included 1455 participants from the DEXLIFE consortium and 400 participants in the intervention group. In the future, this comprehensive approach may provide some more insights on the contribution of DNA methylation sites as predictive markers.

4.2. Histone Modifications

Although a few studies have been conducted to examine the association between histone modifications and T2D [73-75], none of these markers have been proposed as possible novel biomarkers to identify subjects at high risk for T2D. The lack of consistent evidence and replicated studies may explain the absence of a reliable candidate marker serving for clinical purposes.

5. EPIGENETIC CHANGES ASSOCIATED WITH DIABETIC COMPLICATIONS

Diabetes is associated with significantly accelerated rates of several debilitating microvascular complications such as nephropathy, retinopathy, and macrovascular complications such as cardiovascular events. While several studies have been investigating genetic factors related to diabetes and associated complications, little is known about epigenetic changes that occur without alterations in the DNA sequence.

5.1. Epigenetic modifications in cardiovascular disease

Prospective studies have shown that diabetic patients have a two- to four fold risk to develop coronary artery disease, establishing that T2D is an independent risk factor for cardiovascular disease (CVD) [85]. About 70% of T2D patients at an age ≥65 years die from CVD, while T2D cases with no history of coronary artery disease have an equal cardiovascular risk as patients with previous myocardial infarction [86]. CVD and T2D share several common pathophysiological features like the classical cardiovascular risk factors, such as dyslipidaemia, hypertension and obesity. However, all of the known pathways do not explain the complex pathophysiology behind cardiovascular complications of diabetes. Up to date, the underlying mechanisms are often addressed within a specific pathological context, whereas an integrated approach should be preferred in order to capture all potential interlinks between T2D and CVD. New research investigations have linked the participation of epigenetic mechanisms in the process of inflammation, oxidative stress, and endothelial dysfunction, all representing the hallmark of cardiovascular complications of diabetes.

5.1.1. DNA methylation

Using human aortic endothelial cells exposed to high glucose and aortas of diabetic mice, one study found that the mitochondrial adaptor *p66Shc* was epigenetically upregulated by promoter CpG demethylation and H3 acetylation [87]. Moreover the overexpression continued even after returning to normoglycaemia and could only be inhibited after pharmacologic intervention, providing molecular insights for the progression of diabetic vascular complications despite glycaemic control, which might help to define novel therapeutic targets [87].

Although emerging data has linked some aspects of hypertrophy, heart failure, and arrhythmias in cardiomyocytes to DNA methylation and *PTMs* of histones, less evidence has been reported in hearts of diabetic patients [88]. Monkemann et al. reported that oxidative stress damages cardiomyocytes via *p53*-dependent apoptosis in diabetic cardiomyopathy [89]. Interestingly, in animal studies, the methylation of the *p21(WAF1/*

CIP1) gene that encodes several protein kinases at *p53* showed the later to be an early step in the development of hyperglycemia-induced cardiomyopathy in diabetic rats [89].

Other epigenetic marks are linked to intermediate risk factors common to CVD and T2D and that could contribute in discovering diagnostic and prognostic factors. One study, investigating the association between *IGF2* methylation and lipid profile, showed that higher triglyceride/HDL-cholesterol ratio were associated with hypermethylation of *IGF2* gene, indicating that this gene might be an important marker of metabolic risk [90]. The *IGF2* gene provides instructions for making a protein called insulin-like growth factor 2, which plays an essential role in cell growth and insulin mechanisms. Another study that combined genome-wide transcriptome and CpG methylation profiling by array, reported many differentially methylated predicted sites in adipose tissue from insulin-resistant patients compared to controls, which included genes involved in insulin signalling and in the interaction with integrins [91].

Current therapies for diabetes are aimed to optimize glycaemic control and reduce the associated cardiovascular risk. Some preliminary studies have shown that DNA methylation plays an important role in the reversibility and treatment of diabetic complications such as CVD, including vascular inflammation [92]. Resveratrol is a polyphenol with antioxidative and anti-inflammatory properties. Numerous studies have shown that resveratrol might have cardiovascular protective effects and also might contribute in improving insulin sensitivity, reducing plasma glucose levels and reducing inflammation [92]. Lou et al. aimed to investigate the effects of resveratrol (trans-3, 5, 40-trihydroxystilbene) on the expression of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and IFN-γ in diabetic rat aortas and the potential epigenetic mechanisms involved [92]. It showed that the expression levels of pro-inflammatory cytokines were significantly lower in the resveratrol-treated diabetic group. Moreover, the untreated group showed reduced levels of DNA methylation at the specific cytosine phosphate guanosine sites of IL-1β, IL-6, TNF-α and IFN-γ and these levels were reversed by resveratrol [92]. Furthermore, incretins such as glucagon-like peptide 1 receptor *(GLP1R)* agonist are shown to have cardiovascular protection beyond glycaemic control in diabetes subjects[93]. Recent data show that methylation of *GLP1R* is associated with glycaemic control but also cardiometabolic risk factors, such as obesity [94]. Thus, although the studies of epigenetics marks and CVD in diabetes are scarce, they provide some insight on epigenetic modifications as possible targets to develop novel therapeutic agents at preventing and treating vascular complications.

5.1.2. Histone modifications

Gaikwad et al. reported deacetylation and dephosphorylation of histone H3 in the heart and kidney of diabetic Sprague-Dawley rats leading to changes in gene expression in the extracellular matrix and therefore hypertrophy [95]. A recent study used peripheral

blood mononuclear cells to measure histone deacetylases (*HDACs*) activity and expression in relation to glycaemia, inflammation and insulin resistance in patients with T2D. Low-grade chronic inflammation and insulin resistance induced *HDAC3* activity and expression, and correlated positively with circulating levels of TNF-α, IL-6, and other proinflammatory markers, and negatively with *Sirt1* expression [96]. Using aortic endothelial cells, another study showed that exposure to high glucose correlates with the inverse acetylation of the histone *H3K9/K14* and modified DNA methylation patterns [97]. Several histone lysine modifications have also been described following transient high glucose levels that may account for a persistent transcriptional induction of the *RELA* gene, encoding for the *p65* subunit of *NF-kB*, even after subsequent incubation of endothelial cells with normal glucose concentrations [98]. Miao et al. recently compared patients previously included in the conventional treatment arm of the Diabetes Control and Complications Trial who developed diabetic microangiopathy (cases) to patients who were allocated the intensive treatment and had no progression of microvascular complications (controls) [99]. They reported a significantly greater number of promoter regions with enrichment in *H3K9A*c (hyperacetylation) in monocytes, but not in lymphocytes, in cases versus controls [99]. These findings further support the existence of an epigenetic component in the metabolic memory—the concept that early glycaemic control is a major determinant of diabetic complications later in life.

5.2. Epigenetic modifications in diabetic nephropathy

Diabetic nephropathy (DN) is a major chronic complication of diabetes and the most common cause of end-stage kidney disease [100]. Approximately 50% of patients who have end-stage renal disease needing painful and costly dialysis are diabetic [100]. The underlying molecular mechanisms leading to DN are not fully elucidated. High glucose levels adversely impact all renal cell types including mesangial cells, tubular cells, podocytes and endothelial cells, and augment monocyte and macrophage infiltration [101]. High glucose conditions also increase the formation of advanced glycation end-products and production of growth factors such as transforming growth factor β1 (TGF-β1) and angiotensin II in renal cells [101]. Although several classic mechanisms and pathways leading to DN have been described over the years, new molecular and epigenetic mechanisms are emerging [102].

5.2.1. DNA methylation

The role of DNA methylation in DN has elicited much interest [102] mainly because most genome-wide association studies of DN have yielded few susceptibility loci. Studies of DNA methylation profiles in genomic DNA of diabetic patients with or without DN revealed differential methylation levels in several genes, including *UNC13B*, which has been suggested to mediate apoptosis in glomerular cells as a result of hyperglycaemia,

and hence the association could be relevant to the initiation and pathogenesis of DN [103]. In DN, prolonged exposure to hyperglycemia induces production of cytokines, chemokines, and growth factors including *TGFβ1* and connective tissue growth factors, which leads to abnormal glomerular pathology. Brennan et al. measured DNA methylation in 192 candidate genes previously identified to be differentially expressed in *in vitro* models of DN and in renal biopsies from individuals with DN. The study found that 301 CpGs in 38 out of 192 genes were differentially methylated [104]. The gene ontology analysis of the differentially methylated genes revealed that the predominant biological function of the affected genes was organism development [104]. Additional studies using various DNA methylation assays and DNA collected from peripheral blood samples or saliva identified specific DNA methylation profiles for diabetic patients with and without nephropathy [105 106]. These studies proposed using DNA methylation profiles as biomarkers to help predict disease status and progression; however, they did not report associated gene expression data.

5.2.2. Histone modifications

Growing evidence suggests that histone post-translational modifications can have key roles in the pathogenesis of diabetes. *TGF-β1*-induced expression of plasminogen activator inhibitor-1 (*PAI-1*) and *p21* in renal mesangial cells plays a major role in glomerulosclerosis and hypertrophy, key events in the pathogenesis of diabetic nephropathy. However, the involvement of histone acetyl transferases (*HATs*) and *HDACs* that regulate epigenetic histone lysine acetylation, and their interaction with *TGF-β1*-responsive transcription factors, are not clear. *In vitro* studies in rat mesangial cells, have shown that *TGF-β1,* which is a central mediator of fibrogenesis and high glucose treatment, increased *H3K9/14ac* enrichment near Smad and SP1 binding sites (proteins related to the control of gene expression and cell growth) at the plasminogen activator inhibitor 1 and *p21* gene promoters, together with *HATs p300* and *CREB*-binding protein [107]. Histone *H3K9/14ac* was found to have a key role in transcription of these genes in response to *TGF-β1* [107]. High glucose treatment also elicited similar histone post-translational modifications at fibrotic and cell-cycle gene (*p21*) promoters, which were blocked by an anti-*TGF-β* antibody [107]. Therefore, it is conceivable that histone hyperacetylation and related chromatin events involved in *TGF-β1*-mediated *PAI-1* and *p21* expression play important roles in the pathogenesis of DN and could therefore serve as potential therapeutic targets for diabetes-induced renal dysfunction. *In vivo* animal models of DN have also demonstrated changes in histone post-translational modifications. *NF-κB*-dependent inflammatory gene expression has been extensively studied due to the involvement of these target genes in the pathology of several inflammatory diseases, including atherosclerosis, insulin resistance, diabetes and its complications. Studies demonstrated that *H3K4me HMT SET7* could be a NF-κB coactivator at a subset of inducible inflammatory

genes in monocytes[108]. *SET7/9* may therefore be a novel therapeutic target for inflammatory diseases, including diabetes, DN and related metabolic disorders. Interestingly, treatment of mice with losartan, an Ang II type 1 receptor blocker, ameliorated key indices of diabetic nephropathy, and reversed key changes in epigenetic enzymes and *H3K9ac* enrichment at promoters of genes encoding *PAI-1* and *RAGE*, but did not reverse all the diabetes-induced epigenetic changes [109]. Thus, the relative inefficiency of drugs commonly used for diabetic nephropathy, such as Ang II type 1 receptor blockers, to prevent progression to renal failure, in many patients could be due to the incomplete reversal of diabetic nephropathy-associated epigenetic changes [109]. Also, several clinical trials (NCT01038089, NCT00937222) have been conducted testing resveratrol, (class III histone deacetylases group) which is thought to protect against development of diabetic nephropathy via changes in phosphorylation of histone H3 and Sir-2. The activity of resveratrol shows great potential in the prevention and therapy of diabetes and its complications especially to DN, nevertheless the compound is still in its experimental phases. These and other studies emphasize the role of epigenetic mechanisms in the regulation of possible biological and genetic pathways relevant to DN.

5.3. Epigenetic modifications in diabetic retinopathy

Retinopathy, a sight-threatening disease, remains one of the most feared complications of diabetes. Although altered levels of glucose are the main initiators, progression of diabetic retinopathy continues even after the hyperglycaemic insult is reversed by good glycaemic control, suggesting a 'metabolic memory' phenomenon [110 111].

5.3.1. DNA methylation

Recent studies have implicated epigenetic modification in the metabolic memory phenomenon associated with the continued progression of diabetic complications. T2D activates the enzymes responsible for maintaining DNA methylation status in the retina, increasing the activities of DNA methyltransferases (DNMTs) and ten-eleven translocation enzymes (Tets) which can lead to hypo- or hypo- DNA methylation of many genes responsible for mitochondrial homeostasis [112-114]. A dynamic DNA methylation process of the matrix metalloproteinase gene *MMP-9* is shown to maintain its transcriptional activation, though the transcription factor binding sites of the *MMP-9* promoter, which are hypermethylated in diabetes. Due to concomitant increased binding of Tet at the same site, *MMP-9* DNA remains hypomethylated resulting in its transcriptional activation [114] and this continues to fuel into the mitochondrial damage [110]. Abrogation of *MMP-9* gene protects against the development of retinopathy and it is considered to play an important role in the apoptosis of retinal capillary cells.

5.3.2. Histone modifications

Histone acetylation and methylation (*HDAs, HATs, SETs* and *LSD1*) play also a crucial role in epigenetic modifications that occur during diabetic retinopathy. However, experimental studies of diabetic retinopathy have provided contradictory results for histone acetylation, with some showing increased global acetylation of retinal histones with activation of *HAT* and inhibition of *HDACs* [115], and others reporting significant increase in retinal histone acetylation [116 117]. The reasons for such discrepancies are still unknown. Although histone modifications and DNA methylation generally regulate gene transcription independently, DNA methylation and histone modifications are also shown to work in concordance; e.g., cooperation of DNA methylation with histone methyltransferase *SETDB1/ESET* results in trimethylation of *H3K9* (known for its function in condensing the chromatin) [110]. DNMTs and histone modifying enzymes, *SUV39h1* and *EZH2* lysine histone methyltransferase, can work in coordination, and the agents that interact with histone methyl transferases, in addition to regulating histone methylation, also regulate DNA methylation [118].

Some of the studies have shown that even after the reverse of the hyperglycemic insult, *H3K4* and *H4K20* at *Sod2* methylation levels continue to be altered. Also, the enzymes responsible for histone methylation (*SUV420h2*) and demethylation (*LSD1*) remain dysfunctional [116]. Upregulation of *MMP-9*, which encodes another enzyme implicated in diabetic retinopathy, was associated with reduced promoter *H3K9me2* and increased *H3K9ac* levels, along with increased recruitment of NF-κB in retinal endothelial cells from diabetic rats [119]. Furthermore, mass spectrometry studies demonstrated that hyperglycaemia causes acetylation of retinal histones, which was associated with increases in proinflammatory proteins [120]. The *HAT p300* was implicated in endothelial fibronectin expression related to diabetic retinopathy, and in gene expression relevant to diabetic cardiac hypertrophy [120].

5.4. Epigenetic modifications in diabetic neuropathy

Diabetic neuropathy is a common but irreversible complication that develops in up to 50% of patients with diabetes and results in sensory loss, pain, and risk of amputation [121]. The molecular mechanisms involved in the development of diabetic neuropathy is a complex process that includes activation of the polyol pathway, exaggerated oxidative stress, over activity of protein kinase C and increased formation of advanced glycation end-products in the presence of hyperglycaemia. Diabetic neuropathy entails decreased nerve conduction velocity, which indicates a prominent role for Schwann cells because they ensheath peripheral nerves and provide support for nerve conduction and axon regeneration [122]. Moreover, high glucose induces oxidative damage in Schwann cells, considered a major factor in diabetic complications [122]. With respect to diabetic neuropa-

thy, several biological mechanisms have been studied, although the role of epigenetics has only recently been suggested [122].

5.4.1. DNA methylation

Previous studies have suggested that diabetic neuropathy involves dysregulation of the transcription factor peroxisome proliferator-activated receptor (*PPRG* or *PPARγ*) [123]. Although drugs that activate *PPARγ* improve glycaemic control in T2D, its role in metabolic memory has not been extensively examined. Because high glucose produces changes in gene expression that persist after cell division [124], a plausible mechanism for these stable changes is DNA methylation. Using quantitative PCR arrays for glucose and fatty acid metabolism, one study found that chronic high glucose induced a persistent increase in genes that promote glycolysis, while inhibiting those that oppose glycolysis and alternate metabolic pathways such as fatty acid metabolism, the pentose phosphate pathway, and trichloroacetic acid cycle [122]. These sustained effects were associated with decreased *PPARγ* binding and persistently increased reactive oxygen species, cellular NADH, and altered DNA methylation [122]. Their results suggest that Schwann cells might exhibit features of metabolic memory that may be regulated at the transcriptional level therefore, targeting *PPAR* may prevent metabolic memory and the development of diabetic complications such as diabetic neuropathy.

5.4.2. Histone modifications

With respect to diabetic neuropathy and histone modifications, little evidence is available. Nevertheless, factors like dyslipidaemia, oxidative stress and inflammation have been reported to be particularly important for the development of neuropathy [125]. Therefore, one could speculate that some of the reported mechanisms linking diabetes with dyslipidaemia, oxidative stress and inflammation through histone modifications, could play a role also in diabetic neuropathy. Nevertheless, future studies are needed to shed light about the role that epigenetic mechanisms such as histone modifications play in diabetic neuropathy.

6. EPIGENETIC BASED BIOMARKERS FOR DIAGNOSIS AND PROGNOSIS OF T2D

Extensive research over the last decades, has shown that our genome is not the only determinant of disease risk, and that epigenetic marks induced by lifestyle and environmental factors are associated with altered gene expression patterns in important tissues, leading to altered susceptibility to disease in later life [8]. Thus, it should be possible to detect these altered epigenetic marks and use them as predictors of future metabolic

capacity, early detection of disease and better prognosis (**Figure 5.2.2**). A premature diagnosis of disease is crucial as it might greatly improve clinical outcomes for patients. For example, population screening for cancers and the surveillance of high-risk patients allows an early diagnosis of cancer and therefore reduces morbidity by using less invasive treatment, resulting in fewer complications and side effects. However, constant screening for a broad range of diseases across whole populations is not routine and realistic and it poses a high economic burden to governments [77]. Hence, the detection of epigenetic alterations has become a promising tool in many health areas for the diagnosis and prognosis of disease and for the prediction of drug response.

6.1 Epigenetic based biomarkers for diagnosis of T2D

Although great progress has been made in the description of epigenetic modifications in normal and diseased tissues, the studies so far have been mainly focused on cancer research. In prostate cancer, DNA hypermethylation at the glutathione S-transferase pi 1 (*GSTP1*) enzyme gene has been suggested as the most relevant candidate biomarker. It has been detected in 69% of proliferative inflammatory atrophy lesions, that are considered precursor lesions for the development of prostate cancer and/or high-grade prostatic intraepithelial neoplasia, underlining its importance for diagnosis [126]. This epigenetic mark was consistently validated in many studies, showing a sensitivity of 82% and a specificity of 95% [127], compared to serum prostate-specific antigen (PSA, 20%), which is the only biomarker currently used for the detection and monitoring of this cancer. However, *GSTP1* can also be hypermethylated in some other types of cancers. Therefore, combinations with additional biomarker genes and PSA testing have been recommended in order to increase specificity. Nevertheless, later studies that combined DNA hypermethylation of *GSTP1, APC, RASSF1, PTGS2, MDR1* and *TIG1* resulted in both sensitivity and specificity up to 100% [128 129]. Furthermore, for the detection of glioblastoma, the hypermethylation at the promoter of the gene O-6-Methylguanine-DNA Methyltransferase (*MGMT*) has been established as a promising biomarker, since it was possible to detect glioblastomas with a very high sensitivity and specificity (95% and 60% respectively) in serum samples [130]. Also, researchers were able to predict a lack of cancer progression and overall survival of the patient [130]. A step further, in colorectal cancer (CRC), the Food and Drugs Administration (FDA) has already approved the use of epigenetic markers like *SEPT9* (ColoVantage) and vimentin (ColoSure)[131] in clinical practice, after showing a high sensitivity and a specificity for CRC diagnosis [132 133].

As the field has grown, efforts are made also in discovering candidate biomarkers in the diagnosis and prognosis of other types of diseases such as type 2 diabetes, but this research field is still in its early phases. Twin studies might be a powerful approach as, despite the identical genetic background, discordant twins for T2D have differences in DNA methylation [134 135].

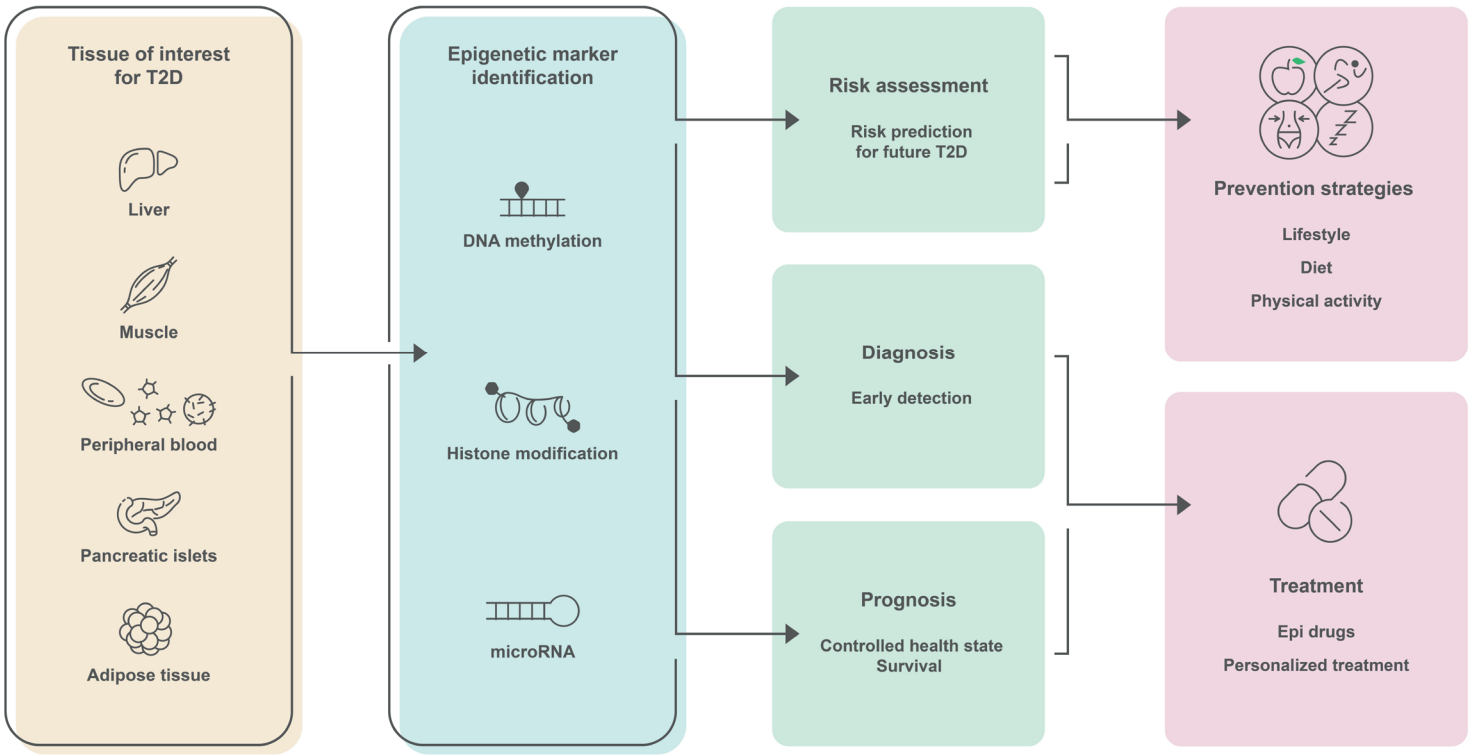


Figure 5.2.2. Characteristic epigenetic patterns for T2D and glycemic traits can be investigated in key tissues for glucose homeostasis and energy balance in the body. After the identification of an epigenetic signature, it can be used as a biomarker for the detection of subjects at high risk of developing T2D, before the onset of the disease. Thus, the patient can receive healthcare advice and is still on time to implement strategies in order to prevent the progression towards T2D. A biomarker can also be of use after the onset of the disease, to make a more accurate diagnose, to choose the best treatment strategy and to monitor the progression of T2D and its complications in order to augment patient´s survival and reduce co-morbidities.

Recently a new classification of diabetes types has been proposed. Using a data-driven cluster analysis approach, newly diagnosed diabetic patients were stratified into five subgroups with differing disease progression and risk of diabetic complications [136]. The study identified five replicable clusters of patients with diabetes, who had significantly different patient characteristics and risk of diabetic complications. Cluster 1 included severe autoimmune diabetes patients; cluster 2 included patients with severe insulin-deficient diabetes; cluster 3 included severe insulin-resistant diabetes patients; cluster 4 was labelled as mild obesity-related diabetes and cluster 5 was labelled as mild age-related diabetes. They found that individuals in cluster 3 had significantly higher risk of diabetic kidney disease than individuals in clusters 4 and 5, but had been prescribed similar diabetes treatment. While, individuals in cluster 2 had the highest risk of retinopathy compared to the other clusters. Moreover, genetic associations in the clusters differed from those seen in traditional T2D [136]. Considering the high heterogeneity of T2D, epigenetics might be a promising path that could help to better disentangle the differences in diabetic phenotypes, which would provide more information on treatment target, as well in prevention strategies individualized by diabetes type, thus evolving towards personalized medicine.

In type 1 diabetes mellitus, recent findings propose the circulating β cell-derived unmethylated insulin (*INS*) DNA as a potential diagnostic tool for the early detection of type 1 diabetes (T1D) [137]. This is based in previous evidence showing that the autoimmune destruction conducted by immune cells in T1D leads to the release of unmethylated *INS* DNA from pancreatic β cells, into the circulation. Thus it is possible the detection of the circulating DNA in blood samples, and the consequent assessment of the unique methylation pattern present in the promotor of the *INS* gene in β cells from patients with early T1D, or in patients with islet transplantation therapy [138].

6.2 Epigenetic based biomarkers for prognosis of T2D

In addition to its diagnostic potential, DNA methylation could be informative for patient prognosis in terms of disease progression/recurrence, complications, treatment and survival. High resolution data usually used for cancer, subtypes-specific profiles can also be used for the identification of powerful single epigenetic biomarker genes and gene combinations at various stages of disease. This advanced screening strategy can be beneficial for many types of clinical applications.

Initially, the potential prognostic profile of DNA methylation has been reported in childhood acute lymphoblastic leukaemia [139]. DNA methylation profiling classified these patients in lymphoblastic leukaemia subtypes and stratified them with high hyperdiploidy and translocation t(12;21) into two subgroups with different probabilities of relapse. DNA methylation profiling therefore resulted in subtype-specific and predictive signatures with potential use as future prognostic biomarkers for this disease [139]. In breast

cancer, DNA methylation profiling identified a previously unrecognized subtype associated with T lymphocyte infiltration. Importantly, profiling immune genes determined a prognostic value of the profile. In particular, the hypermethylation at the promoter of lymphocyte transmembrane adaptor 1 (*LAX1*) and *CD3D* significantly correlated with survival in certain breast cancer subtypes [140]. Another study discovered and validated the epigenetic signature of *NEFH* and *HS3ST2* as an independent prognostic factor for type II ovarian cancer [141]. Moreover, they showed that 3-0 sulfation of HS was important to oncogenic signalling, such as IL-6 and EGF signalling, which could render useless current targeted therapies for ovarian cancer without further patient stratification [141].

However, when it comes to the role of epigenetics used as a prognostic marker for T2D the data are very limited. Recent published studies have suggested that the differentially methylated circulating DNA might be a promising novel biomarker which reflects beta cell death and could predict the progression of diabetes [142]. The novel beta cell death marker unmethylated insulin (*INS*) DNA has been studied in TPIAT (total pancreatectomy with islet autotransplantation) patients before and immediately after islet infusion, and also 90 days post-TPIAT concurrent with metabolic functional assessments [143]. Universal early elevations in the beta cell death marker *INS* DNA after TPAIT were observed, with pronounced elevations in the islet supernatant pre-infusion, likely reflecting beta cell death induced by islet isolation. In addition, persistent post-transplant elevation of INS DNA predicted greater hyperglycaemia at 90 days [143]. Although, more studies are needed to identify the best methylation target sites in the *INS* gene, differentially methylated circulating DNA may be a good method to evaluate progression, diagnosis and prognosis of islet related diseases and in diabetes patients for whom insulin production is impaired.

Aging is also an important risk factor for metabolic disorders, including obesity, impaired glucose tolerance, and T2D [144]. Almost one third of the elderly in the United States of America have diabetes and three quarters have diabetes or prediabetes [145]. Recent attempts have been done in identifying biomarkers of aging, which are thought of as individual-level measures of aging that capture inter-individual differences in the timing of disease onset, functional decline and death over the life course. In this context, DNA methylation has gained a lot of interest as a potential biomarker that could predict mortality [146]. Successfully, a DNA methylation based biomarker of aging (PhenoAge) was developed, which is highly predictive of nearly every morbidity and mortality outcome tested, especially cardiovascular disease and coronary heart disease . In addition, the study observed that higher DNA methylation PhenoAge was associated with increases in the activation of proinflammatory pathways, such as NF-κB, increased interferon signalling and decreases in damage recognition and repair pathways [146]. Given this, one could hypothesize that DNA methylation PhenoAge might have an influence also on T2D. Future studies could test this hypothesis in diabetes patients which would provide

some insights on whether methylation age of specific methylation sites would identify T2D patients who are at risk of early death.

7. CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

Epigenetics promises an auspicious future in its role as clinical instrument to complement the current practice. Current evidence in the field suggests that study of the role of epigenetic changes in the onset and progression of the diseases is a promising opportunity for the development of prediction, diagnostic and disease progression monitoring tools, as well as novel therapeutic targets (**Figure 5.2.2**). Overall, changes in global DNA methylation, CpG islands methylation, and histone acetylation and methylation have been found in relevant genes for aorta, heart, kidney, retina, nerves and glia cells function in samples from diabetic human and rodent donors or after hyperglycemic stimuli of the same type of tissues, albeit some of them exhibit contrary results. However, since epigenetic changes have shown to be dynamic and vary in response of environmental stimulations, epigenetic mechanisms are important to study also because it is believed that they may play a significant role in the reversibility and treatment of diabetic complications, contributing even as a protective mechanism against them, though which drugs and other therapeutic tools may exert their effect. Furthermore, not solely for risk prediction, epigenetic changes also arise as a promising tool in the diagnosis, stratification of the patients, prognosis and monitoring of therapy response. Various research methodologies and DNA methylation assessment methods have been used to disentangle the epigenetic network; however, the technological advances in DNA methylation arrays have permitted EWAS approach to analyze a higher number of CpGs, a larger samples size and a more standardized method to assess DNA methylation. Nevertheless, identifying optimal methods of detecting possible epigenetic biomarkers and implementing appropriate reference standards are rapidly evolving. The use of common plans in the study designs, methylation assessment methods and statistical models for data analysis with similar strategies for confounding control, would allow comparability and independent validation of the findings, thus better quality evidence to identify biomarkers for T2D precision medicine, reliable for both clinicians and the patients.

REFERENCES

1. Zghebi SS, Steinke DT, Carr MJ, et al. Examining trends in type 2 diabetes incidence, prevalence and mortality in the UK between 2004 and 2014. Diabetes Obes Metab 2017;**19**(11):1537-45.
2. Wild S, Roglic G, Green A, et al. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004;**27**(5):1047-53.
3. Federation ID. *IDF Diabetes Atlas, 8th edn*. Brussels, Belgium: International Diabetes Federation, 2017. 2017.
4. Animaw W, Seyoum Y. Increasing prevalence of diabetes mellitus in a developing country and its related factors. PLoS One 2017;**12**(11):e0187670.
5. Guthrie RA, Guthrie DW. Pathophysiology of diabetes mellitus. Crit Care Nurs Q 2004;**27**(2):113-25.
6. American Diabetes A. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2018. Diabetes Care 2018;**41**(Suppl 1):S13-S27.
7. Bonora E, Tuomilehto J. The Pros and Cons of Diagnosing Diabetes With A1C. Diabetes Care 2011;**34**:S184-S90.
8. Gilbert ER, Liu D. Epigenetics: the missing link to understanding beta-cell dysfunction in the pathogenesis of type 2 diabetes. Epigenetics 2012;**7**(8):841-52.
9. Volkmar M, Dedeurwaerder S, Cunha DA, et al. DNA methylation profiling identifies epigenetic dysregulation in pancreatic islets from type 2 diabetic patients. Embo J 2012;**31**(6):1405-26.
10. Bonora E, Tuomilehto J. The pros and cons of diagnosing diabetes with A1C. Diabetes Care 2011;**34 Suppl 2**:S184-90.
11. International Expert C. International Expert Committee report on the role of the A1C assay in the diagnosis of diabetes. Diabetes Care 2009;**32**(7):1327-34.
12. Zhuo X, Zhang P, Selvin E, et al. Alternative HbA1c cutoffs to identify high-risk adults for diabetes prevention: a cost-effectiveness perspective. Am J Prev Med 2012;**42**(4):374-81.
13. Tabak AG, Jokela M, Akbaraly TN, et al. Trajectories of glycaemia, insulin sensitivity, and insulin secretion before diagnosis of type 2 diabetes: an analysis from the Whitehall II study. Lancet 2009;**373**(9682):2215-21.
14. Weisenberger DJ, Campan M, Long TI, et al. Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 2005;**33**(21):6823-36.
15. Muka T, Nano J, Voortman T, et al. The role of global and regional DNA methylation and histone modifications in glycemic traits and type 2 diabetes: A systematic review. Nutr Metab Cardiovas 2016;**26**(7):553-66.
16. Ribel-Madsen R, Fraga MF, Jacobsen S, et al. Genome-wide analysis of DNA methylation differences in muscle and fat from monozygotic twins discordant for type 2 diabetes. PLoS One 2012;**7**(12):e51302.
17. Keller M, Kralisch S, Rohde K, et al. Global DNA methylation levels in human adipose tissue are related to fat distribution and glucose homeostasis. Diabetologia 2014;**57**(11):2374-83.
18. Simar D, Versteyhe S, Donkin I, et al. DNA methylation is altered in B and NK lymphocytes in obese and type 2 diabetic human. Metabolism 2014;**63**(9):1188-97.
19. Pearce MS, McConnell JC, Potter C, et al. Global LINE-1 DNA methylation is associated with blood glycaemic and lipid profiles. Int J Epidemiol 2012;**41**(1):210-17.
20. Canivell S, Ruano EG, Siso-Almirall A, et al. Gastric Inhibitory Polypeptide Receptor Methylation in Newly Diagnosed, Drug-Naive Patients with Type 2 Diabetes: A Case-Control Study. Plos One 2013;**8**(9).

21. Gillberg L, Jacobsen S, Ribel-Madsen R, et al. Does DNA methylation of PPARGC1A influence insulin action in first degree relatives of patients with type 2 diabetes? Diabetologia 2012;**55**:S130-S30.
22. Hall E, Dekker Nitert M, Volkov P, et al. The effects of high glucose exposure on global gene expression and DNA methylation in human pancreatic islets. Mol Cell Endocrinol 2017.
23. Su SY, Zhu HD, Xu XJ, et al. DNA Methylation of the LY86 Gene is Associated With Obesity, Insulin Resistance, and Inflammation. Twin Res Hum Genet 2014;**17**(3):183-91.
24. Bouchard L, Hivert MF, Guay SP, et al. Placental adiponectin gene DNA methylation levels are associated with mothers' blood glucose concentration. Diabetes 2012;**61**(5):1272-80.
25. Xie XM, Gao HJ, Zeng WJ, et al. Placental DNA methylation of peroxisome-proliferator-activated receptor-gamma co-activator-1 alpha promoter is associated with maternal gestational glucose level. Clin Sci 2015;**129**(4):385-94.
26. Desgagne V, Hivert MF, St-Pierre J, et al. Epigenetic dysregulation of the IGF system in placenta of newborns exposed to maternal impaired glucose tolerance. Epigenomics 2014;**6**(2):193-207.
27. Hidalgo B, Irvin MR, Sha J, et al. Epigenome-wide association study of fasting measures of glucose, insulin, and HOMA-IR in the Genetics of Lipid Lowering Drugs and Diet Network study. Diabetes 2014;**63**(2):801-7.
28. Kriebel J, Herder C, Rathmann W, et al. Association between DNA Methylation in Whole Blood and Measures of Glucose Metabolism: KORA F4 Study. Plos One 2016;**11**(3).
29. Lempradl A, Pospisilik JA, Penninger JM. MODES OF TRANSCRIPTIONAL REGULATION Exploring the emerging complexity in transcriptional regulation of energy homeostasis. Nat Rev Genet 2015;**16**(11):665-81.
30. Chambers JC, Loh M, Lehne B, et al. Epigenome-wide association of DNA methylation markers in peripheral blood from Indian Asians and Europeans with incident type 2 diabetes: a nested case-control study. Lancet Diabetes Endocrinol 2015;**3**(7):526-34.
31. Cha-Molstad H, Saxena G, Chen JQ, et al. Glucose-stimulated Expression of Txnip Is Mediated by Carbohydrate Response Element-binding Protein, p300, and Histone H4 Acetylation in Pancreatic Beta Cells. J Biol Chem 2009;**284**(25):16898-905.
32. Bompada P, Atac D, Luan C, et al. Histone acetylation of glucose-induced thioredoxin-interacting protein gene expression in pancreatic islets. Int J Biochem Cell B 2016;**81**:82-91.
33. Samuel VT, Shulman GI. The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. J Clin Invest 2016;**126**(1):12-22.
34. Piyathilake CJ, Badiga S, Alvarez RD, et al. A Lower Degree of PBMC L1 Methylation Is Associated with Excess Body Weight and Higher HOMA-IR in the Presence of Lower Concentrations of Plasma Folate. Plos One 2013;**8**(1).
35. Zhao JY, Goldberg J, Bremner JD, et al. Global DNA Methylation Is Associated With Insulin Resistance A Monozygotic Twin Study. Diabetes 2012;**61**(2):542-46.
36. Simar D, Versteyhe S, Donkin I, et al. DNA methylation is altered in B and NK lymphocytes in obese and type 2 diabetic human. Metabolism-Clinical and Experimental 2014;**63**(9):1188-97.
37. Manning AK, Hivert MF, Scott RA, et al. A genome-wide approach accounting for body mass index identifies genetic variants influencing fasting glycemic traits and insulin resistance. Nat Genet 2012;**44**(6):659-U81.
38. Kulkarni H, Kos MZ, Neary J, et al. Novel epigenetic determinants of type 2 diabetes in Mexican-American families. Hum Mol Genet 2015;**24**(18):5330-44.
39. Hidalgo B, Irvin MR, Sha J, et al. Epigenome-Wide Association Study of Fasting Measures of Glucose, Insulin, and HOMA-IR in the Genetics of Lipid Lowering Drugs and Diet Network Study. Diabetes 2014;**63**(2):801-07.

40. Das S, Dey JK, Prabhu N, et al. Association Between 5-HTR2C-759C/T (rs3813929) and-697G/C (rs518147) Gene Polymorphisms and Risperidone-Induced Insulin Resistance Syndrome in an Indian Population (Retraction of, vol 57, 2017) (Retraction of Vol 57, 10.1002/JCPH.1012, 2017). J Clin Pharmacol 2018;**58**(3):399-99.
41. Gillberg L, Jacobsen S, Ribel-Madsen R, et al. Does DNA Methylation of PPARGC1A Influence Insulin Action in First Degree Relatives of Patients with Type 2 Diabetes? Plos One 2013;**8**(3).
42. Maier B, Thimme W, Kallischnigg G, et al. Does diabetes mellitus explain the higher hospital mortality of women with acute myocardial infarction? Results from the Berlin Myocardial Infarction Registry. J Investig Med 2006;**54**(3):143-51.
43. Kabra DG, Gupta J, Tikoo K. Insulin induced alteration in post-translational modifications of histone H3 under a hyperglycemic condition in L6 skeletal muscle myoblasts. Biochim Biophys Acta 2009;**1792**(6):574-83.
44. Uusitupa M. Gene-diet interaction in relation to the prevention of obesity and type 2 diabetes: evidence from the Finnish Diabetes Prevention Study. Nutr Metab Cardiovasc Dis 2005;**15**(3):225-33.
45. Ling C, Groop L. Epigenetics: a molecular link between environmental factors and type 2 diabetes. Diabetes 2009;**58**(12):2718-25.
46. Zhang H, Cai X, Yi B, et al. Correlation of CTGF gene promoter methylation with CTGF expression in type 2 diabetes mellitus with or without nephropathy. Mol Med Rep 2014;**9**(6):2138-44.
47. Kato S, Lindholm B, Stenvinkel P, et al. DNA hypermethylation and inflammatory markers in incident Japanese dialysis patients. Nephron Extra 2012;**2**(1):159-68.
48. Luttmer R, Spijkerman AM, Kok RM, et al. Metabolic syndrome components are associated with DNA hypomethylation. Obes Res Clin Pract 2013;**7**(2):e106-e15.
49. Gu HF, Gu T, Hilding A, et al. Evaluation of IGFBP-7 DNA methylation changes and serum protein variation in Swedish subjects with and without type 2 diabetes. Clin Epigenetics 2013;**5**(1):20.
50. Gu T, Gu HF, Hilding A, et al. Increased DNA methylation levels of the insulin-like growth factor binding protein 1 gene are associated with type 2 diabetes in Swedish men. Clin Epigenetics 2013;**5**(1):21.
51. Remely M, Aumueller E, Jahn D, et al. Microbiota and epigenetic regulation of inflammatory mediators in type 2 diabetes and obesity. Benef Microbes 2014;**5**(1):33-43.
52. Seman NA, Mohamud WN, Ostenson CG, et al. Increased DNA methylation of the SLC30A8 gene promoter is associated with type 2 diabetes in a Malay population. Clin Epigenetics 2015;**7**:30.
53. Tang L, Ye H, Hong Q, et al. Elevated CpG island methylation of GCK gene predicts the risk of type 2 diabetes in Chinese males. Gene 2014;**547**(2):329-33.
54. Zou L, Yan S, Guan X, et al. Hypermethylation of the PRKCZ Gene in Type 2 Diabetes Mellitus. J Diabetes Res 2013;**2013**:721493.
55. Ling C, Del Guerra S, Lupi R, et al. Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion. Diabetologia 2008;**51**(4):615-22.
56. Yang BT, Dayeh TA, Volkov PA, et al. Increased DNA methylation and decreased expression of PDX-1 in pancreatic islets from patients with type 2 diabetes. Mol Endocrinol 2012;**26**(7):1203-12.
57. Yang BT, Dayeh TA, Kirkpatrick CL, et al. Insulin promoter DNA methylation correlates negatively with insulin gene expression and positively with HbA(1c) levels in human pancreatic islets. Diabetologia 2011;**54**(2):360-7.
58. Jun Z, Wangqiang Z, Yulei D, et al. [Correlation between type 2 diabetes and DNA methylation and mRNA expression of APN in abdominal adipose tissues in Xinjiang Uygur population]. Yi Chuan 2015;**37**(3):269-75.

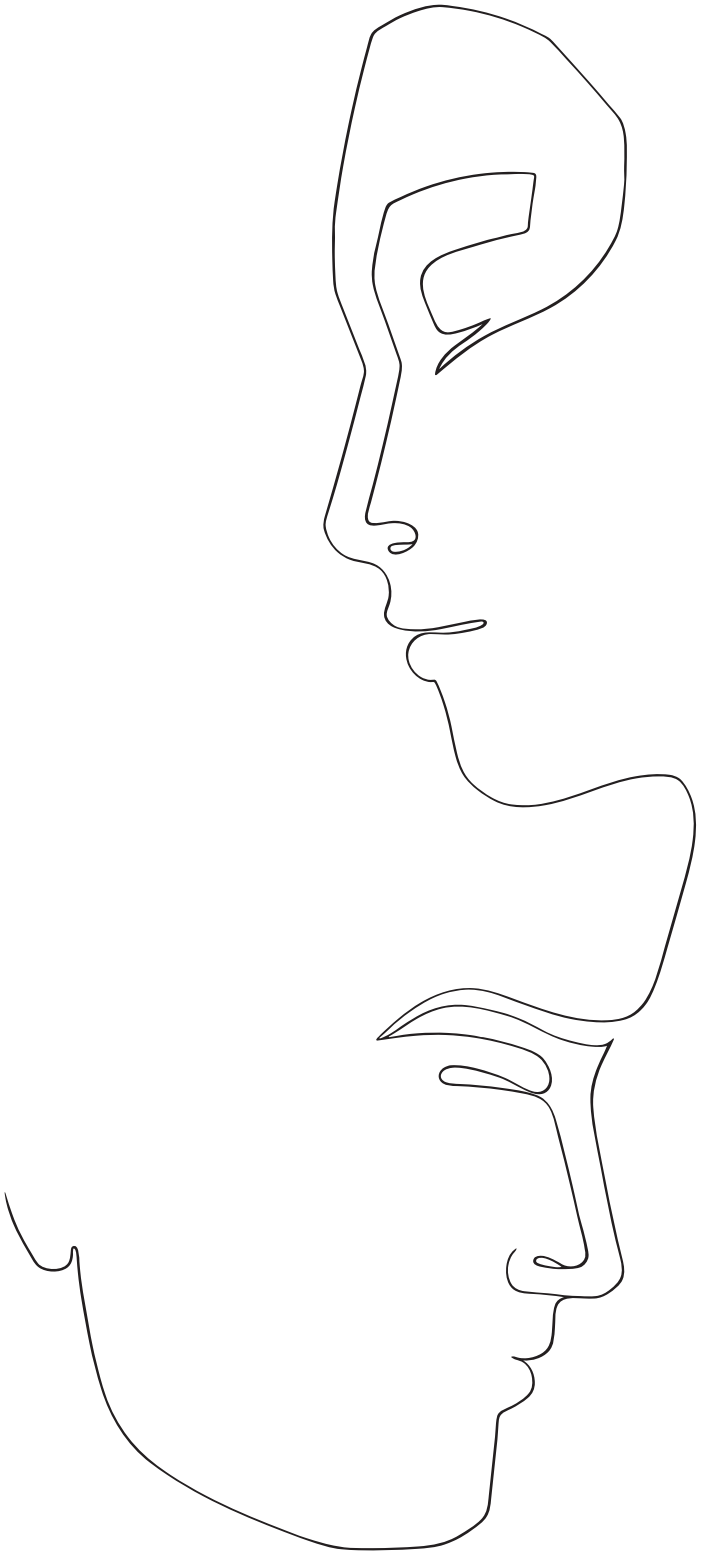
59. Canivell S, Ruano EG, Siso-Almirall A, et al. Gastric inhibitory polypeptide receptor methylation in newly diagnosed, drug-naive patients with type 2 diabetes: a case-control study. PLoS One 2013;**8**(9):e75474.
60. Cheng J, Tang L, Hong Q, et al. Investigation into the promoter DNA methylation of three genes (CAMK1D, CRY2 and CALM2) in the peripheral blood of patients with type 2 diabetes. Exp Ther Med 2014;**8**(2):579-84.
61. Liu ZH, Chen LL, Deng XL, et al. Methylation status of CpG sites in the MCP-1 promoter is correlated to serum MCP-1 in Type 2 diabetes. J Endocrinol Invest 2012;**35**(6):585-9.
62. Tros F, Meirhaeghe A, Hadjadj S, et al. Hypomethylation of the promoter of the catalytic subunit of protein phosphatase 2A in response to hyperglycemia. Physiol Rep 2014;**2**(7).
63. Kulkarni SS, Salehzadeh F, Fritz T, et al. Mitochondrial regulators of fatty acid metabolism reflect metabolic dysfunction in type 2 diabetes mellitus. Metabolism 2012;**61**(2):175-85.
64. Canivell S, Ruano EG, Siso-Almirall A, et al. Differential methylation of TCF7L2 promoter in peripheral blood DNA in newly diagnosed, drug-naive patients with type 2 diabetes. PLoS One 2014;**9**(6):e99310.
65. Ma J, Cheng J, Wang L, et al. No association between IRS1 promoter methylation and type 2 diabetes. Mol Med Rep 2013;**8**(3):949-53.
66. Hall E, Dayeh T, Kirkpatrick CL, et al. DNA methylation of the glucagon-like peptide 1 receptor (GLP1R) in human pancreatic islets. BMC Med Genet 2013;**14**:76.
67. Gillberg L, Jacobsen SC, Ribel-Madsen R, et al. Does DNA methylation of PPARGC1A influence insulin action in first degree relatives of patients with type 2 diabetes? PLoS One 2013;**8**(3):e58384.
68. Dayeh T, Volkov P, Salo S, et al. Genome-wide DNA methylation analysis of human pancreatic islets from type 2 diabetic and non-diabetic donors identifies candidate genes that influence insulin secretion. PLoS Genet 2014;**10**(3):e1004160.
69. Kulkarni H, Kos MZ, Neary J, et al. Novel epigenetic determinants of type 2 diabetes in Mexican-American families. Hum Mol Genet 2015;**24**(18):5330-44.
70. Toperoff G, Aran D, Kark JD, et al. Genome-wide survey reveals predisposing diabetes type 2-related DNA methylation variations in human peripheral blood. Hum Mol Genet 2012;**21**(2):371-83.
71. Strahl BD, Allis CD. The language of covalent histone modifications. Nature 2000;**403**(6765):41-5.
72. Sims RJ, 3rd, Nishioka K, Reinberg D. Histone lysine methylation: a signature for chromatin function. Trends Genet 2003;**19**(11):629-39.
73. Paneni F, Costantino S, Battista R, et al. Adverse epigenetic signatures by histone methyltransferase Set7 contribute to vascular dysfunction in patients with type 2 diabetes mellitus. Circ Cardiovasc Genet 2015;**8**(1):150-8.
74. Miao F, Wu X, Zhang L, et al. Genome-wide analysis of histone lysine methylation variations caused by diabetic conditions in human monocytes. J Biol Chem 2007;**282**(18):13854-63.
75. Hou C, Zhao M, Li X, et al. [Histone H3 acetylation of tumor necrosis factor-alpha and cyclooxygenase-2 in patients with type 2 diabetes]. Zhonghua Yi Xue Za Zhi 2011;**91**(26):1805-8.
76. Roglic G. WHO Global report on diabetes: A summary. International Journal of Noncommunicable Diseases 2016;**1**(1):3-8.
77. Muka T, Imo D, Jaspers L, et al. The global impact of non-communicable diseases on healthcare spending and national income: a systematic review. European Journal of Epidemiology 2015;**30**(4):251-77.
78. Noble D, Mathur R, Dent T, et al. Risk models and scores for type 2 diabetes: systematic review. BMJ 2011;**343**:d7163.

79. Meigs JB, Shrader P, Sullivan LM, et al. Genotype score in addition to common risk factors for prediction of type 2 diabetes. N Engl J Med 2008;**359**(21):2208-19.
80. Talmud PJ, Hingorani AD, Cooper JA, et al. Utility of genetic and non-genetic risk factors in prediction of type 2 diabetes: Whitehall II prospective cohort study. BMJ 2010;**340**:b4838.
81. Stancakova A, Laakso M. Genetics of Type 2 Diabetes. Endocr Dev 2016;**31**:203-20.
82. Martin-Nunez GM, Rubio-Martin E, Cabrera-Mulero R, et al. Type 2 diabetes mellitus in relation to global LINE-1 DNA methylation in peripheral blood: a cohort study. Epigenetics 2014;**9**(10):1322-8.
83. Dayeh T, Tuomi T, Almgren P, et al. DNA methylation of loci within ABCG1 and PHOSPHO1 in blood DNA is associated with future type 2 diabetes risk. Epigenetics 2016;**11**(7):482-8.
84. Andersen GS, Thybo T, Cederberg H, et al. The DEXLIFE study methods: identifying novel candidate biomarkers that predict progression to type 2 diabetes in high risk individuals. Diabetes Res Clin Pract 2014;**106**(2):383-9.
85. Kannel WB, McGee DL. Diabetes and cardiovascular disease. The Framingham study. JAMA 1979;**241**(19):2035-8.
86. Kim JA, Koh KK, Quon MJ. The union of vascular and metabolic actions of insulin in sickness and in health. Arterioscl Throm Vas 2005;**25**(5):889-91.
87. Paneni F, Mocharla P, Akhmedov A, et al. Gene silencing of the mitochondrial adaptor p66(Shc) suppresses vascular hyperglycemic memory in diabetes. Circ Res 2012;**111**(3):278-89.
88. Abi Khalil C. The emerging role of epigenetics in cardiovascular disease. Ther Adv Chronic Dis 2014;**5**(4):178-87.
89. Monkemann H, De Vriese AS, Blom HJ, et al. Early molecular events in the development of the diabetic cardiomyopathy. Amino Acids 2002;**23**(1-3):331-6.
90. Deodati A, Inzaghi E, Liguori A, et al. IGF2 Methylation Is Associated with Lipid Profile in Obese Children. Horm Res Paediat 2013;**79**(6):361-67.
91. Arner P, Sahlqvist AS, Sinha I, et al. The epigenetic signature of systemic insulin resistance in obese women (vol 59, pg 2393, 2016). Diabetologia 2016;**59**(12):2728-28.
92. Lou XD, Wang HD, Xia SJ, et al. Effects of Resveratrol on the Expression and DNA Methylation of Cytokine Genes in Diabetic Rat Aortas. Arch Immunol Ther Ex 2014;**62**(4):329-40.
93. Madsbad S. Liraglutide Effect and Action in Diabetes (LEAD™) trial. Expert Review of Endocrinology & Metabolism 2009;**4**(2):119-29.
94. Hall E, Dayeh T, Kirkpatrick CL, et al. DNA methylation of the glucagon-like peptide 1 receptor (GLP1R) in human pancreatic islets. Bmc Med Genet 2013;**14**.
95. Gaikwad AB, Gupta J, Tikoo K. Epigenetic changes and alteration of Fbn1 and Col3A1 gene expression under hyperglycaemic and hyperinsulinaemic conditions. Biochem J 2010;**432**(2):333-41.
96. Sathishkumar C, Prabu P, Balakumar M, et al. Augmentation of histone deacetylase 3 (HDAC3) epigenetic signature at the interface of proinflammation and insulin resistance in patients with type 2 diabetes. Clin Epigenetics 2016;**8**.
97. Pirola L, Balcerczyk A, Tothill RW, et al. Genome-wide analysis distinguishes hyperglycemia regulated epigenetic signatures of primary vascular cells. Genome Research 2011;**21**(10):1601-15.
98. Brasacchio D, Okabe J, Tikellis C, et al. Hyperglycemia Induces a Dynamic Cooperativity of Histone Methylase and Demethylase Enzymes Associated With Gene-Activating Epigenetic Marks That Coexist on the Lysine Tail. Diabetes 2009;**58**(5):1229-36.
99. Miao F, Chen Z, Genuth S, et al. Evaluating the role of epigenetic histone modifications in the metabolic memory of type 1 diabetes. Diabetes 2014;**63**(5):1748-62.

100. Jones CA, Krolewski AS, Rogus J, et al. Epidemic of end-stage renal disease in people with diabetes in the United States population: Do we know the cause? Kidney Int 2005;**67**(5):1684-91.
101. Kato M, Natarajan R. Diabetic nephropathy-emerging epigenetic mechanisms. Nat Rev Nephrol 2014;**10**(9):517-30.
102. Reddy MA, Park JT, Natarajan R. Epigenetic Modifications in the Pathogenesis of Diabetic Nephropathy. Semin Nephrol 2013;**33**(4):341-53.
103. Bell CG, Teschendorff AE, Rakyan VK, et al. Genome-wide DNA methylation analysis for diabetic nephropathy in type 1 diabetes mellitus. Bmc Med Genomics 2010;**3**.
104. Brennan EP, Ehrich M, O'Donovan H, et al. DNA methylation profiling in cell models of diabetic nephropathy. Epigenetics 2010;**5**(5):396-401.
105. Sapienza C, Lee J, Powell J, et al. DNA methylation profiling identifies epigenetic differences between diabetes patients with ESRD and diabetes patients without nephropathy. Epigenetics 2011;**6**(1):20-28.
106. Smyth LJ, McKay GJ, Maxwell AP, et al. DNA hypermethylation and DNA hypomethylation is present at different loci in chronic kidney disease. Epigenetics 2014;**9**(3):366-76.
107. Yuan H, Reddy MA, Sun G, et al. Involvement of p300/CBP and epigenetic histone acetylation in TGF-beta1-mediated gene transcription in mesangial cells. Am J Physiol Renal Physiol 2013;**304**(5):F601-13.
108. Li Y, Reddy MA, Miao F, et al. Role of the histone H3 lysine 4 methyltransferase, SET7/9, in the regulation of NF-kappaB-dependent inflammatory genes. Relevance to diabetes and inflammation. J Biol Chem 2008;**283**(39):26771-81.
109. Reddy MA, Sumanth P, Lanting LT, et al. Losartan reverses permissive epigenetic changes in renal glomeruli of diabetic db/db mice. Kidney International 2014;**85**(2):362-73.
110. Kowluru RA. Diabetic retinopathy, metabolic memory and epigenetic modifications. Vision Res 2017;**139**:30-38.
111. Diabetes C, Complications Trial/Epidemiology of Diabetes I, Complications Research G, et al. Retinopathy and nephropathy in patients with type 1 diabetes four years after a trial of intensive therapy. N Engl J Med 2000;**342**(6):381-9.
112. Tewari S, Zhong Q, Santos JM, et al. Mitochondria DNA replication and DNA methylation in the metabolic memory associated with continued progression of diabetic retinopathy. Invest Ophthalmol Vis Sci 2012;**53**(8):4881-8.
113. Mishra M, Kowluru RA. Epigenetic Modification of Mitochondrial DNA in the Development of Diabetic Retinopathy. Invest Ophthalmol Vis Sci 2015;**56**(9):5133-42.
114. Kowluru RA, Shan Y, Mishra M. Dynamic DNA methylation of matrix metalloproteinase-9 in the development of diabetic retinopathy. Lab Invest 2016;**96**(10):1040-9.
115. Mishra M, Zhong Q, Kowluru RA. Epigenetic modifications of Nrf2-mediated glutamate-cysteine ligase: implications for the development of diabetic retinopathy and the metabolic memory phenomenon associated with its continued progression. Free Radic Biol Med 2014;**75**:129-39.
116. Zhong Q, Kowluru RA. Epigenetic modification of Sod2 in the development of diabetic retinopathy and in the metabolic memory: role of histone methylation. Invest Ophthalmol Vis Sci 2013;**54**(1):244-50.
117. Kadiyala CS, Zheng L, Du Y, et al. Acetylation of retinal histones in diabetes increases inflammatory proteins: effects of minocycline and manipulation of histone acetyltransferase (HAT) and histone deacetylase (HDAC). J Biol Chem 2012;**287**(31):25869-80.
118. Cedar H, Bergman Y. Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet 2009;**10**(5):295-304.

119. Zhong Q, Kowluru RA. Regulation of Matrix Metalloproteinase-9 by Epigenetic Modifications and the Development of Diabetic Retinopathy. Diabetes 2013;**62**(7):2559-68.
120. Reddy MA, Zhang EL, Natarajan R. Epigenetic mechanisms in diabetic complications and metabolic memory. Diabetologia 2015;**58**(3):443-55.
121. Zochodne DW. Diabetes mellitus and the peripheral nervous system: Manifestations and mechanisms. Muscle Nerve 2007;**36**(2):144-66.
122. Kim ES, Isoda F, Kurland I, et al. Glucose-Induced Metabolic Memory in Schwann Cells: Prevention by PPAR Agonists. Endocrinology 2013;**154**(9):3054-66.
123. Pande M, Hur J, Hong Y, et al. Transcriptional Profiling of Diabetic Neuropathy in the BKS db/db Mouse A Model of Type 2 Diabetes. Diabetes 2011;**60**(7):1981-89.
124. El-Osta A, Brasacchio D, Yao D, et al. Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia (vol 205, pg 2409, 2008). J Exp Med 2008;**205**(11):2683-83.
125. Yagihashi S, Mizukami H, Sugimoto K. Mechanism of diabetic neuropathy: Where are we now and where to go? J Diabetes Invest 2011;**2**(1):18-32.
126. Nakayama M, Bennett CJ, Hicks JL, et al. Hypermethylation of the human glutathione S-transferase-pi gene (GSTP1) CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate - A detailed study using laser-capture-microdissection. Am J Pathol 2003;**163**(3):923-33.
127. Van Neste L, Herman JG, Otto G, et al. The epigenetic promise for prostate cancer diagnosis. Prostate 2012;**72**(11):1248-61.
128. Yegnasubramanian S, Kowalski J, Gonzalgo ML, et al. Hypermethylation of CpG islands in primary and metastatic human prostate cancer. Cancer Res 2004;**64**(6):1975-86.
129. Ellinger J, Bastian PJ, Jurgan T, et al. CpG island hypermethylation at multiple gene sites in diagnosis and prognosis of prostate cancer. Urology 2008;**71**(1):161-67.
130. Balana C, Carrato C, Ramirez JL, et al. Tumour and serum MGMT promoter methylation and protein expression in glioblastoma patients. Clin Transl Oncol 2011;**13**(9):677-85.
131. Gyparaki MT, Basdra EK, Papavassiliou AG. DNA methylation biomarkers as diagnostic and prognostic tools in colorectal cancer. J Mol Med 2013;**91**(11):1249-56.
132. Warren JD, Xiong W, Bunker AM, et al. Septin 9 methylated DNA is a sensitive and specific blood test for colorectal cancer. Bmc Medicine 2011;**9**.
133. Lofton-Day C, Model F, DeVos T, et al. DNA methylation biomarkers for blood-based colorectal cancer screening. Clinical Chemistry 2008;**54**(2):414-23.
134. Fraga MF, Ballestar E, Paz MF, et al. Epigenetic differences arise during the lifetime of monozygotic twins. P Natl Acad Sci USA 2005;**102**(30):10604-09.
135. Rakyan VK, Beyan H, Down TA, et al. Identification of Type 1 Diabetes-Associated DNA Methylation Variable Positions That Precede Disease Diagnosis. Plos Genetics 2011;**7**(9).
136. Ahlqvist E, Storm P, Karajamaki A, et al. Novel subgroups of adult-onset diabetes and their association with outcomes: a data-driven cluster analysis of six variables. Lancet Diabetes Endocrinol 2018;**6**(5):361-69.
137. Zhang K, Lin G, Han Y, et al. Circulating unmethylated insulin DNA as a potential non-invasive biomarker of beta cell death in type 1 Diabetes: a review and future prospect. Clin Epigenetics 2017;**9**:44.
138. Husseiny MI, Kaye A, Zebadua E, et al. Tissue-specific methylation of human insulin gene and PCR assay for monitoring beta cell death. PLoS One 2014;**9**(4):e94591.

139. Milani L, Lundmark A, Kiialainen A, et al. DNA methylation for subtype classification and prediction of treatment outcome in patients with childhood acute lymphoblastic leukemia. Blood 2010;**115**(6):1214-25.
140. Dedeurwaerder S, Desmedt C, Calonne E, et al. DNA methylation profiling reveals a predominant immune component in breast cancers. Embo Mol Med 2011;**3**(12):726-41.
141. Huang RL, Chen HJ, Chen LY, et al. Epigenetic loss of heparan sulfate 3-O-sulfation sensitizes ovarian carcinoma to oncogenic signals and predicts prognosis. Int J Cancer 2018.
142. Liu Y, Tan Q, Liu F. Differentially methylated circulating DNA: A novel biomarker to monitor beta cell death. J Diabetes Complications 2018;**32**(3):349-53.
143. Bellin MD, Clark P, Usmani-Brown S, et al. Unmethylated Insulin DNA Is Elevated After Total Pancreatectomy With Islet Autotransplantation: Assessment of a Novel Beta Cell Marker. Am J Transplant 2017;**17**(4):1112-18.
144. Gong Z, Muzumdar RH. Pancreatic function, type 2 diabetes, and metabolism in aging. Int J Endocrinol 2012;**2012**:320482.
145. Record Owner NLM. Full accounting of diabetes and pre-diabetes in the U.S. population in 1988-1994 and 2005-2006.
146. Levine ME, Lu AT, Quach A, et al. An epigenetic biomarker of aging for lifespan and healthspan. Aging-Us 2018;**10**(4):573-91.
147. Safran M, Dalah I, Alexander J, et al. GeneCards Version 3: the human gene integrator. Database (Oxford) 2010;**2010**:baq020.



6

General Discussion

MAIN FINDINGS

The main objective of the work described in this thesis was to study traditional and novel sex specific risk factors of cardiometabolic diseases and longevity. First we investigated the association of reproductive factors, mainly focusing on age at menopause, menopausal status and sex hormones with various cardiometabolic disease outcomes. We further focused on lifestyle and major CVD risk factors and investigated their association with cardiometabolic risk and longevity in a sex specific manner. Upon the solid framework of value based health care principles, we attempted to create a comprehensive overview of the burden of these risk factors on cardiometabolic health outcomes important to CVD and diabetes patients and valuable to health care policies. The next step was to explore epigenetic determinants for clinical diagnosis and prognosis of T2D and identifying epigenetic sex differences in the overall spectrum of cardiometabolic diseases and its traditional risk factors by comprehensively investigating current literature. In this discussion the main findings are summarized. Furthermore, some methodological considerations are addressed, and potential clinical implications of the findings together with directions for future research are discussed.

Reproductive health

In *chapter 2.1* we investigated the direction of the association and causality between age at natural menopause and blood pressure 3,994 postmenopausal women participants of the Rotterdam Study. Age at menopause categories were defined as follows: early (44 y old), normal (45-54 y old), and late (55 y old).Using genetic variants related to systolic blood pressure, diastolic blood pressure and age at menopause, we created genetic risk scores and performed a bi-directional Mendelian Randomization analysis. This approach is discussed in details in the section of methodological considerations. Our findings suggested that higher blood pressure, or environmental exposure related with higher blood pressure, such as use of antihypertensive medications, might be causally associated with a later onset of natural menopause but we didn't find any evidence that higher blood pressure could affect age at menopause. Further, in *chapters 2.2 and 2.3* we evaluated the association of age at menopause and T2D risk. We included 3 650 postmenopausal women aged 45+ years from the Rotterdam Study, a prospective population-based cohort study. Early onset of natural menopause was associated with an increased risk of type 2 diabetes, independent of potential intermediate risk factors for type 2 diabetes (including BMI, glucose and insulin levels) and of levels of endogenous sex hormones and SHBG. Moreover, our findings suggested that shared genetic factors might not explain the association between age at natural menopause and risk of type 2 diabetes. Therefore, we further explored the impact of age at menopause and T2D on total life expectancy and translated it in number of years that our participants lived

with and without T2D, depending on the age of onset of their menopause. To calculate life expectancy and number of years lived with and without T2D, we used multistate life tables. We constructed three different health states: 1) free of T2D, 2) T2D and 3) death and evaluated how participants in our study moved from one state to the other. Details about this method are discussed in the section of methodological considerations. Our findings showed that, at the age of 50 years, women who experienced early menopause lived fewer years and spent fewer years without diabetes than women who experienced normal or late menopause. Compared with women with normal or late age at menopause, women with early menopause lived at least 3.1 years fewer overall and at least 3.3 years fewer without diabetes, respectively. In *chapters 2.4 and 2.5* we investigated the association between sex hormones and cardiometabolic risk. NT-proBNP has a well-documented prognostic value for CVD and sex hormones are suggested to modulate NT-proBNP levels. Hence, we examined the associations of estradiol, androgens and SHBG with pro-BNP levels in postmenopausal women. In a cross-sectional analysis among 4,112 postmenopausal women free of CVD, lower levels of serum androgens (testosterone, free androgen index, DHEA and DHEAs) and higher level of SHBG were associated with higher levels of serum NT-proBNP, irrespective of known confounders. We further investigated whether the association between DHEAs and NT-proBNP was causal. In a large sample of individuals free of CVD from the Rotterdam Study (7 390 men and women), we found inverse associations between DHEA and DHEAs and serum NT-proBNP. Using a Mendelian Randomization approach, genetically predisposed higher levels of DHEAs were associated with lower NT-proBNP concentrations, providing evidence for potential causal effects of higher DHEAs on lower NT-proBNP.

Lifestyle and other major CVD risk factors

In *chapter 3.1* we investigated whether total life expectancy and life expectancy with and without type 2 diabetes differs between smokers and non-smokers, and between normal-weight smokers and overweight or obese ex-smokers. In a large longitudinal data analysis, 10 738 participants aged 50+ years old from the population-based Rotterdam Study were included. We developed multistate life tables to calculate life expectancy for individuals who were (i) current, former and never smokers as well as for (ii) normal-weight current smokers, overweight and obese ex-smokers. Current smoker men and women lived up to 6.3 years shorter than never smokers and spent less years free of diabetes. No difference was observed in years lived with diabetes.

Similarly, men who were former smoker, but not women, lived overall 1.9 years less than never smokers, and spent fewer years free of diabetes. However, former smokers, both men and women, who were overweight or obese presented a higher risk of developing diabetes but also an extended number of years lived with diabetes, when compared with normal weight current smokers. Moreover, overweight but not obese

ex-smokers spend more years free of diabetes. On average, for men, total life expectancy of an obese ex-smoker was 4.8 years higher than an current normal weight smoker ; and this difference was even larger (8.3 years) in women. Most of these extra years were years spent with diabetes. Further, in chapter 3.3 we tested the common assumption that people who experienced recent weight gain are more likely to be diagnosed with diabetes and explored more generally the patterns of body weight and composition in the years before developing diabetes. We used latent class trajectory analysis to identify several groups among all the people who eventually developed diabetes each with a distinct pattern of BMI development (with BMI measured based on a person's weight and height). Latent class trajectory analysis subdivides a number of people into groups that differ based on specified parameters. By using this method, in our population, we identified three distinct trajectories of change in BMI before the diagnosis of diabetes. Notably, the majority of individuals who developed diabetes were progressively gaining weight within the overweight range but their Framingham 8 year diabetes risk showed a decreasing trend throughout the period of follow-up suggesting that the diagnosis of diabetes might be biased towards enhanced screening efforts reserved to obese individuals rather than overweight. In chapter 3.2 we investigated the impact of the most important lifestyle factors (diet, smoking, alcohol use, physical activity and BMI) altogether combined in a lifestyle score in regard to T2D and longevity. We again created multistate life tables to calculate the differences in life expectancy in the: healthier, moderate and unhealthier lifestyle score. Compared to men in the unhealthier lifestyle category, the total life expectancy of 45-y-old men in the healthier lifestyle group was 6.0 years longer and for women, the difference was 4.6 years longer. The difference in life expectancy free of diabetes for both men and women, in the healthier category was 5.1 and 4.2 years. Moreover, compared to the unhealthier lifestyle group, the difference in life expectancy with diabetes for the healthier lifestyle category was 0.8 for men and 0.8 for women.

Epigenetic and cardiometabolic health

Sex is a major determinant of cardiometabolic risk. DNA methylation and histone modifications, important epigenetic mechanisms that differ between sexes, have been recently involved in the development of cardiometabolic diseases. Therefore, in chapter 5.1 we aimed to systematically review the observational studies in humans investigating the sex-specific associations of epigenetic mechanisms (DNA methylation and histone modifications) with intermediate cardiometabolic traits, T2D and CVD. We focused on 33 studies that had either stratified the analyses by sex or specified that their results did not differ among sexes. The relative importance of epigenetic modifications to the establishment of sex differences in cardio-metabolic health remains an open question, but that there is some role cannot be denied. Overall, our review suggests that epigenetic chang-

es in specific individual genes could be differently associated with cardiometabolic traits in men and women. However, it must be kept in mind that although DNA methylation may modulate sex differences in cardiometabolic diseases, an inverse relationship may equally occur. In chapter 5.2, we discuss the topics of epigenetic alterations, particularly DNA methylation and histone modifications and the importance of epigenetic biomarkers for risk prediction, diagnosis and prognosis of T2D. Current evidence in the field suggests that study of the role of epigenetic changes in the onset and progression of the diseases is a promising opportunity for the development of prediction, diagnostic and disease progression monitoring tools, as well as novel therapeutic targets. Overall, changes in global DNA methylation, CpG islands methylation, and histone acetylation and methylation have been found in relevant genes for aorta, heart, kidney, retina, nerves, and glia cells function in samples from diabetic human and rodent donors or after hyperglycaemic stimuli of the same type of tissues, albeit some of them exhibit contrary results.

METHODOLOGICAL CONSIDERATIONS

Multistate life tables

Most of the studies in this thesis are characterized by a prospective design with a long term follow-up time. As often observed in clinical practice also in cohort studies, participants can switch between different health statuses through follow-up time, sometimes leaving and then returning to the same health status. Such changes in health status include switching to obese or overweight for normal weight ranges; hypertension for normotensives; and changes between healthy, diseased and dead. However, for some states, such as death, severe stages of heart failure or other CVDs, the transition is irreversible. Multistate life table is an appropriate and useful method to properly describe the complex transitions among multiple heath states in cohort studies[1]. It is a demographic tool that is often used to estimate the total life expectancy and disease-specific life expectancies[2].

In this thesis, the life tables method was used in several studies (*chapter 2.3, chapter 3.1, chapter 3.3 and chapter 4.1*). The overall concept of the method as described in more details in the respective chapters of this thesis included three different health states at baseline: "free of disease", "disease," and "death". Participants can experience the following transitions moving from state to state without return[3]: 1) from being free of disease to developing the disease, 2) from diseased to death and 3) from free of disease to death (**Figure 6.1**) . The age of the participants is calculated when entering the study (when the event occurs) and at the end of the follow-up[4]. Total life expectancy and disease-specific

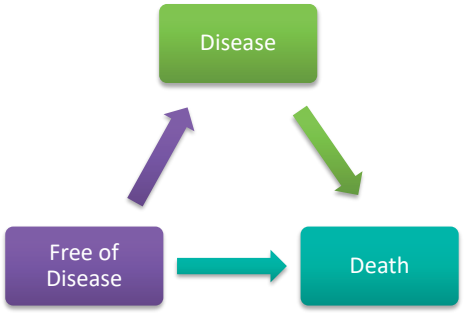


Figure 6.1. Graphic representation of health states in multistate life tables.

life expectancy are calculated from age and sex-specific probabilities for mortality and rates of disease in every transition.

One of the model's strengths is using epidemiological data within a single homogenous cohort such as Rotterdam Study. In this case, the multistate life table enables estimation of the potential burden of the disease in terms of years of life lost and lived with disease due to a risk factor of interest. However, this is also the subject of one of its major limitations. Because of the long follow-up and the broad age range at onset of the cohort, the impact of mortality and disease incidence by age are a mixture of cohort and period effects[4]. Consequently, people at younger ages have different disease and mortality rates from participants entering the study some decades ago. In addition, as people are surviving longer with cardiovascular disease, we expect the life expectancies with cardiovascular disease presented in this thesis to be less than those for current low mortality populations. Another factor that could contribute to different life expectancies calculated in cohort studies compared to the current general population might be due to the healthy volunteer bias in cohort studies[5].

A second limitation of this approach lies in the interpretation of the potential burden of morbidity, which can only be an approximation of the disease burden since this method does not take into account the severity or the improvement (in the case of T2D) of the disease over time. This is particularly an issue for the burden of CVD, which is derived from a very heterogeneous group of conditions. A great improvement of the method will be to add in one further layer of complexity: estimations of the severity of disease, disability, specific treatments associated with the specific health states.

One further limitation is that the current structure of the method is more useful for descriptive rather than interventional purposes. Here a unidirectional flow is used as the simplest way to capture all time spent with a history of disease without the creation of further mixed disease states. While this structure is appropriate for the descriptive analyses like the ones presented in *chapter 2.3, chapter 3.1, chapter 3.3 and chapter 4.1*, a more biological pathway would be preferred (for example allowing coronary heart

disease to congestive heart failure transitions or reversing from T2D to non-T2D) for any interventional analyses.

Altogether, these findings approximately quantify the population burden of cardiometabolic diseases. Decisions regarding which treatment and prevention strategies to follow and how best to respond to changing secular trends are made difficult by the complexity of the interactions between mortality and morbidity at older ages. Multistate life tables are a simple and transparent method to enable meaningful conclusions about the potential impact on population morbidity and mortality of specific interventions and societal trends.

Mendelian Randomization

Establishing causality in observational epidemiological studies is often challenging as the observed associations can be hampered by reverse causation and residual confounding. Mendelian Randomization (MR) is a method that can be used to discover causal relationships between an exposure and outcome in the presence of such limitations using genetic variants as proxies for the exposure of interest (**Figure 6.2**). Following Mendel's Laws of Inheritance, the meiotic alleles segregation happens randomly from parents to offspring. Thus, offspring genotypes are unlikely to be associated with confounders in the population. In addition, germline genotypes are fixed at genesis, therefore, temporally precede the variables under observation, avoiding the issue of reverse causality. Nevertheless, the following considerations need to be taken into account.

First, MR studies are based on the assumption that the genetic variants should be associated with the exposure. Given the small effect size of the SNPs in general, it is required for future studies to explore larger proportions of heritability for various phenotypes, such as age at menopause, blood pressure and DHEAs studied in this thesis. In addition, a way to increase the strength of the variants is to use multiple SNPs combined into a single genetic risk score (GRS) associated with the phenotype of interest that can be used as an instrumental variable (IV). In *chapters 2.1 and 2.5*, we constructed the GRS using a big number of SNPs from public available GWAS associated with DHEAs, age at menopause or blood pressure. The strength of the GRS as an instrument, measured by the F-statistic was satisfactory for both our MR studies (*chapters 2.1 and 2.5)*.

Second, genetic variants themselves may affect various phenotypes and potential risk factors for disease, such as age at menopause, blood pressure and DHEAs, and can therefore be used as an IV assuming these genetic variants do not affect disease risk through other pathways or are not involved in other diseases. These multiple phenotypic effects are also known as pleiotropic effects[6]. Due to the incomplete knowledge in the underlying biology of the genes, this assumption cannot be avoided formally in practice. In attempt to minimize the possibility of pleiotropic associations in *chapter 2.1*, we performed sensitivity analyses excluding the most significantly SNPs associated

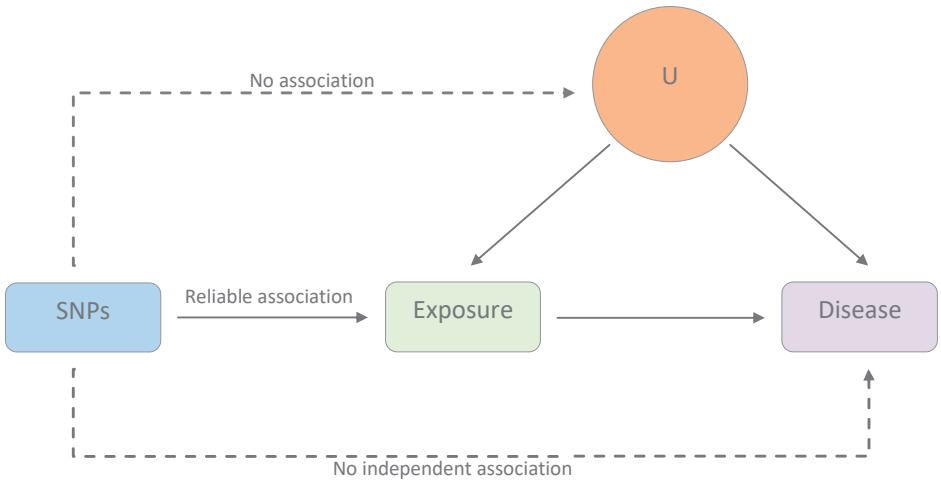


Figure 6.2. Schematic presentation of the MR method
In this figure "U" represents the Unknown/Confounders. The MR approach is based in three assumptions: "No association"- genetic variants should not be associated with confounding variables. "Reliable association"- genetic variants should be associated with the exposure. "No independent association"- genetic variants are only associated with the outcome (disease) through the exposure.

with age at menopause/blood pressure traits. Furthermore, for the SNPs that showed evidence of a causal association with the corresponding outcome, we also performed a "leave one out" analysis to avoid the possibility that the causal association was driven by a single SNP.

Third, MR studies require very large sample sizes in order to have sufficient statistical power. In the case of quantitative approaches to MR, sample size calculations need to consider the magnitude of the predicted effect of the intermediate phenotype on disease outcome[7]. Failure to recognize the sample sizes required to detect plausible or predicted effects of genotype on disease can lead to studies being uninformative[7].

Fourth, the effects of genetic variants could be buffered by compensatory developmental processes. The developmental compensation may occur during foetal development in which, a polymorphic genotype could influence development in such a way as to buffer against the effect of the polymorphism itself. Such compensatory processes have been discussed since C.H. Waddington introduced the notion of canalization in the 1940s[8].

Selection bias

Most studies included in this thesis were performed in the Rotterdam study, a large prospective population based cohort study. the study included in chapter 4.1 was performed in the THIN database, a large primary care database in the UK. In the Rotterdam Study, participants were included in the original cohort if they were 55 years or older.

In this case, only persons that survived up to that age could participate in the study. Although the response rate was 72% at baseline (14,926 of 20,744 invited individuals), which is considered quite good, the included participants might not be a good representation of the general population since individuals that are frail, disabled or hospitalized are less likely to participate. Hence, it might be that chronic diseases such as CVD and T2D could be more prevalent among non-participants. It has been shown that cohort studies tend to represent a healthier population than the underlying general population that was eligible for inclusion in the study[5]. In the Rotterdam Study, participants had a slightly lower cardiovascular risk compared to all individuals that were invited, and non-participation was associated with mortality risk[5]. Therefore, our results on chapters 2.2, 2.3, 2.4, 2.5, 3.1, 3.2, 3.3, and 4.2 might have been underestimated. However, it is unlikely to influence our findings in chapters 2.4 and 2.5 since participants with prevalent CVD were excluded. Furthermore, if selection bias were present, then the true point estimate for the relationship between our exposure (e.g. early menopause) and outcomes (e.g. type 2 diabetes) might be larger than we observed.

Generalizability

Another concern that requires attention in cohort studies such as the Rotterdam Study is the generalizability of the results. The Rotterdam Study population is homogenous, consisting of middle aged and elderly Caucasians. Therefore, our results might be applied to populations with similar characteristics. However, since participants are invited based on the postal code, they can be considered as a random sample of the general Dutch population. Also, in *chapter 2* we included only postmenopausal women in the analysis, and therefore our findings cannot be generalized to pre-menopausal women or women in the menopausal transition. Further, in our studies calculating life expectancies, the mortality rates and the numbers of new cases of CVD and T2D could be lower than in the general population due to the healthy cohort effect. However, generalizability was less of a problem in our study in *chapter 4.1* were we used data from the THIN database with routinely collected data from UK. THIN currently covers approximately 6.0% of the overall UK population encompassing participants of different ethnicities and a wide age range group (18-118 years old). Moreover, it has been shown that disease prevalence and mortality rates in THIN are very similar to the general population in UK[9].

Assessment of menopause

Age at menopause in the Rotterdam Study is defined as the age at final menstrual period, which was followed by cessation of menses that lasted at least 12 months[10]. It is self-reported and was assessed retrospectively during the baseline interview using a questionnaire. The assessment of age at menopause by questionnaires could be subject to some measurement error, while the reliance on retrospective self-report of age at

menopause could be subject to memory and reporting bias, particularly in older women. Moreover, inaccuracy from reporting bias would have been nondifferential in relation to the menopausal categories. Hence, it is unlikely that this would have created any difference between the menopausal groups (early, normal and late menopause). Because the outcomes (such as T2D, CVD and mortality) were assessed prospectively, the subjective measure of age at menopause would, however, likely lead to nondifferential misclassification with respect to the outcome, and therefore would likely bias our estimates toward the null. Furthermore, studies have reported that the validity and reproducibility of self-reported age at menopause are fairly good[11 12]. Moreover, the women of the Rotterdam Study and interviewers administering the women's health questionnaire were unaware of the research questions under study. We therefore reasonably expect that any misclassification of the exposure would be non-differential, and if any, would provide a conservative approach given that the effect estimates would be less strong than if no misclassification would have occurred.

In most of the studies investigating age at menopause analyses were restricted to postmenopausal women with a natural menopause. This was done because health status' can differ greatly between pre, peri and postmenopausal women, given that the menopausal transition is characterized by significant changes at the hormonal, physiological, and metabolic level[10 13]. To what extent these changes affect later life health further differs according to menopausal type (i.e. natural vs. surgical menopause). These restrictions were applied to reduce the amount of bias in our effect estimates, since menopausal age, menopausal status and menopausal type are considered factors that are associated with both the exposures and the outcomes under study in this thesis.

IMPLICATIONS AND DIRECTIONS FOR FUTURE RESEARCH

Timing of menopause as an independent risk factor in women's diseased risk

Our findings from studies in reproductive factors and cardiometabolic factors suggest that age at menopause could be in itself an important independent risk factor for future cardiometabolic risk and mortality in women, and that menopause could be a period to evaluate women's risk for CVD and introduce prevention strategies. For women undergoing menopause before age of 45, general physicians should consider screening for the levels of risk factors such as obesity, glucose and insulin to identify women at risk of developing diabetes who could benefit from early interventions.

In the future, holistic comprehensive approaches on menopause are needed. Well-designed large scale individual participant data meta-analysis are necessary to establish whether age of menopause is an independent risk factor of cardiometabolic health and could contribute to improvement of risk prediction models in T2D and CVD. In addition,

such studies could help to set a cut-off value of age of menopause for which the risk of cardiometabolic risk is increased, which currently is based on arbitrary categories. Mechanisms linking age of menopause with cardiometabolic risk remain unclear, and studies employing mediation analysis could investigate whether and to what extent certain pathways such as inflammation, lipids, atherosclerosis, sex hormone metabolism, iron and oxidative stress mediate the association. Further, up to date, most studies have not assessed directionality of the association between age at menopause and risk of chronic diseases. Although increased risk of chronic disease, for instance CVD, has been proposed as consequence of early menopause, the alternative hypothesis, that fluctuations of cardiovascular risk factors in premenopausal women may promote early menopause, could also be true[14 15]. In *chapter 2.1* we attempted to assess directionality and causality between age at menopause and blood pressure by using a bi-directional MR. However, our results need replication in studies with more statistical power in order to avoid possible false positive results. Recently, another hypothesis has emerged suggesting that several other factors, such as epigenetic mechanisms could play a role in the relationship between menopause and appearance of chronic diseases. Epigenetic modifications such as DNA methylation of cytosine residues in CpG dinucleotides histone modification and micro RNAs might constitute an additional pathway linking the timing when menopause occurs with co-morbidity and longevity[16 17].

Lifestyle as "medicine"; can we reverse T2D?

Besides and/or through epigenetics, other modifiable factors such as lifestyle factors are thought to play an important role in the onset of menopause[18-20]. As such, lifestyle changes in order to improve future disease risk and symptoms targeting menopausal women have been recently suggested to be incorporated in some clinical guidelines[21]. Overall healthy lifestyle like, a healthy diet, avoiding alcohol and smoking, maintaining a healthy body weight and being physically active are not only important for women during menopause but are known to be important predictors of good health in both sexes and in all age ranges as also shown from our results in *chapter 3.1 and 3.3*. Despite their relevance especially in T2D, recommendations of switching to a healthier lifestyle are mainly given as an advice in the clinical practice. Indeed, current guidelines for management of type 2 diabetes focus heavily on multiple drug treatments, generally to reduce blood glucose and the associated elevated risks of cardiovascular disease. In addition to pharmaceutical interventions, lifestyle changes can also play a large role in managing and even reversing T2D[22 23]. Indeed, recent scientific evidence for reversibility, remission and even cure of T2D is accumulating, both from scientific studies and 'case reports'[23-25]. The rationale behind it is that since type 2 diabetes involves several organs and biological processes making it a 'systems disease', it also needs to be treated as a system. This could be of great importance for both patients and healthcare providers

since it is "natural" and less costly than pharmaceutical treatments, has less side effects and it could be important for a better overall health. Therefore, future larger randomized clinical trials and observational studies should investigate more the influence, costs and benefits of a healthier lifestyle in type 2 diabetes. Further, healthcare providers need to be educated on reversal options so they can actively engage in counselling patients who may desire this approach to their disease.

Sex differences and sex stratification in research

Despite the knowledge that sex is a strong modulator in the pathophysiology, risk, diagnosis and response to treatment in cardiovascular disease, very often, sex is not considered in clinical practice when it comes to decision making[26 27]. The application of research evidence in clinical practice often takes time and it is challenging, but efforts to increase awareness of the importance of sex in disease and health among clinicians and the general public are essential. There is still a belief that research performed mostly in men is applicable to women as well. Also, because men die quicker from heart diseases while women live longer but even though they get sicker it is believed that heart diseases are not a big problem for women. Hence, increasing awareness and skills among researchers in all biomedical scientific fields will improve the quality of health research. A way to go, is to improve the enrolment of women in clinical trials, to report study results by sex, and to consider sex in both research and clinical practice could be important steps that can improve the evidence for prevention, diagnosis and treatment of cardiometabolic diseases in women by making it more personalized and accurate and could ultimately improve the quality of health care for both men and women. Indeed, a more broader inclusion of women must happen at all stages and fields of research. For example, sex must be considered in all phases of drug development, especially in the areas of efficacy and safety since we still use drugs that are tested predominantly in men[26]. This could have lead also to sex disparities in the prognosis and management of diabetes; while diabetes mortality over the last decades has decreased in diabetic men, in women, it has remained invariable. Even when women are included in clinical trials, the numbers are too low, so the lack of differences mainly reflects unpowered studies rather than a true absence of difference[27 28]. This was also the scope of our systematic review in chapter 5.2 were we underscore the need to investigate the effects of sex on epigenetic mechanisms as current research reflects a strong influence of sex on epigenetic regulatory processes.

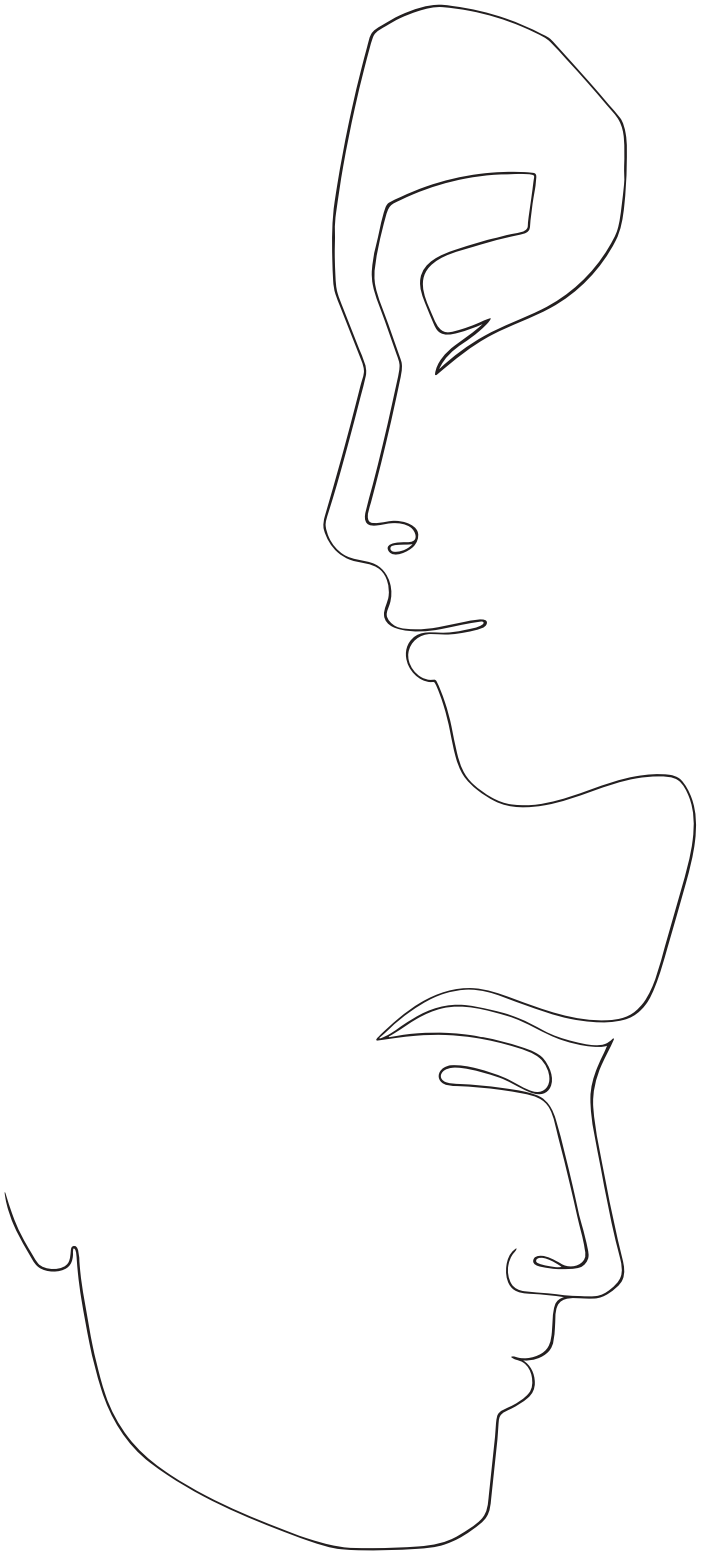
CONCLUSIONS

Overall, men and women have different cardiometabolic risks, which influences longevity in a sex-specific manner. More specifically, we showed that lifestyle factors could influence men and women differently, highlighting the need for sex-specific approaches. We found that the benefits of quitting smoking outweigh the risk of weight gain in terms of overall life expectancy and a great part of this increased life expectancy remains with T2D. However, the effects were more profound in women than in men. In addition, a healthier lifestyle was associated with a longer overall life expectancy of 5 years longer. Sex-specific factors are another factor to be considered when investigating cardiometabolic risk. In women, reproductive factors such as age at menopause and alterations in sex hormones could be independent markers of cardiometabolic disease and ageing. Women experiencing menopause at an earlier age might have an increased risk of T2D and live shorter than women who experience menopause after the age of 55 years. DNA methylation on the other hand, might be a promising tool to understand sex differences in the pathophysiology, diagnosis and prognosis of cardiometabolic diseases and why environmental factors can have sex-specific effects. However, the research on DNA methylation and cardiometablic disease is on its infancy and future studies on this topic should provide sex-specific estimates.

REFERENCES

1. Peeters A, Mamun AA, Willekens F, et al. A cardiovascular life history. A life course analysis of the original Framingham Heart Study cohort. Eur Heart J 2002;**23**(6):458-66.
2. Dhana K, Nano J, Ligthart S, et al. Obesity and Life Expectancy with and without Diabetes in Adults Aged 55 Years and Older in the Netherlands: A Prospective Cohort Study. Plos Medicine 2016;**13**(7).
3. Franco OH, de Laet C, Peeters A, et al. Effects of physical activity on life expectancy with cardiovascular disease. Arch Intern Med 2005;**165**(20):2355-60.
4. Peeters A, Mamun AA, Willekens F, et al. A cardiovascular life history - A life course analysis of the original Framingham Heart Study cohort. European Heart Journal 2002;**23**(6):458-66.
5. Leening MJG, Heeringa J, Deckers JW, et al. Healthy Volunteer Effect and Cardiovascular Risk. Epidemiology 2014;**25**(3):470-71.
6. Smith GD, Ebrahim S. Mendelian randomization: prospects, potentials, and limitations. Int J Epidemiol 2004;**33**(1):30-42.
7. Smith GD, Ebrahim S. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? Int J Epidemiol 2003;**32**(1):1-22.
8. Wilkins AS. Canalization: A molecular genetic perspective. Bioessays 1997;**19**(3):257-62.
9. Blak BT, Thompson M, Dattani H, et al. Generalisability of The Health Improvement Network (THIN) database: demographics, chronic disease prevalence and mortality rates. Inform Prim Care 2011;**19**(4):251-5.
10. Harlow SD, Gass M, Hall JE, et al. Executive Summary of the Stages of Reproductive Aging Workshop+10: Addressing the Unfinished Agenda of Staging Reproductive Aging. J Clin Endocr Metab 2012;**97**(4):1159-68.
11. Muka T, Oliver-Williams C, Kunutsor S, et al. Association of Age at Onset of Menopause and Time Since Onset of Menopause With Cardiovascular Outcomes, Intermediate Vascular Traits, and All-Cause Mortality A Systematic Review and Meta-analysis. Jama Cardiology 2016;**1**(7):767-76.
12. denTonkelaar I. Validity and reproducibility of self-reported age at menopause in women participating in the DOM-project. Maturitas 1997;**27**(2):117-23.
13. van Dijk GM, Kavousi M, Troup J, et al. Health issues for menopausal women: The top 11 conditions have common solutions. Maturitas 2015;**80**(1):24-30.
14. Kok HS, van Asselt KM, van der Schouw YT, et al. Heart disease risk determines menopausal age rather than the reverse. J Am Coll Cardiol 2006;**47**(10):1976-83.
15. Sarnowski C, Kavousi M, Isaacs S, et al. Genetic variants associated with earlier age at menopause increase the risk of cardiovascular events in women. Menopause 2018;**25**(4):451-57.
16. Muka T, Nano J, Voortman T, et al. The role of global and regional DNA methylation and histone modifications in glycemic traits and type 2 diabetes: A systematic review. Nutr Metab Cardiovasc Dis 2016;**26**(7):553-66.
17. Laven JSE, Visser JA, Uitterlinden AG, et al. Menopause: Genome stability as new paradigm. Maturitas 2016;**92**:15-23.
18. Gold EB, Bromberger J, Crawford S, et al. Factors associated with age at natural menopause in a multiethnic sample of midlife women. Am J Epidemiol 2001;**153**(9):865-74.
19. Castelo-Branco C, Blumel JE, Chedraui P, et al. Age at menopause in Latin America (vol 13, pg 706, 2006). Menopause 2006;**13**(5):850-50.
20. Luoto R, Kaprio J, Uutela A. Age at Natural Menopause and Sociodemographic Status in Finland. Am J Epidemiol 1994;**139**(1):64-76.

21. Meeta, Digumarti L, Agarwal N, et al. Clinical practice guidelines on menopause: An executive summary and recommendations. J Midlife Health 2013;**4**(2):77-106.
22. Lean ME, Leslie WS, Barnes AC, et al. Primary care-led weight management for remission of type 2 diabetes (DiRECT): an open-label, cluster-randomised trial. Lancet 2018;**391**(10120):541-51.
23. Lim EL, Hollingsworth KG, Aribisala BS, et al. Reversal of type 2 diabetes: normalisation of beta cell function in association with decreased pancreas and liver triacylglycerol. Diabetologia 2011;**54**(10):2506-14.
24. Taylor R. Pathogenesis of type 2 diabetes: tracing the reverse route from cure to cause. Diabetologia 2008;**51**(10):1781-9.
25. Steven S, Hollingsworth KG, Al-Mrabeh A, et al. Very Low-Calorie Diet and 6 Months of Weight Stability in Type 2 Diabetes: Pathophysiological Changes in Responders and Nonresponders (vol 39, pg 808, 2016). Diabetes Care 2018;**41**(6).
26. Yang Y, Carlin AS, Faustino PJ, et al. Participation of women in clinical trials for new drugs approved by the food and drug administration in 2000-2002. J Womens Health (Larchmt) 2009;**18**(3):303-10.
27. Rosano GM, Lewis B, Agewall S, et al. Gender differences in the effect of cardiovascular drugs: a position document of the Working Group on Pharmacology and Drug Therapy of the ESC. Eur Heart J 2015;**36**(40):2677-80.
28. Humphries KH, Pilote L. Research in Women's Cardiovascular Health-Progress at Last? Canadian Journal of Cardiology 2018;**34**(4):349-53.



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Appendices

SUMMARY

The aim of this thesis as described in *chapter 1*, was to investigate the role that sex specific reproductive factors, lifestyle, and epigenetics play in cardiometabolic health and longevity.

In *chapter 2* we mainly focus on the role of age at menopause and sex hormones in cardiometabolic risk. In *chapter 2.1* we investigated the causality and direction of the relation between age at natural menopause and blood pressure. For this aim we used using genetic variants as instrumental variables in a bi-directional Mendelian Randomization analysis. Our results suggest that higher blood pressure, or environmental exposures related to higher blood pressure, such as use of antihypertensive medications, might result in a later onset of natural menopause, but not vice versa. In *chapters 2.2 and 2.3*, we examined the association between age at natural menopause and risk of type 2 diabetes (T2D) and with total life expectancy and the number of years lived with and without T2D. Data from 3 650 postmenopausal women followed for a median of 9 years in the Rotterdam Study showed that early onset of menopause might be an independent risk factor for diabetes risk and mortality. Therefore, we further explored the impact of age at menopause and T2D on total life expectancy and translated it in number of years that our participants lived with and without T2D, depending on the age of onset of their menopause. Women who experienced early menopause lived less long and spent fewer years without diabetes than women who experienced normal or late menopause. In *chapter 2.4* we used data from 4,112 postmenopausal women of the Rotterdam Study cohort and showed that some androgens such as testosterone, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate (DHEAs) and free androgen index but not estradiol might be responsible for lower cardioprotective natriuretic peptides levels in postmenopausal women. In *chapter 2.5* we further analysed the data on DHEAs and amino-terminal-B-type-natriuretic peptide (NT-proBNP) in both male and female participants of the Rotterdam Study and observed that being genetically predisposed to higher DHEAs levels was associated with lower NT-proBNP levels. These findings suggest that androgens could in part play some role in the risk of developing cardiometabolic diseases after menopause while altering levels of DHEAs could be important in the prevention of chronic heart failure.

Chapter 3 focuses in the association between lifestyle, cardiometabolic risk and longevity. For several of these studies, we used some novel epidemiological methodologies such as multistate lifetables and latent class trajectories to investigate the data. In *chapter 3.1*, we showed that the benefits of quitting smoking outweigh the risk of weight gain in terms of overall life expectancy. However, a great part of this increased life expectancy is spent with T2D. Hypothesizing that individuals who experience recent weight gain are more likely to be diagnosed with diabetes in *chapter 3.2*, we explored patterns of body

mass index changes before developing diabetes. We identified three distinct patterns of change in body mass index (BMI); "progressive overweight", "persistent obese" and "progressive weight loss". In general, the majority of individuals with diabetes within the overweight range, were characterized by gaining weight prior diagnosis of diabetes, suggesting that could be important to focus in small weight reductions for the total population rather than only in the high risk groups. Further, in *chapter 3.3* we combined five most important lifestyle factors (BMI, diet, smoking, alcohol use and physical activity) in a score to see the combined effects of these modifiable risk factors in overall life expectancy and diabetes risk. Our findings showed that overall, men and women in the healthier lifestyle category lived up to 5 years longer compared to their counterparts in the unhealthier lifestyle category. In addition, the difference in life expectancy free of diabetes for both men and women, in the healthier category was up to 5 years, while the difference in life expectancy with diabetes was up to 1 year.

Chapter 4 describes the association of major cardiovascular disease (CVD) risk factors with mortality. In *chapter 4.1* we analysed 6.5 million participants from the THIN database in the United Kingdom and explored their disease course from healthy or with T2D to being diagnosed with CVD and dying. We assessed their CVD risk and life expectancy starting from the age of 50 years and older. Our results suggest that people with diabetes have a higher risk of developing CVD and CVD mortality. However, when quantifying these risks into life expectancies we did not observe significant differences in years spent with and without CVD among diabetics and non-diabetics, suggesting that management, care and treatment of CVD in patients with diabetes might have improved in recent years.

In *chapter 5* we conducted a systematic review on the role that epigenetics mechanisms play on sex differences in cardiometabolic health and in the diagnosis and prognosis of T2D. In *chapter 5.1* we conclude that only a small number of studies in the field of cardiometabolic health stratify or present their results by sex. However, our findings suggested that DNA methylation might be a promising molecular strategy for understanding sex differences in the pathophysiology of cardiometabolic disease, and that future studies in the topic should provide sex-specific estimates. In *chapter 5.2* we summarize that epigenetic mechanisms might play a significant role in the reversibility and treatment of diabetic complications. Further, these "non-permanent" changes could be great targets for example for developing more effective drugs. They might also arise as a promising tool in the diagnosis, stratification of the patients, prognosis, and monitoring of therapy response.

In *chapter 6*, we discuss in a broader perspective the findings from the studies described in this thesis. Moreover, methodological considerations, clinical implications and future research directions are discussed.

NEDERLANDSE SAMENVATTING

Het doel van dit proefschrift zoals beschreven in hoofdstuk 1, was het onderzoeken welke rol geslacht specifieke reproductieve factoren, leefstijl, en epigenetica spelen in cardiometabole gezondheid en levensduur.

In hoofdstuk 2 richten we voornamelijk op de rol van leeftijd van menopauze en geslachtshormonen. In hoofdstuk 2.1 hebben we de causaliteit en richting van de relatie tussen leeftijd van menopauze en bloeddruk. For dit doel gebruikten we genetische varianten als instrumentele variabele in een bi-directionele Mendelian Randomisatie analyse. Onze resultaten suggereren dat een hogere bloeddruk of omgevingsfactoren gerelateerd aan een hoge bloeddruk, zoals het gebruik van antihypertensiva, mogelijk leiden tot een latere start van menopauze, maar niet andersom. In hoofstukken 2.2 en 2.3 onderzochten we de associatie tussen leeftijd van natuurlijke menopauze en het risico op type 2 diabetes (T2D). Data van 3650 postmenopauzale vrouwen gevolgd over een mediaan van 9 jaar in de Rotterdam Studie liet zien dat een vroeg begin van de menopauze mogelijk een onafhankelijk risicofactor is van diabetes en mortaliteit. We hebben om deze reden verder onderzocht wat de impact is van leeftijd van menopauze en T2D op totale levensverwachting. Dit vertaalden we naar aantal jaren dat onze deelnemers leefden met en zonder T2D, afhangend van de leeftijd van het begin van de menopauze. Vrouwen met een vroeg begin van de menopauze leefden korter en hadden minder jaren zonder diabetes dan vrouwen met een normaal of laat begin van de menopauze. In hoofdstuk 2.4 gebruikten we data van 4,112 postmenopauzale vrouwen van de Rotterdam Studie en lieten we zien dat sommige androgenen zoals testosteron, dehydroepiandrosteron (DHEA), dehydroepiandrosteronsulfaat (DHEA's) en vrije androgeenindex, maar niet estradiol, wellicht de reden zijn voor lagere cardioprotectieve niveaus van natriuretische peptiden. In hoofdstuk 2.5 analyseerden we de data van DHEAs en amino-terminaal-B-type-natriuretisch peptide (NT-proBNP) in zowel mannelijke als vrouwelijk deelnemers van de Rotterdam Studie en we observeerden dat een genetische aanleg voor hogere DHEA waarden geassocieerd was met lagere NT-proBNP waarden. Deze bevindingen suggereren dat androgenen gedeeltelijk een rol kunnen spelen in het risico van het ontwikkelen van cardiometabole ziekten na menopauze, terwijl het veranderen van DHEAs belangrijk kan zijn in de preventie van chronisch hartfalen.

Hoofdstuk 3 richt zich op de associatie tussen leefstijl, cardiometabool risico en levensduur. Voor een aantal van deze studies gebruikten we nieuwe epidemiologische methodologie, zoals multistate lifetables en latent class trajectories om de data te onderzoeken. In hoofdstuk 3.1 lieten we zien dat de voordelen van stoppen met roken zwaarder wegen dan het risico van gewichtstoename met betrekking tot verwachte levensduur. Echter is een groot gedeelte van deze toename in verwachte levensduur

gespendeerd met T2D. Met de hypothese dat individuen wie recent in gewicht zijn toegenomen ook vaker met diabetes worden gediagnosticeerd onderzochten we in hoofdstuk 3.2 de patronen van verandering in BMI voor het ontwikkelen van diabetes. We identificeerden 3 patronen van verandering in BMI: "progressief overgewicht", "aanhoudend obesitas", en "progressief gewichtsafname". De meerderheid van individuen met diabetes in de overgewichtsklasse werden gekarakteriseerd door gewichtstoename voor de diagnose van diabetes, wat suggereert dat het belangrijk kan zijn om te richten op kleine gewichtsreducties in voor de totale populatie in plaats van alleen te richten op de groepen met een hoog risico. In hoofdstuk 3.3 combineerden we de vijf meest belangrijke leefstijlfactoren (BMI, voeding, roken, alcohol en fysieke activiteit) in een score om te zien of er een gecombineerd effect is van deze aanpasbare risicofactoren in verwachte levensduur en risico op diabetes. Onze bevindingen lieten zien dat mannen en vrouwen in de gezondere leefstijl categorie tot 5 jaar langer leefden vergeleken met degene in een ongezondere leefstijl categorie. Daarnaast was het verschil in verwachte levensduur zonder diabetes tot 5 jaar voor mannen en vrouwen in de gezondere categorie, terwijl het verschil in verwachte levensduur met diabetes tot 1 jaar was.

Hoofdstuk 4 beschrijft de associatie van risicofactoren van hart en vaat ziekten met mortaliteit. In hoofdstuk 4.1 analyseerden we 6.5 miljoen deelnemers van de THIN database in het Verenigd Koninkrijk en onderzochten we het ziekteverloop van gezond of met T2D naar gediagnostiseerd worden met hart- en vaatziekten of sterfte. We beoordeelden het risico op hart- en vaatziekten en verwachte levensduur vanaf de leeftijd van 50 jaar oud. Onze resultaten suggereren dat mensen met diabetes een hoger risico hebben op het ontwikkelen van hart- en vaatziekten en mortaliteit door hart- en vaatziekten. Echter wanneer we deze risico's kwantificeerden in verwachte levensduur zagen we geen significant verschil in jaren met en zonder hart- en vaatziekten onder diabeten en niet-diabeten, wat suggereert dat management, zorg en behandeling van hart- en vaatziekten bij patiënten met diabetes mogelijk verbeterd is over de laatste jaren.

In hoofdstuk 5 voerden we een systematisch literatuuronderzoek uit naar de rol van epigenetische mechanismen bij geslachtsverschillen in cardiometabole gezondheid en de diagnose en prognose van T2D. in Hoofdstuk 5.1 concludeerden we dat slechts een klein aantal studies in het veld van cardiometabole gezondheid resultaten stratificeren of presenteren per geslacht. Echter suggereren onze bevindingen dat DNA methylatie mogelijk een veelbelovende moleculaire strategie is voor het begrijpen van geslachtsverschillen in de pathofysiologie van cardiometabole ziekten en dat toekomstige studies van dit onderwerp geslacht specifieke resultaten zouden moeten laten zien. In hoofdstuk 5.2 vatten we samen dat epigenetische mechanismen mogelijk een aanzienlijke rol spelen in de omkeerbaarheid en behandeling van complicaties bij diabetes. Daarnaast kunnen deze "niet-permanente" veranderingen targets zijn, door medicatie.

Deze veranderingen kunnen ook een veelbelovende tool zijn in diagnose, stratificatie van patiënten, prognose, en monitoren van de reactie op therapie.

In hoofdstuk 6, discussiëren we in een breder perspectief de bevindingen van studies beschreven in dit proefschrift. Daarnaast worden methodologische afwegingen, klinische implicaties en richtingen voor vervolgonderzoek beschreven.

PUBLICATIONS AND MANUSCRIPTS

Asllanaj E, Bano A, Glisic M, Jaspers L, Ikram MA, Laven JSE, Vőlzke H, Muka T, Franco OH. Age at natural menopause and life expectancy with and without diabetes. Menopause. 2018 Oct 8. doi: 10.1097/GME.0000000000001246.

Asllanaj E*, Muka T*, Avazverdi N, Jaspers L, Stringa N, Milic J, Ligthart S, Ikram MA, Laven JSE, Kavousi M, Dehghan A, Franco OH. Age at natural menopause and risk of type 2 diabetes: a prospective cohort study. Diabetologia. 2017 Oct;60(10):1951-1960. doi: 10.1007/s00125-017-4346-8

E Asllanaj*, C Ochoa-Rosales*, M Glisic, J Nano, T Muka, OH. Franco. Chromatin landscape and epigenetic biomarkers for clinical diagnosis and prognosis of type 2 diabetes mellitus. Prognostic Epigenetics

Volume 15 in Translational Epigenetics 2019, Pages 289-324. doi: 10.1016/B978-0-12-814259-2.00012-1

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

Asllanaj E, Zhang X*, Rosales C.O*, Bramer W.H, Nano J, Voortman T, Fernandez E.P, Braun K, Gonzalez V, Ghanbari M, Ahrens W, Ikram MA, Franco O.H, Muka T*, Glisic M*. Sexually Dimorphic DNA-methylation in Cardio-metabolic Health: a Systematic Review. Maturitas 13-FEB-2020 DOI: 10.1016/j.maturitas.2020.02.005

Asllanaj E, Vőlzke H, Glisic M, Voortman T, Maas S.C, Ikram M.A, Franco O.H, Muka T. Obese ex-smokers live longer overall and in good health, but more years with diabetes than normal weight smokers: Findings from The Rotterdam Study. *Revision at International Journal of Obesity*

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

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

Milic J, Glisic M, Voortman T, Borba LP, **Asllanaj E**, Rojas LZ, Troup J, Kiefte-de Jong JC, van Beeck E, Muka T, Franco OH. Menopause, ageing, and alcohol use disorders in women. Maturitas. 2018 May;111:100-109. doi: 10.1016/j.maturitas.2018.03.006. Epub 2018 Mar 13. Review.

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

Glisic M, Kastrati N, Musa J, Milic J, **Asllanaj E**, Portilla Fernandez E, Nano J, Ochoa Rosales C, Amiri M, Kraja B, Bano A, Bramer WM, Roks AJM, Danser AHJ, Franco OH, Muka T. Phytoestrogen supplementation and body composition in postmenopausal women: A systematic review and meta-analysis of randomized controlled trials. Maturitas. 2018 Sep;115:74-83. doi: 10.1016/j.maturitas.2018.06.012. Epub 2018 Jun 22. Review.

Wolters M, **Asllanaj* E,** Dejanovic***** GM, Günther K, Pohlabeln H, Bramer WM, Ahrens J, Nagrani R, Pigeot I, Franco OH, Ahrens W, Muka T, Glisic M. Phytoestrogen supplementation and Intermediate Cardiovascular Disease Risk Factors among Postmenopausal Women: a Meta-analysis of Randomized Controlled Trials. *Revision at Menopause*

Asllanaj E*, Rojas L.R*, Rueda-Ochoa O.L*, FernandezE.P, Ochoa-Rosales C, Day F, Trajanoska K, Nano J, Ikram M.A, Burgess S, Franco O.H, Glisic M*, Muka T*. Mendelian randomization provides evidence for a causal role of dehydroepiandrosterone sulfate in decreasing NT-proBNP levels in a Caucasian population. *Revision at Circulation Research*

Nano J*, Dhana K*, **Asllanaj E**, Sijbrand E, Ikram M.A, Dehghan A, Muka T*, Franco O.H* Trajectories of Body Mass Index before the Diagnosis of Type 2 Diabetes: The Rotterdam Study. *Accepted for publication at Obesity*

Asllanaj E, Nano J, Koromani F, Laven J.S.E, Deghan A, Ikram M.A, Franco O.H, Muka T. Age At Natural Menopause and Blood Pressure: a Bi-Directional Mendelian Randomization Analysis. *Submitted for publication*

Asllanaj E, Zhang X, Amiri M, Ikram MA, Ghanbari M. Epigenetics and non-alcoholic fatty liver disease: a systematic review. *Manuscript in preparation*

Limpens M, **Asllanaj E**, Ikram MA, Kavousi M, Voortman T. Healthy lifestyle score and life expectancy with and without heart failure. *Manuscript in preparation*

Zhang X, **Asllanaj E**, Amiri M, Nano J, Glisic M, Franco OH, Ikram MA, Ghanbari M. Association of sex hormones and non-alcoholic fatty liver disease. *Manuscript in preparation*

Praveen Surendran , Elena V. Feofanova, Najim Lahrouchi, Ioanna Ntalla, Savita Karthikeyan, James Cook, Lingyan Chen, Borbala Mifsud, Chen Yao, Aldi T. Kraja, James H. Cartwright, Jacklyn N. Hellwege, Ayush Giri, Vinicius Tragante, Gudmar Thorleifsson, Dajiang J. Liu, Bram P. Prins, Isobel D. Stewart, Claudia P. Cabrera, James M. Eales, Artur Akbarov, Paul L. Auer, Lawrence F. Bielak, Joshua C. Bis, Vickie S. Braithwaite, Jennifer A. Brody, E. Warwick Daw, Fotios Drenos, Sune Fallgaard Nielsen, Jessica D. Faul, Eric B. Fauman, Cristiano Fava, Teresa Ferreira, Christopher N. Foley, Nora Franceschini, He Gao, Olga Giannakopoulou, Franco Giulianini, Daniel F. Gudbjartsson, Xiuqing Guo, Sarah E. Harris, Aki S. Havulinna, Anna Helgadottir, Jennifer E. Huffman, Shih-Jen Hwang, Stavroula Kanoni, Jukka Kontto, Martin G. Larson, Ruifang Li-Gao, Jaana Lindström, Luca A. Lotta, Yingchang Lu, Jian'an Luan, Anubha Mahajan, Giovanni Malerba, Nicholas GD. Masca, Hao Mei, Cristina Menni, Dennis O. Mook-Kanamori, David Mosen-Ansorena, Martina Müller-Nurasyid, Guillaume Paré, Dirk S. Paul, Markus Perola, Alaitz Poveda, Rauramaa Rainer, Melissa Richard, Tom G. Richardson, Nuno Sepúlveda, Xueling Sim, Albert V. Smith, Jennifer A. Smith, James R. Staley, Alena Stanáková, Patrick Sulem, Sébastien Thériault, Unnur Thorsteinsdottir, Stella Trompet, Tibor V. Varga, Digna R. Velez Edwards, Giovanni Veronesi, Stefan Weiss, Sara M. Willems, Jie Yao, Robin Young, Bing Yu, Weihua Zhang, Jing-Hua Zhao, Wei Zhao, Wei Zhao, Evangelos Evangelou, Stefanie Aeschbacher, **Eralda Asllanaj**, Stefan Blankenberg, Lori L. Bonnycastle, Jette Bork-Jensen, Ivan Brandslund, Peter S. Braund, Stephen Burgess, Kelly Cho, Cramer Christensen, John Connell, Renée de Mutsert, Anna F. Dominiczak, Marcus Dörr, Gudny Eiriksdottir, Aliki-Eleni Farmaki, J. Michael Gaziano, Niels Grarup, Megan L. Grove-Gaona, Göran Hallmans, Torben Hansen, Christian T. Have, Gerardo Heiss, Marit E. Jørgensen, Pekka Jousilahti, Eero Kajantie, Mihir Kamat, AnneMari Käräjämäki, Fredrik Karpe, Heikki A. Koistinen, Csaba P. Kovesdy, Kari Kuulasmaa, Tiina Laatikainen, Lars Lannfelt, I-Te Lee, Wen-Jane Lee, LifeLines Cohort Study, Allan Linneberg, Lisa W. Martin, Marie Moitry, Girish Nadkarni, Matthew Neville, Colin NA. Palmer, George J. Papanicolaou, Oluf Pedersen, James Peters, Neil Poulter, Asif Rasheed, Katrine L. Rasmussen, N. William Rayner, Mägi Reedik, Frida Renström, Rainer Rettig, Jacques Rossouw, Pamela J. Schreiner, Peter J. Sever, Emil L. Sigurdsson, Tea Skaaby, Yan V. Sun, Johan Sundstrom, Gudmundur Thorgeirsson, Esko Tõnu, Elisabetta Trabetti, Philip S. Tsao, Tiinamaija Tuomi, Stephen T. Turner, Ioanna Tzoulaki, Ilonca Vaartjes, Anne-Claire Vergnaud, Helen R. Warren, Cristen J. Willer, Peter WF. Wilson, Daniel R. Witte, Ekaterina Yonova-Doing, He Zhang, Naheed Aliya, Peter Almgren, Philippe Amouyel, Folkert W. Asselbergs, Michael R. Barnes, Alexandra I. Blakemore, Michael Boehnke, Michiel L. Bots, Erwin P. Bottinger, Julie E. Buring, John C. Chambers, Yii-Der Ida Chen, Rajiv Chowdhury, David Conen, Adolfo Correa, George Davey Smith, Rudolf A. de Boer, Ian J. Deary, George Dedoussis, Panos Deloukas, Emanuele Di Angelantonio, Paul Elliott, EPIC-CVD, EPIC-InterAct, Stephan B. Felix, Jean Ferrières, Ian Ford, Myriam Fornage, Paul W. Franks, Stephen Franks, Philippe Frossard, Giovanni Gambaro, Tom R. Gaunt, Leif

Groop, Vilmundur Gudnason, Tamara B. Harris, Caroline Hayward, Branwen J. Hennig, Karl-Heinz Herzig, Erik Ingelsson, Tuomilehto Jaakko, Marjo-Riitta Jarvelin, J. Wouter Jukema, Sharon LR. Kardia, Frank Kee, Jaspal S. Kooner, Charles Kooperberg, Lenore J. Launer, Lars Lind, Ruth J.F. Loos, Abdulla al Shafi. Majumder, Laakso Markku, Mark I. McCarthy, Olle Melander, Karen L. Mohlke, Alison D. Murray, Børge Grønne Nordestgaard, Marju Orho-Melander, Chris J.. Packard, Sandosh Padmanabhan, Walter Palmas, Ozren Polasek , David J. Porteous, Andrew M. Prentice, Michael A. Province, Caroline L. Relton, Kenneth Rice, Paul M. Ridker, Olov Rolandsson, Frits R. Rosendaal, Jerome I. Rotter, Igor Rudan, Veikko Salomaa, Nilesh J. Samani, Naveed Sattar, Wayne H-H Sheu, Blair H. Smith, Nicole Soranzo, Timothy D. Spector, John M. Starr, Sebert Sylvain, Kent D. Taylor, Lakka A. Timo, Nicholas J. Timpson, Martin D. Tobin, Understanding Society Scientific Group, Pim van der Harst, Peter van der meer, Vasan S. Ramachandran, Niek Verweij, Jarmo Virtamo, Uwe Völker, David R. Weir, Eleftheria Zeggini, Fadi J. Charchar, Million Veteran Program, Nicholas J. Wareham, Claudia Langenberg, Maciej Tomaszewski, Adam S. Butterworth, Mark J. Caulfield, John Danesh, Todd L. Edwards, Hilma Holm, Adriana M. Hung, Cecilia M. Lindgren, Chunyu Liu, Alisa K. Manning, Andrew P. Morris, Alanna C. Morrison, Chris O'Donnell, Bruce M. Psaty, Danish Saleheen, Kari Stefansson, Eric Boerwinkle, Daniel I. Chasman, Daniel Levy, Christopher Newton-Cheh, Patricia B. Munroe, Joanna M. M. Howson. 87 rare variants associated with blood pressure regulation in meta-analysis of ~1.3 million individuals. *Revision in Nature Genetics*

Asllanaj E, Dhana A, Ikram MA, Muka T, Dhana K, Franco OH. Hypertension and life expectancy with and without cardiovascular disease. *Submitted for publication*

Asllanaj E, Subramanian A, Muka T, Gokhale K, Franco OH, Nirantharakumar K. Type 2 diabetes, life expectancy and the number of years lived with and without cardiovascular disease. *Manuscript*

Glisic M, Zaimi I, M. Arfan Ikram, Laven J.S.E., Kavousi M, Muka T, Franco O.H, **Asllanaj E**. Endogenous dehydroepiandrosterone levels and its derivatives modifies the association between total estradiol and type 2 diabetes risk in postmenopausal women: The Rotterdam Study.

Asllanaj E, Kastrati N, Meun C, Sedaghat S, Ikram M. A, Laven J.S.E, Nirantharankumar K, Franco OH, Muka T, Glisic M. Prognostic value of dehydroepiandrosterone in type 2 diabetes: The Rotterdam Study

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Seminars at the department of Epidemiology, Erasmus MC	2015-2019	2.0
2020 meetings, Erasmus MC	2015-2019	2.0
SHIP-WIP, Greifswald, Germany	2018	1.0
Conferences and presentations		
Nutritional Science Days (voormalig NWO Voeding) in Heeze- *Oral Presentation*	2019	0.3
American Diabetes Association (ADA) in Orlando, Florida- *Poster Presentation*	2018	0.3
XXXII meeting GISED meeting, in Florence, Italy- *Oral presentation*	2018	0.3
Automated Clinical Epidemiology Studies (ACES) to bridge Public Health and Basic Science Research, Birmingham, UK-*Oral presentation*	2017	0.3
the CardiOvascular Systems MEdicine COME "From disease to p-values and back to the patient", Hamburg, Germany- *Poster presentation*	2017	0.3
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Biostatistical Methods I	2017	1.0
Principles of Research in Medicine and Epidemiology	2016	1.0
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Volunteering		
European Menopause and Andropause Society (EMAS), 2017		

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Eralda Asllanaj was born on May 26th 1988 in Berat, Albania. She completed her medical studies in 2013 at the Faculty of Medicine, University of Tirana, Albania. In 2015, Eralda moved to the Netherlands to pursue a Master of Science in Genetic Epidemiology at the Netherlands Institute of Sciences (NIHES). Her master thesis investigated the causality of the association of age when natural menopause occurs with blood pressure. In August 2016, she received an ERAWEB scholarship to start her PhD trajectory. In parallel, from August 2016 till August 2017 she completed a Doctor of Science program in Clinical Epidemiology at NIHES, investigating the role of reproductive and lifestyle factors in type 2 diabetes and longevity. Subsequently, she continued working at the department of Epidemiology at Erasmus MC and expanded her studies to her PhD research in collaboration with the department of Community Medicine at the University of Greifswald in Germany. In the upcoming years she will pursue her post-doctoral research at the department of Health Evidence at Radboud University Medical Center in Nijmegen.

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In a world where you can be anything,
be kind