Novel Risk Markers for Type 2 Diabetes

Inflammation, Body Fat and Sex Hormones

ADELA BRAHIMAJ

Novel Risk Markers for Type 2 Diabetes

Inflammation, Body Fat and Sex Hormones

ADELA BRAHIMAJ

ACKNOWLEDGEMENTS

The work presented in this thesis was conducted at the Cardiovascular Group of the Department of Epidemiology, Erasmus Medical Center, Rotterdam, the Netherlands.

All of the studies described in this thesis involved the Rotterdam Study, which is supported by the Erasmus Medical Center and the Erasmus University Rotterdam, the Netherlands Organization for Scientific Research (NOW), the Netherlands Organization for Health Research and Development (ZonMw), the Dutch Heart Foundation, the Research Institute for Diseases in Elderly (RIDE), the Ministry of Education, Culture, and Science, the Ministry of Health, Welfare and Sports, the European Commission, and the municipality of Rotterdam.

Publication of this thesis was kindly supported by the Department of Epidemiology of Erasmus Medical Center. Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged. Further financial support was kindly provided by ChipSoft, Ortholon bv and Boehringer Ingelheim bv.

ISBN: 978-94-6361-081-0
Cover design: Adela Brahimaj & Optima Grafische Communicatie
Thesis layout and printing: Optima Grafische Communicatie

Novel Risk Markers for Type 2 Diabetes
Inflammation, Body Fat and Sex Hormones

Nieuwe Risicomarkers Voor Diabetes Type 2
Ontsteking, Lichaamsvet en Geslachtshormonen

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof. dr. H. A. P. Pols
en volgens besluit van het College voor Promoties
De openbare verdediging zal plaatsvinden op
25 april 2018 om 11.30 uur

door

Adela Brahimaj
geboren te Vlorë, Albanië

Erasmus University Rotterdam

DOCTORAL COMMITTEE

Promotor:	Prof. dr. O. H. Franco
Other members:	Prof. dr. E. J. G. Sijbrands Prof. dr. E. F. C. van Rossum Prof. dr. C. D. A. Stehouwer
Co-promotors:	Dr. M. Kavousi Dr. A. Dehghan

Paranymphs:	B. Dhamo E. Shevroja

To my loved ones

CONTENTS

MANUSCRIPTS THAT FORM THE BASIS OF THIS THESIS

1. **Brahimaj A**, Ligthart S, Ikram MA, Hofman A, Franco OH, Sijbrands EJ, Kavousi M, Dehghan A: Serum Levels of Apolipoproteins and Incident Type 2 Diabetes: A Prospective Cohort Study. Diabetes Care 2017;40:346-351
2. **Brahimaj A**, Muka T, Kavousi M, Laven JS, Dehghan A, Franco OH: Serum dehydroepiandrosterone levels are associated with lower risk of type 2 diabetes: the Rotterdam Study. Diabetologia 2017;60:98-106
3. **Brahimaj A**, Ligthart S, Ghanbari M, Ikram MA, Hofman A, Franco OH, Kavousi M, Dehghan A: Novel inflammatory markers for incident pre-diabetes and type 2 diabetes: the Rotterdam Study. Eur J Epidemiol 2017;32:217-226
4. van der Schaft N, **Brahimaj A**, Wen KX, Franco OH, Dehghan A: The association between serum uric acid and the incidence of prediabetes and type 2 diabetes mellitus: The Rotterdam Study. PLoS One 2017;12:e0179482
5. Stringa N, **Brahimaj A**, Zaciragic A, Dehghan A, Ikram MA, Hofman A, Muka T, Kiefte-de Jong JC, Franco OH: Relation of antioxidant capacity of diet and markers of oxidative status with C-reactive protein and adipocytokines: a prospective study. Metabolism 2017;71:171-181
6. **Brahimaj A**, Rivadeneira F, Muka T, Sijbrands E.J.G., Franco OH, Dehghan A, Kavousi M: Novel metabolic indices and incident type 2 diabetes among women and men: The Rotterdam Study. (submitted)
7. **Brahimaj A**, Bos D, Vernooij MW, Ikram MA, Dehghan A, Franco OH, Kavousi M: Epicardial fat volume and the risk for incident type 2 diabetes and cardiovascular disease: the Rotterdam Study. (manuscript)
8. Stringa N, **Brahimaj A**, Trajanoska K, Kiefte-de Jong JC, Ikram MA, Laven J, Rivadeneira F, Muka T, Franco OH: Endogenous sex hormone levels relate with 5-year changes in body composition in postmenopausal women: the Rotterdam Study. (submitted)

Chapter 1

General introduction

INTRODUCTION

Definition of type 2 diabetes and prediabetes

Type 2 diabetes (T2D), the most common form of diabetes mellitus, is a serious (1-4), chronic metabolic disease, characterized by hyperglycemia that occurs due to reductions in both insulin sensitivity and beta-cell function (5; 6). The current World Health Organization (WHO) diagnostic criteria define diabetes as fasting plasma glucose ≥ 7.0mmol/l (126mg/dl), or 2–h plasma glucose ≥ 11.1mmol/l (200mg/dl), or on medication for raised blood glucose, or with a history of diagnosis of diabetes (7). The precursor condition before diabetes is called prediabetes. Prediabetes should not be viewed as a clinical entity in its own, but rather as an increased risk for diabetes (8), in which not all of the symptoms that are required to diagnose diabetes have to be present, but blood glucose level is abnormally high. WHO opted to keep its upper limit of normal at under 110 mg/dl (6.1 mmol/l) for fear of causing too many people to be diagnosed with prediabetes (9), whereas the American Diabetes Association (ADA) lowered the upper limit of normal to a fasting plasma glucose under 100 mg/dl (5.6 mmol/l) (10).

Epidemiology of type 2 diabetes

In recent decades, both the number of cases and the prevalence of T2D have been steadily increasing in epidemic proportions worldwide (11). The global prevalence of diabetes has nearly doubled since 1980, rising from 4.7% (108 millions) to 8.5% (422 millions) in the adult population (12), becoming one of the most challenging public health issues of 21st century (13). The prevalence of T2D vastly exceeds that of type 1 diabetes (T1D), accounting for > 95% of diabetes (14). However, separate global estimates of diabetes prevalence for T1D and T2D do not exist, because sophisticated laboratory tests are usually required. Diabetes, the fifth leading cause of death worldwide in 2015, is expected to be the seventh leading cause of death in 2030 according to WHO (15).

Due to its adverse effect on people's health, diabetes imposes an economic burden not only on individuals affected and their families, but also on healthcare systems and the whole society (16; 17). Besides the chronic nature of diabetes, its many complications make it a costly disease. Much of the burden of diabetes is due to the development of vascular complications, divided into microvascular (due to damage to small blood vessels) and macrovascular (due to damage to larger blood vessels). These are major causes of disability, reduced quality of life, and death (18). T1D cannot be prevented with current knowledge, but effective approaches are available to prevent T2D or delay its onset (12; 13; 19). A better prevention requires a better investigation of its etiology and identification of T2D risk markers, preferably valuable for the earliest stages of the disease.

Pathogenesis of type 2 diabetes

The etiology of T2D is complex and multifactorial. Although T2D has a strong genetic component (20-27), behavioral/environmental factors (prenatal factors, obesity, physical inactivity, dietary and socioeconomic factors) have a significant role in triggering this condition (28-30). The complex interaction between genes and environment through epigenetic modifications

(DNA methylation or histone modifications) makes a person susceptible to develop T2D (31; 32). It is generally agreed that all these factors trigger both tissue insulin resistance (in muscle, liver, adipose tissue) or beta-cell dysfunction, the two major pathophysiologic events driving type 2 diabetes, coming into play with different time courses (5; 33; 34). Together, these abnormalities result in increased rates of glucose release by the liver and glucose filtration in kidney as well as decreased clearance from the circulation (35). The latter, insulin resistance in muscle is mostly recognized as the earliest detectable abnormality that persist leading the long run into prediabetes and type 2 diabetes (36; 37). However, it is well accepted that for hyperglycemia to exist in type 2 diabetes, β-cell dysfunction has to be present (38), probably before the development of hyperglycemia, and may commence many years before diagnosis of the disease (39; 40). Despite the large number of previous studies, the mechanisms controlling the interplay of these two impairments remain unclear. A number of factors related to specific pathways have been suggested as possibly linking insulin resistance and beta-cell dysfunction in the pathogenesis of type 2 diabetes. In this regard, the ongoing investigation on traditional or novel type 2 diabetes risk factors is crucial. Therefore, in this thesis, we focused in three sets of type 2 diabetes risk factors: inflammatory markers, lipids and body fat and sex hormones.

Inflammation and type 2 diabetes

There has been growing evidence that chronic low-grade systemic inflammation is a key component in the development of T2D, adding further weight to the concept of type 2 diabetes as an inflammatory disease (41-46). Chronic low-grade inflammation is an ongoing, destructive process, which instead of turning off when it should, it turns harmful acting like a slow-burning fire, continuing to stimulate pro-inflammatory immune cells that attack healthy areas of the body. It differs from normal inflammation in that there are no typical signs of inflammation, but it is similar in that it shares the disorders generated by typical inflammation mediators and signaling pathways (47). The proposed inflammation related mechanisms to explain impaired insulin secretion and sensitivity in type 2 diabetes include oxidative stress (48; 49), endoplasmic reticulum stress (50-52), amyloid deposition in pancreas (53), lipotoxicity and ectopic lipid deposition in the muscle, liver and pancreas (54; 55) and glucotoxicity (55). These mechanisms may induce an inflammatory response or are exacerbated by inflammation. They are strongly linked and have a role in both insulin resistance and islet β-cell failure (41), except for amyloid deposition, which mainly leads to progressive loss of β-cells (56; 57). The mechanisms thought to be responsible for the inflammatory state in type 2 diabetes include reduced oxygenation (58), apoptosis of expanding adipose tissue (59) and β-cells (60), transcriptional pathways (JNK, IKK-β/ NF-κB or PKR pathways) (61-63), activation of interleukin-1β (64), and recruitment and activation of immune cells. The most common inflammatory state in type 2 diabetes is the metabolic one, termed metaflammation and defined as low-grade, chronic inflammation, orchestrated by metabolic cells in response to excess nutrients and energy (44; 65). Overnutrition (lipotoxicity and glucotoxicity) stresses the pancreatic islets and insulin sensitive tissues (adipose tissue, the liver and muscle), leading to the local production and release of cytokines such as interleukins, adipocytokines, chemokines (66). The release of all the mediators from the adipose tissues into the circulation promotes inflammation in other tissues, including the islets (67; 68). In this regard,

many inflammatory markers (TNF-α, CRP, IL-1β, IL-6 etc.) are associated with type 2 diabetes and are suggested as risk predictors for the disease (69-73). Similarly, IL-1Ra serum levels are elevated in obesity and prediabetes with an accelerated increase before the onset of T2D (74; 75). However, the immune response involved in each phase of T2D development might be different (76). So far, the focus has been on a limited number of inflammatory markers that predict the progression from normoglycemia to T2D. The specific inflammatory profile of different phases of T2D development (prediabetes, T2D, insulin therapy initiation) remain unknown. Newly identified inflammatory markers may be key players in the induction of T2D and will shed light on the pathophysiology of the disease. Furthermore, missing links between lifestyle (mainly diet and physical activity), inflammation and T2D may be discovered, increasing the knowledge on T2D etiology and permitting more timely better prevention (77; 78). Among the modifiable lifestyle factors, diet seems to be of great importance for the earliest natural prevention of T2D, given that certain dietary patterns may increase chronic inflammation and raise the risk of developing type 2 diabetes (79).

Body fat, lipids and type 2 diabetes

A vast body of evidence indicates that obesity is a strong risk factor for developing insulin resistance, prediabetes and T2D and may play a causal role in their development (80-83). Moreover, the term "diabesity", indicating the coexistence of both T2D and obesity has been created to highlight the importance of obesity as an etiologic cause of T2D (84). Although most T2D patients are obese, yet most obese individuals do not develop T2D (85). This fact emphasizes the role of the body fat distribution, rather than obesity itself in the development of the unfavorable metabolic profiles, such as T2D (82; 86).

There are generally two types of fat storage: the visceral (surrounding internal organs) and the subcutaneous (beneath the skin, about 80% of all body fat). There are two basic areas of body fat location: around the buttocks and thighs or "pear-shaped", mostly found in women and around the abdomen or "apple-shaped", mostly found in men.

Central (intra-abdominal) obesity is observed in the majority of patients with T2D (85). While the dose relationship between general obesity and T2D risk is mainly defined by BMI (87), the most common measures of central obesity are waist circumference and waist-to- hip ratio, which have also been implicated as T2D risk markers in previous studies (88; 89). Although the clinical perspective focusing on central obesity is appealing, these three obesity indicators have similar associations with incident diabetes (89; 90).

Recently, excess visceral fat, but not general adiposity, has been independently associated with incident prediabetes and type 2 diabetes in obese adults (91; 92). Furthermore, ectopic and visceral fat deposition were reported to be high in diabetics with or without obesity (93; 94). Given that the golden standards to measure visceral fat such as MRI and CT are expensive, the identification of a routinely applicable indicator for the evaluation of visceral adipose function, with higher sensitivity and specificity than classical parameters (BMI, WC) could be useful for T2D risk assessment (95-97).

Insulin resistance is induced by fat deposited intracellularly and the secretory products of the expanded adipocyte mass, which has been recognized as the body's most prolific endocrine organ

(98). The result is the release of a host of inflammatory adipokines and excessive amounts of free fatty acids that promote ectopic fat deposition and lipotoxicity in muscle, liver, and pancreatic β cells. Hence, T2D as outcome is the sum total of multiple components, one of which is disturbances in lipid metabolism (99; 100), present also in prediabetes (101).Thus, the optimal indicator of visceral fat should preferably reflect both its quantitative part and its metabolic activities, with particular regard to effects on serum lipids and lipoproteins (102). In this regard, the investigation of associations between different lipoprotein components (such as apolipoproteins), newly proposed metabolic/body composition indices (such as visceral adiposity index) or specific visceral fat portions (such as epicardial fat) and T2D may shed light on the underlying mechanisms of the disease and help better discrimination of subjects at high risk for T2D. Moreover, there are controversial conclusions on the value of traditional body composition parameters for T2D risk prediction (103-105). Although the strong association between visceral fat and increased risk of T2D (106) suggests that measures of central fat distribution (WC) may be better than measures of general obesity (BMI)(107), further research is needed to clarify the issue.

Sex hormones and type 2 diabetes

Previous literature indicates that endogenous sex hormones may differentially modulate glycemic status and risk of type 2 diabetes in men and women (108). Moreover, the association between adiposity and T2D risk was reported to be stronger in postmenopausal women than in men (109). This might be explained by hormonal changes during menopause, which contribute to an increase in visceral adiposity and therefore may influence the risk of T2D (110; 111).

Emerging evidence from observational studies shows that, irrespective of sex, higher levels of sex-hormone binding globulin (SHBG) are associated with lower risk of developing T2D (112). Increasing genetic evidence is showing that SHBG and sex hormones are involved in the etiology of T2D (113; 114). However, literature on the associations of steroid sex hormones, such as endogenous estradiol and testosterone with T2D is scarce. Studies have shown that high testosterone levels are associated with higher risk of type 2 diabetes in women but with lower risk in men (108; 115). The previous literature on the association between estradiol and T2D risk remains controversial (116). Higher concentrations of total estradiol are associated with increased risk of T2D (117-119), while randomized controlled trials have consistently shown decreased T2D incidence in women assigned to menopausal treatment with estrogens (120).

Less is known about the relation between dehydroepiandrosterone (DHEA) and the risk of T2D development in humans. Most of the evidence on the positive role of DHEA in glucose metabolism comes from animal studies (121), while the results from human studies are rare, controversial (122-124) and mainly conducted in postmenopausal women .

Despite extensive research on sex hormones and the risk of T2D development, the evidence on the role of upstream hormones such as DHEA from longitudinal studies including both healthy women and men remains scarce. Moreover, further research is needed on the way sex hormones affect T2D etiological components such as obesity and body fat distribution.

Outline of this thesis

In this thesis, I attempt to provide additional evidence from the large prospective population-based Rotterdam Study, regarding the role of already known or novel risk markers for prediabetes and type 2 diabetes risk. I mainly focus on markers of inflammation, body fat and lipids as well as sex hormones role in the development of T2D. The second chapter is focused on the role of inflammation in T2D. **Chapter 2.1** investigates the association between several novel inflammatory markers and incident prediabetes, T2D as well as insulin therapy start. **Chapter 2.2** examines the role of total antioxidant capacity of diet and plasma markers of oxidant-antioxidant status in low-grade chronic inflammation. The objective of **chapter 2.3** is to determine whether serum uric acid is associated with incident prediabetes among normoglycaemic individuals and incident T2D among prediabetic individuals.

The third chapter focuses on the role of body fat and lipids in type 2 diabetes risk. **Chapter 3.1**investigates the capacity of different HDL apolipoproteins as biomarkers for incident type 2 diabetes. In **chapter 3.2** novel metabolic indices are studied as potential markers for the risk of T2D separately in women and men from the Rotterdam Study. Moreover, this chapter assesses the associations of truncal fat depot measured by DXA with incident type 2 diabetes.

Chapter 3.3 examines the associations between epicardial fat volume and incident type 2 diabetes as well as stroke and hard coronary heart disease.

In the fourth chapter, I focus on the role of sex hormones in T2D risk. **Chapter 4.1** aims to assess the associations between serum levels of DHEA, its main derivatives- DHEAS and androstenedione as well as the DHEAS-to-DHEA ratio with the risk of type 2 diabetes. **Chapter 4.2** aims to explore if endogenous sex hormone levels relate to changes in body composition in postmenopausal women.

Finally, the general discussion (**chapter 5**) summarizes the key findings of the studies included in this thesis, places the results in the context of the current literature, elaborates on their potential clinical implications and discusses the directions for future research.

REFERENCES

1. Emerging Risk Factors C, Seshasai SR, Kaptoge S, Thompson A, Di Angelantonio E, Gao P, Sarwar N, Whincup PH, Mukamal KJ, Gillum RF, Holme I, Njolstad I, Fletcher A, Nilsson P, Lewington S, Collins R, Gudnason V, Thompson SG, Sattar N, Selvin E, Hu FB, Danesh J: Diabetes mellitus, fasting glucose, and risk of cause-specific death. N Engl J Med 2011;364:829-841
2. King H, Aubert RE, Herman WH: Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. Diabetes Care 1998;21:1414-1431
3. Zhang P, Gregg E: Global economic burden of diabetes and its implications. Lancet Diabetes Endocrinol 2017;5:404-405
4. Bommer C, Heesemann E, Sagalova V, Manne-Goehler J, Atun R, Barnighausen T, Vollmer S: The global economic burden of diabetes in adults aged 20-79 years: a cost-of-illness study. Lancet Diabetes Endocrinol 2017;5:423-430
5. Kahn SE PDJ: Pathophysiology of type II diabetes mellitus. 1996;In: Diabetes Mellitus. Porte D Jr, Sherwin RS, Eds. Stamford, CT: Appleton & Lange: p. 487–512
6. Kahn SE: The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. Diabetologia 2003;46:3-19
7. World Health Organization G: Definition, diagnosis and classification of diabetes mellitus and its complications : report of a WHO consultation. Part 1, Diagnosis and classification of diabetes mellitus. 1999;
8. American Diabetes A: 2. Classification and Diagnosis of Diabetes. Diabetes Care 2017;40:S11-S24
9. World Health Organization G: Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation. 2006;
10. American Diabetes A: Diagnosis and classification of diabetes mellitus. Diabetes Care 2005;28 Suppl 1:S37-42
11. Boyle JP, Thompson TJ, Gregg EW, Barker LE, Williamson DF: Projection of the year 2050 burden of diabetes in the US adult population: dynamic modeling of incidence, mortality, and prediabetes prevalence. Popul Health Metr 2010;8:29
12. World Health Organization G: Global report on diabetes. 2016;
13. Iqbal N: The burden of type 2 diabetes: strategies to prevent or delay onset. Vasc Health Risk Manag 2007;3:511-520
14. Prevention. CfDCa: National Diabetes Fact Sheet: General Information and National Estimates on Diabetes in the United States, 2005. Atlanta, GA: US Department of Health and Human Services, Centers for Disease Control and Prevention.
15. Mathers CD, Loncar D: Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med 2006;3:e442
16. Ettaro L, Songer TJ, Zhang P, Engelgau MM: Cost-of-illness studies in diabetes mellitus. Pharmacoeconomics 2004;22:149-164
17. Ng CS, Lee JY, Toh MP, Ko Y: Cost-of-illness studies of diabetes mellitus: a systematic review. Diabetes Res Clin Pract 2014;105:151-163
18. Chaturvedi N: The burden of diabetes and its complications: trends and implications for intervention. Diabetes Res Clin Pract 2007;76 Suppl 1:S3-12
19. Taylor R: Type 2 diabetes: etiology and reversibility. Diabetes Care 2013;36:1047-1055
20. Rich SS: Mapping genes in diabetes. Genetic epidemiological perspective. Diabetes 1990;39:1315-1319
21. Prokopenko I, McCarthy MI, Lindgren CM: Type 2 diabetes: new genes, new understanding. Trends Genet 2008;24:613-621
22. Voight BF, Scott LJ, Steinthorsdottir V, Morris AP, Dina C, Welch RP, Zeggini E, Huth C, Aulchenko YS, Thorleifsson G, McCulloch LJ, Ferreira T, Grallert H, Amin N, Wu G, Willer CJ, Raychaudhuri S, McCarroll SA, Langenberg C, Hofmann OM, Dupuis J, Qi L, Segre AV, van Hoek M, Navarro P, Ardlie K, Balkau B, Benediktsson R, Bennett AJ, Blagieva R, Boerwinkle E, Bonnycastle LL, Bengtsson Bostrom K, Bravenboer B, Bumpstead S, Burtt NP, Charpentier G, Chines PS, Cornelis M, Couper DJ, Crawford G, Doney AS, Elliott KS, Elliott AL, Erdos MR, Fox CS, Franklin CS, Ganser M, Gieger C, Grarup N, Green T, Griffin S, Groves CJ, Guiducci C, Hadjadj S, Hassanali N, Herder C, Isomaa B, Jackson AU, Johnson PR, Jorgensen T, Kao WH, Klopp N, Kong A, Kraft P, Kuusisto J, Lauritzen T, Li M, Lieverse A, Lindgren CM, Lyssenko V, Marre M, Meitinger T, Midthjell K, Morken MA, Narisu N, Nilsson P, Owen KR, Payne F, Perry JR, Petersen AK, Platou C, Proenca C, Prokopenko I, Rathmann W, Rayner NW, Robertson NR, Rocheleau G, Roden M, Sampson MJ, Saxena R, Shields BM, Shrader P, Sigurdsson G, Sparso T, Strassburger K, Stringham

HM, Sun Q, Swift AJ, Thorand B, Tichet J, Tuomi T, van Dam RM, van Haeften TW, van Herpt T, van Vliet-Ostaptchouk JV, Walters GB, Weedon MN, Wijmenga C, Witteman J, Bergman RN, Cauchi S, Collins FS, Gloyn AL, Gyllensten U, Hansen T, Hide WA, Hitman GA, Hofman A, Hunter DJ, Hveem K, Laakso M, Mohlke KL, Morris AD, Palmer CN, Pramstaller PP, Rudan I, Sijbrands E, Stein LD, Tuomilehto J, Uitterlinden A, Walker M, Wareham NJ, Watanabe RM, Abecasis GR, Boehm BO, Campbell H, Daly MJ, Hattersley AT, Hu FB, Meigs JB, Pankow JS, Pedersen O, Wichmann HE, Barroso I, Florez JC, Frayling TM, Groop L, Sladek R, Thorsteinsdottir U, Wilson JF, Illig T, Froguel P, van Duijn CM, Stefansson K, Altshuler D, Boehnke M, McCarthy MI, investigators M, Consortium G: Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. Nat Genet 2010;42:579-589

23. Meigs JB, Cupples LA, Wilson PW: Parental transmission of type 2 diabetes: the Framingham Offspring Study. Diabetes 2000;49:2201-2207
24. Altshuler D, Hirschhorn JN, Klannemark M, Lindgren CM, Vohl MC, Nemesh J, Lane CR, Schaffner SF, Bolk S, Brewer C, Tuomi T, Gaudet D, Hudson TJ, Daly M, Groop L, Lander ES: The common PPARgamma Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes. Nat Genet 2000;26:76-80
25. Grant SF, Thorleifsson G, Reynisdottir I, Benediktsson R, Manolescu A, Sainz J, Helgason A, Stefansson H, Emilsson V, Helgadottir A, Styrkarsdottir U, Magnusson KP, Walters GB, Palsdottir E, Jonsdottir T, Gudmundsdottir T, Gylfason A, Saemundsdottir J, Wilensky RL, Reilly MP, Rader DJ, Bagger Y, Christiansen C, Gudnason V, Sigurdsson G, Thorsteinsdottir U, Gulcher JR, Kong A, Stefansson K: Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. Nat Genet 2006;38:320-323
26. Yasuda K, Miyake K, Horikawa Y, Hara K, Osawa H, Furuta H, Hirota Y, Mori H, Jonsson A, Sato Y, Yamagata K, Hinokio Y, Wang HY, Tanahashi T, Nakamura N, Oka Y, Iwasaki N, Iwamoto Y, Yamada Y, Seino Y, Maegawa H, Kashiwagi A, Takeda J, Maeda E, Shin HD, Cho YM, Park KS, Lee HK, Ng MC, Ma RC, So WY, Chan JC, Lyssenko V, Tuomi T, Nilsson P, Groop L, Kamatani N, Sekine A, Nakamura Y, Yamamoto K, Yoshida T, Tokunaga K, Itakura M, Makino H, Nanjo K, Kadowaki T, Kasuga M: Variants in KCNQ1 are associated with susceptibility to type 2 diabetes mellitus. Nat Genet 2008;40:1092-1097
27. Diabetes Genetics Initiative of Broad Institute of H, Mit LU, Novartis Institutes of BioMedical R, Saxena R, Voight BF, Lyssenko V, Burtt NP, de Bakker PI, Chen H, Roix JJ, Kathiresan S, Hirschhorn JN, Daly MJ, Hughes TE, Groop L, Altshuler D, Almgren P, Florez JC, Meyer J, Ardlie K, Bengtsson Bostrom K, Isomaa B, Lettre G, Lindblad U, Lyon HN, Melander O, Newton-Cheh C, Nilsson P, Orho-Melander M, Rastam L, Speliotes EK, Taskinen MR, Tuomi T, Guiducci C, Berglund A, Carlson J, Gianniny L, Hackett R, Hall L, Holmkvist J, Laurila E, Sjogren M, Sterner M, Surti A, Svensson M, Svensson M, Tewhey R, Blumenstiel B, Parkin M, Defelice M, Barry R, Brodeur W, Camarata J, Chia N, Fava M, Gibbons J, Handsaker B, Healy C, Nguyen K, Gates C, Sougnez C, Gage D, Nizzari M, Gabriel SB, Chirn GW, Ma Q, Parikh H, Richardson D, Ricke D, Purcell S: Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. Science 2007;316:1331-1336
28. Adeghate E, Schattner P, Dunn E: An update on the etiology and epidemiology of diabetes mellitus. Ann N Y Acad Sci 2006;1084:1-29
29. Murea M, Ma L, Freedman BI: Genetic and environmental factors associated with type 2 diabetes and diabetic vascular complications. Rev Diabet Stud 2012;9:6-22
30. Hu FB, Manson JE, Stampfer MJ, Colditz G, Liu S, Solomon CG, Willett WC: Diet, lifestyle, and the risk of type 2 diabetes mellitus in women. N Engl J Med 2001;345:790-797
31. Ling C, Groop L: Epigenetics: a molecular link between environmental factors and type 2 diabetes. Diabetes 2009;58:2718-2725
32. Bird A: Perceptions of epigenetics. Nature 2007;447:396-398
33. Saltiel AR, Kahn CR: Insulin signalling and the regulation of glucose and lipid metabolism. Nature 2001;414:799-806
34. Weir GC: Which comes first in non-insulin-dependent diabetes mellitus: insulin resistance or beta-cell failure? Both come first. JAMA 1995;273:1878-1879
35. Kolterman OG, Gray RS, Shapiro G, Scarlett JA, Griffin J, Olefsky JM: The acute and chronic effects of sulfonylurea therapy in type II diabetic subjects. Diabetes 1984;33:346-354
36. Petersen KF, Dufour S, Morino K, Yoo PS, Cline GW, Shulman GI: Reversal of muscle insulin resistance by weight reduction in young, lean, insulin-resistant offspring of parents with type 2 diabetes. Proc Natl Acad Sci U S A 2012;109:8236-8240
37. Goldstein BJ: Insulin resistance: from benign to type 2 diabetes mellitus. Rev Cardiovasc Med 2003;4 Suppl 6:S3-10

38. Kahn SE: Clinical review 135: The importance of beta-cell failure in the development and progression of type 2 diabetes. J Clin Endocrinol Metab 2001;86:4047-4058
39. Holman RR: Assessing the potential for alpha-glucosidase inhibitors in prediabetic states. Diabetes Res Clin Pract 1998;40 Suppl:S21-25
40. Weir GC, Bonner-Weir S: Five stages of evolving beta-cell dysfunction during progression to diabetes. Diabetes 2004;53 Suppl 3:S16-21
41. Donath MY, Shoelson SE: Type 2 diabetes as an inflammatory disease. Nat Rev Immunol 2011;11:98-107
42. Brooks-Worrell BM, Reichow JL, Goel A, Ismail H, Palmer JP: Identification of autoantibody-negative autoimmune type 2 diabetic patients. Diabetes Care 2011;34:168-173
43. Pickup JC, Mattock MB, Chusney GD, Burt D: NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. Diabetologia 1997;40:1286-1292
44. Hotamisligil GS: Inflammation, metaflammation and immunometabolic disorders. Nature 2017;542:177-185
45. Schmidt MI, Duncan BB, Sharrett AR, Lindberg G, Savage PJ, Offenbacher S, Azambuja MI, Tracy RP, Heiss G: Markers of inflammation and prediction of diabetes mellitus in adults (Atherosclerosis Risk in Communities study): a cohort study. Lancet 1999;353:1649-1652
46. Wellen KE, Hotamisligil GS: Inflammation, stress, and diabetes. J Clin Invest 2005;115:1111-1119
47. Hotamisligil GS: Inflammation and metabolic disorders. Nature 2006;444:860-867
48. Zephy D, Ahmad J: Type 2 diabetes mellitus: Role of melatonin and oxidative stress. Diabetes Metab Syndr 2015;9:127-131
49. Rizzo MR, Barbieri M, Marfella R, Paolisso G: Reduction of oxidative stress and inflammation by blunting daily acute glucose fluctuations in patients with type 2 diabetes: role of dipeptidyl peptidase-IV inhibition. Diabetes Care 2012;35:2076-2082
50. Harding HP, Ron D: Endoplasmic reticulum stress and the development of diabetes: a review. Diabetes 2002;51 Suppl 3:S455-461
51. Hotamisligil GS: Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell 2010;140:900-917
52. Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Glimcher LH, Hotamisligil GS: Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 2004;306:457-461
53. Zraika S, Hull RL, Verchere CB, Clark A, Potter KJ, Fraser PE, Raleigh DP, Kahn SE: Toxic oligomers and islet beta cell death: guilty by association or convicted by circumstantial evidence? Diabetologia 2010;53:1046-1056
54. Cusi K: The role of adipose tissue and lipotoxicity in the pathogenesis of type 2 diabetes. Curr Diab Rep 2010;10:306-315
55. Del Prato S: Role of glucotoxicity and lipotoxicity in the pathophysiology of Type 2 diabetes mellitus and emerging treatment strategies. Diabet Med 2009;26:1185-1192
56. Westermark P: Amyloid in the islets of Langerhans: thoughts and some historical aspects. Ups J Med Sci 2011;116:81-89
57. Jurgens CA, Toukatly MN, Fligner CL, Udayasankar J, Subramanian SL, Zraika S, Aston-Mourney K, Carr DB, Westermark P, Westermark GT, Kahn SE, Hull RL: beta-cell loss and beta-cell apoptosis in human type 2 diabetes are related to islet amyloid deposition. Am J Pathol 2011;178:2632-2640
58. Pasarica M, Sereda OR, Redman LM, Albarado DC, Hymel DT, Roan LE, Rood JC, Burk DH, Smith SR: Reduced adipose tissue oxygenation in human obesity: evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response. Diabetes 2009;58:718-725
59. Strissel KJ, Stancheva Z, Miyoshi H, Perfield JW, 2nd, DeFuria J, Jick Z, Greenberg AS, Obin MS: Adipocyte death, adipose tissue remodeling, and obesity complications. Diabetes 2007;56:2910-2918
60. Tomita T: Apoptosis in pancreatic beta-islet cells in Type 2 diabetes. Bosn J Basic Med Sci 2016;16:162-179
61. Arkan MC, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM, Wynshaw-Boris A, Poli G, Olefsky J, Karin M: IKK-beta links inflammation to obesity-induced insulin resistance. Nat Med 2005;11:191-198
62. Solinas G, Karin M: JNK1 and IKKbeta: molecular links between obesity and metabolic dysfunction. FASEB J 2010;24:2596-2611
63. Solinas G, Becattini B: JNK at the crossroad of obesity, insulin resistance, and cell stress response. Mol Metab 2017;6:174-184
64. Mandrup-Poulsen T: The role of interleukin-1 in the pathogenesis of IDDM. Diabetologia 1996;39:1005-1029
65. Gregor MF, Hotamisligil GS: Inflammatory mechanisms in obesity. Annu Rev Immunol 2011;29:415-445

66. Robertson RP, Harmon J, Tran PO, Poitout V: Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. Diabetes 2004;53 Suppl 1:S119-124
67. Donath MY, Schumann DM, Faulenbach M, Ellingsgaard H, Perren A, Ehses JA: Islet inflammation in type 2 diabetes: from metabolic stress to therapy. Diabetes Care 2008;31 Suppl 2:S161-164
68. Marchetti P: Islet inflammation in type 2 diabetes. Diabetologia 2016;59:668-672
69. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM: C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. JAMA 2001;286:327-334
70. Spranger J, Kroke A, Mohlig M, Hoffmann K, Bergmann MM, Ristow M, Boeing H, Pfeiffer AF: Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. Diabetes 2003;52:812-817
71. Hu FB, Meigs JB, Li TY, Rifai N, Manson JE: Inflammatory markers and risk of developing type 2 diabetes in women. Diabetes 2004;53:693-700
72. Wang X, Bao W, Liu J, Ouyang YY, Wang D, Rong S, Xiao X, Shan ZL, Zhang Y, Yao P, Liu LG: Inflammatory markers and risk of type 2 diabetes: a systematic review and meta-analysis. Diabetes Care 2013;36:166-175
73. Krakoff J, Funahashi T, Stehouwer CD, Schalkwijk CG, Tanaka S, Matsuzawa Y, Kobes S, Tataranni PA, Hanson RL, Knowler WC, Lindsay RS: Inflammatory markers, adiponectin, and risk of type 2 diabetes in the Pima Indian. Diabetes Care 2003;26:1745-1751
74. Herder C, Brunner EJ, Rathmann W, Strassburger K, Tabak AG, Schloot NC, Witte DR: Elevated levels of the anti-inflammatory interleukin-1 receptor antagonist precede the onset of type 2 diabetes: the Whitehall II study. Diabetes Care 2009;32:421-423
75. Carstensen M, Herder C, Kivimaki M, Jokela M, Roden M, Shipley MJ, Witte DR, Brunner EJ, Tabak AG: Accelerated increase in serum interleukin-1 receptor antagonist starts 6 years before diagnosis of type 2 diabetes: Whitehall II prospective cohort study. Diabetes 2010;59:1222-1227
76. Grossmann V, Schmitt VH, Zeller T, Panova-Noeva M, Schulz A, Laubert-Reh D, Juenger C, Schnabel RB, Abt TG, Laskowski R, Wiltink J, Schulz E, Blankenberg S, Lackner KJ, Munzel T, Wild PS: Profile of the Immune and Inflammatory Response in Individuals With Prediabetes and Type 2 Diabetes. Diabetes Care 2015;38:1356-1364
77. Giugliano D, Ceriello A, Esposito K: The effects of diet on inflammation: emphasis on the metabolic syndrome. J Am Coll Cardiol 2006;48:677-685
78. Nowlin SY, Hammer MJ, D'Eramo Melkus G: Diet, inflammation, and glycemic control in type 2 diabetes: an integrative review of the literature. J Nutr Metab 2012;2012:542698
79. Schulze MB, Hoffmann K, Manson JE, Willett WC, Meigs JB, Weikert C, Heidemann C, Colditz GA, Hu FB: Dietary pattern, inflammation, and incidence of type 2 diabetes in women. Am J Clin Nutr 2005;82:675-684; quiz 714-675
80. Kahn SE, Hull RL, Utzschneider KM: Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006;444:840-846
81. Li S, Zhao JH, Luan J, Langenberg C, Luben RN, Khaw KT, Wareham NJ, Loos RJ: Genetic predisposition to obesity leads to increased risk of type 2 diabetes. Diabetologia 2011;54:776-782
82. Ntuk UE, Gill JM, Mackay DF, Sattar N, Pell JP: Ethnic-specific obesity cutoffs for diabetes risk: cross-sectional study of 490,288 UK biobank participants. Diabetes Care 2014;37:2500-2507
83. Abdullah A, Stoelwinder J, Shortreed S, Wolfe R, Stevenson C, Walls H, de Courten M, Peeters A: The duration of obesity and the risk of type 2 diabetes. Public Health Nutr 2011;14:119-126
84. Astrup A, Finer N: Redefining type 2 diabetes: 'diabesity' or 'obesity dependent diabetes mellitus'? Obes Rev 2000;1:57-59
85. Mokdad AH, Ford ES, Bowman BA, Dietz WH, Vinicor F, Bales VS, Marks JS: Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. JAMA 2003;289:76-79
86. Eckel RH, Kahn SE, Ferrannini E, Goldfine AB, Nathan DM, Schwartz MW, Smith RJ, Smith SR, Endocrine S, American Diabetes A, European Association for the Study of D: Obesity and type 2 diabetes: what can be unified and what needs to be individualized? Diabetes Care 2011;34:1424-1430
87. Colditz GA, Willett WC, Stampfer MJ, Manson JE, Hennekens CH, Arky RA, Speizer FE: Weight as a risk factor for clinical diabetes in women. Am J Epidemiol 1990;132:501-513
88. Kaye SA, Folsom AR, Sprafka JM, Prineas RJ, Wallace RB: Increased incidence of diabetes mellitus in relation to abdominal adiposity in older women. J Clin Epidemiol 1991;44:329-334
89. Vazquez G, Duval S, Jacobs DR, Jr., Silventoinen K: Comparison of body mass index, waist circumference, and waist/hip ratio in predicting incident diabetes: a meta-analysis. Epidemiol Rev 2007;29:115-128

90. Freemantle N, Holmes J, Hockey A, Kumar S: How strong is the association between abdominal obesity and the incidence of type 2 diabetes? Int J Clin Pract 2008;62:1391-1396
91. Boyko EJ, Fujimoto WY, Leonetti DL, Newell-Morris L: Visceral adiposity and risk of type 2 diabetes: a prospective study among Japanese Americans. Diabetes Care 2000;23:465-471
92. Neeland IJ, Turer AT, Ayers CR, Powell-Wiley TM, Vega GL, Farzaneh-Far R, Grundy SM, Khera A, McGuire DK, de Lemos JA: Dysfunctional adiposity and the risk of prediabetes and type 2 diabetes in obese adults. JAMA 2012;308:1150-1159
93. Levelt E, Pavlides M, Banerjee R, Mahmod M, Kelly C, Sellwood J, Ariga R, Thomas S, Francis J, Rodgers C, Clarke W, Sabharwal N, Antoniades C, Schneider J, Robson M, Clarke K, Karamitsos T, Rider O, Neubauer S: Ectopic and Visceral Fat Deposition in Lean and Obese Patients With Type 2 Diabetes. J Am Coll Cardiol 2016;68:53-63
94. Shah RV, Murthy VL, Abbasi SA, Blankstein R, Kwong RY, Goldfine AB, Jerosch-Herold M, Lima JA, Ding J, Allison MA: Visceral adiposity and the risk of metabolic syndrome across body mass index: the MESA Study. JACC Cardiovasc Imaging 2014;7:1221-1235
95. Neeland IJ, McGuire DK, Eliasson B, Ridderstrale M, Zeller C, Woerle HJ, Broedl UC, Johansen OE: Comparison of Adipose Distribution Indices with Gold Standard Body Composition Assessments in the EMPA-REG H2H SU Trial: A Body Composition Sub-Study. Diabetes Ther 2015;6:635-642
96. Amato MC, Giordano C: Visceral adiposity index: an indicator of adipose tissue dysfunction. Int J Endocrinol 2014;2014:730827
97. Amato MC, Giordano C, Galia M, Criscimanna A, Vitabile S, Midiri M, Galluzzo A, AlkaMeSy Study G: Visceral Adiposity Index: a reliable indicator of visceral fat function associated with cardiometabolic risk. Diabetes Care 2010;33:920-922
98. Wei X, Song H, Yin L, Rizzo MG, Sidhu R, Covey DF, Ory DS, Semenkovich CF: Fatty acid synthesis configures the plasma membrane for inflammation in diabetes. Nature 2016;539:294-298
99. Ivancova G, Tkacova R, Ivanco I: [Disorders of lipid metabolism in type 2 diabetics] Poruchy metabolizmu lipidov u diabetikov 2. typu. Vnitr Lek 1992;38:759-763
100. Halcox J, Misra A: Type 2 diabetes mellitus, metabolic syndrome, and mixed dyslipidemia: how similar, how different, and how to treat? Metab Syndr Relat Disord 2015;13:1-21
101. Haffner SM, Mykkanen L, Festa A, Burke JP, Stern MP: Insulin-resistant prediabetic subjects have more atherogenic risk factors than insulin-sensitive prediabetic subjects: implications for preventing coronary heart disease during the prediabetic state. Circulation 2000;101:975-980
102. Ahmed H. Kissebah GRK: Effects of Obesity on Lipid Metabolism 1997;Clinical Research in Diabetes and Obesity:73-91
103. Warne DK, Charles MA, Hanson RL, Jacobsson LT, McCance DR, Knowler WC, Pettitt DJ: Comparison of body size measurements as predictors of NIDDM in Pima Indians. Diabetes Care 1995;18:435-439
104. Tulloch-Reid MK, Williams DE, Looker HC, Hanson RL, Knowler WC: Do measures of body fat distribution provide information on the risk of type 2 diabetes in addition to measures of general obesity? Comparison of anthropometric predictors of type 2 diabetes in Pima Indians. Diabetes Care 2003;26:2556-2561
105. Wang Y, Rimm EB, Stampfer MJ, Willett WC, Hu FB: Comparison of abdominal adiposity and overall obesity in predicting risk of type 2 diabetes among men. Am J Clin Nutr 2005;81:555-563
106. Klein S, Allison DB, Heymsfield SB, Kelley DE, Leibel RL, Nonas C, Kahn R, Association for Weight M, Obesity P, Naaso, Obesity S, American Society for N, American Diabetes A: Waist circumference and cardiometabolic risk: a consensus statement from shaping America's health: Association for Weight Management and Obesity Prevention; NAASO, the Obesity Society; the American Society for Nutrition; and the American Diabetes Association. Diabetes Care 2007;30:1647-1652
107. Schulze MB, Heidemann C, Schienkiewitz A, Bergmann MM, Hoffmann K, Boeing H: Comparison of anthropometric characteristics in predicting the incidence of type 2 diabetes in the EPIC-Potsdam study. Diabetes Care 2006;29:1921-1923
108. Ding EL, Song Y, Malik VS, Liu S: Sex differences of endogenous sex hormones and risk of type 2 diabetes: a systematic review and meta-analysis. JAMA 2006;295:1288-1299
109. Wang SL, Pan WH, Hwu CM, Ho LT, Lo CH, Lin SL, Jong YS: Incidence of NIDDM and the effects of gender, obesity and hyperinsulinaemia in Taiwan. Diabetologia 1997;40:1431-1438
110. Liu Y, Ding J, Bush TL, Longenecker JC, Nieto FJ, Golden SH, Szklo M: Relative androgen excess and increased cardiovascular risk after menopause: a hypothesized relation. Am J Epidemiol 2001;154:489-494
111. Brand JS, van der Schouw YT, Onland-Moret NC, Sharp SJ, Ong KK, Khaw KT, Ardanaz E, Amiano P, Boeing H, Chirlaque MD, Clavel-Chapelon F, Crowe FL, de Lauzon-Guillain B, Duell EJ, Fagherazzi G,

Franks PW, Grioni S, Groop LC, Kaaks R, Key TJ, Nilsson PM, Overvad K, Palli D, Panico S, Quiros JR, Rolandsson O, Sacerdote C, Sanchez MJ, Slimani N, Teucher B, Tjonneland A, Tumino R, van der AD, Feskens EJ, Langenberg C, Forouhi NG, Riboli E, Wareham NJ, InterAct C: Age at menopause, reproductive life span, and type 2 diabetes risk: results from the EPIC-InterAct study. Diabetes Care 2013;36:1012-1019

112. Ding EL, Song Y, Manson JE, Hunter DJ, Lee CC, Rifai N, Buring JE, Gaziano JM, Liu S: Sex hormone-binding globulin and risk of type 2 diabetes in women and men. N Engl J Med 2009;361:1152-1163
113. Perry JR, Weedon MN, Langenberg C, Jackson AU, Lyssenko V, Sparso T, Thorleifsson G, Grallert H, Ferrucci L, Maggio M, Paolisso G, Walker M, Palmer CN, Payne F, Young E, Herder C, Narisu N, Morken MA, Bonnycastle LL, Owen KR, Shields B, Knight B, Bennett A, Groves CJ, Ruokonen A, Jarvelin MR, Pearson E, Pascoe L, Ferrannini E, Bornstein SR, Stringham HM, Scott LJ, Kuusisto J, Nilsson P, Neptin M, Gjesing AP, Pisinger C, Lauritzen T, Sandbaek A, Sampson M, Magic, Zeggini E, Lindgren CM, Steinthorsdottir V, Thorsteinsdottir U, Hansen T, Schwarz P, Illig T, Laakso M, Stefansson K, Morris AD, Groop L, Pedersen O, Boehnke M, Barroso I, Wareham NJ, Hattersley AT, McCarthy MI, Frayling TM: Genetic evidence that raised sex hormone binding globulin (SHBG) levels reduce the risk of type 2 diabetes. Hum Mol Genet 2010;19:535-544
114. Wang Q, Kangas AJ, Soininen P, Tiainen M, Tynkkynen T, Puukka K, Ruokonen A, Viikari J, Kahonen M, Lehtimaki T, Salomaa V, Perola M, Davey Smith G, Raitakari OT, Jarvelin MR, Wurtz P, Kettunen J, Ala-Korpela M: Sex hormone-binding globulin associations with circulating lipids and metabolites and the risk for type 2 diabetes: observational and causal effect estimates. Int J Epidemiol 2015;44:623-637
115. Corona G, Monami M, Rastrelli G, Aversa A, Sforza A, Lenzi A, Forti G, Mannucci E, Maggi M: Type 2 diabetes mellitus and testosterone: a meta-analysis study. Int J Androl 2011;34:528-540
116. Mauvais-Jarvis F: Is Estradiol a Biomarker of Type 2 Diabetes Risk in Postmenopausal Women? Diabetes 2017;66:568-570
117. Muka T, Nano J, Jaspers L, Meun C, Bramer WM, Hofman A, Dehghan A, Kavousi M, Laven JS, Franco OH: 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 2017;66:577-586
118. Ding EL, Song Y, Manson JE, Rifai N, Buring JE, Liu S: Plasma sex steroid hormones and risk of developing type 2 diabetes in women: a prospective study. Diabetologia 2007;50:2076-2084
119. Kalyani RR, Franco M, Dobs AS, Ouyang P, Vaidya D, Bertoni A, Gapstur SM, Golden SH: The association of endogenous sex hormones, adiposity, and insulin resistance with incident diabetes in postmenopausal women. J Clin Endocrinol Metab 2009;94:4127-4135
120. Margolis KL, Bonds DE, Rodabough RJ, Tinker L, Phillips LS, Allen C, Bassford T, Burke G, Torrens J, Howard BV, Women's Health Initiative I: Effect of oestrogen plus progestin on the incidence of diabetes in postmenopausal women: results from the Women's Health Initiative Hormone Trial. Diabetologia 2004;47:1175-1187
121. Coleman DL, Leiter EH, Schwizer RW: Therapeutic effects of dehydroepiandrosterone (DHEA) in diabetic mice. Diabetes 1982;31:830-833
122. Veronese N, Trevisan C, De Rui M, Bolzetta F, Maggi S, Zambon S, Corti MC, Baggio G, Perissinotto E, Crepaldi G, Manzato E, Sergi G: Serum Dehydroepiandrosterone Sulfate and Risk for Type 2 Diabetes in Older Men and Women: The Pro.V.A Study. Can J Diabetes 2016;40:158-163
123. Kawano H, Yasue H, Kitagawa A, Hirai N, Yoshida T, Soejima H, Miyamoto S, Nakano M, Ogawa H: Dehydroepiandrosterone supplementation improves endothelial function and insulin sensitivity in men. J Clin Endocrinol Metab 2003;88:3190-3195
124. Basu R, Dalla Man C, Campioni M, Basu A, Nair KS, Jensen MD, Khosla S, Klee G, Toffolo G, Cobelli C, Rizza RA: Two years of treatment with dehydroepiandrosterone does not improve insulin secretion, insulin action, or postprandial glucose turnover in elderly men or women. Diabetes 2007;56:753-766

Chapter 2

Inflammation and type 2 diabetes

Chapter 2.1

Novel inflammatory markers for incident prediabetes and type 2 diabetes mellitus: the Rotterdam Study.

Adela Brahimaj
Symen Ligthart
Mohsen Ghanbari
M. Arfan Ikram
Albert Hofman
Oscar H. Franco
Maryam Kavousi
Abbas Dehghan

Eur J Epidemiol. 2017

ABSTRACT

Background

The immune response involved in each phase of type 2 diabetes (T2D) development might be different. We aimed to identify novel inflammatory markers that predict progression from normoglycemia to pre-diabetes, incident T2D and insulin therapy.

Methods

We used plasma levels of 26 inflammatory markers in 971 subjects from the Rotterdam Study. Among them 17 are novel and 9 previously studied. Cox regression models were built to perform survival analysis.

Main Outcome Measures

During a follow-up of up to 14.7 years (between April 1, 1997, and Jan 1, 2012) 139 cases of pre-diabetes, 110 cases of T2D and 26 cases of insulin initiation were identified.

Results

In age and sex adjusted Cox models, IL13 (HR = 0.78), EN-RAGE (1.30), CFH (1.24), IL18 (1.22) and CRP (1.32) were associated with incident pre-diabetes. IL13 (0.62), IL17 (0.75), EN-RAGE (1.25), complement 3 (1.44), IL18 (1.35), TNFRII (1.27), IL1ra (1.24) and CRP (1.64) were associated with incident T2D. In multivariate models, IL13 (0.77), EN-RAGE (1.23) and CRP (1.26) remained associated with pre-diabetes. IL13 (0.67), IL17 (0.76) and CRP (1.32) remained associated with T2D. IL13 (0.55) was the only marker associated with initiation of insulin therapy in diabetics.

Conclusions

Various inflammatory markers are associated with progression from normoglycemia to pre-diabetes (IL13, EN-RAGE, CRP), T2D (IL13, IL17, CRP) or insulin therapy start (IL13). Among them, EN-RAGE is a novel inflammatory marker for pre-diabetes, IL17 for incident T2D and IL13 for pre-diabetes, incident T2D and insulin therapy start.

Keywords

inflammatory markers, phase-specific, pre-diabetes, type 2 diabetes, insulin therapy, novel, IL13, IL17, EN-RAGE.

INTRODUCTION

There is increasing evidence that inflammation plays a role in the development of type 2 diabetes mellitus (DM) (1-3). In this context, the identification of novel inflammatory markers associated with the risk of type 2 DM will shed light on the pathophysiology of the disease and might also help clinicians to target individuals at highest risk (4; 5). So far, a limited number of inflammatory markers have been investigated. Previous studies reported inflammatory markers including C-reactive protein (CRP), interleukin 6 (IL6) and adiponectin to associate with the risk of type 2 DM (6-11). These studies merely investigated inflammatory markers that predict the conversion from normoglycemia to type 2 DM.
Healthy individuals are thought to experience a pre-diabetes phase before developing type 2 DM. Pre-diabetes is the presence of blood glucose levels higher than normal, but not yet high enough to be classified as diabetes (12). Moreover, type 2 DM could further deteriorate to a stage, where glucose control is only possible by insulin therapy (12; 13). Progression from normoglycemia to pre-diabetes is thought to be driven by insulin resistance, while progression to type 2 DM and need for insulin therapy is further affected by beta cell dysfunction (14-16). Therefore, the immune response involved in each of these phases might be different (17).
We hypothesized that inflammatory markers are phase-specific for conversion from normoglycemia to pre-diabetes, diabetes and need for insulin therapy. We agnostically studied the association of a set of inflammatory markers with progression from normoglycemia to pre-diabetes, type 2 DM and finally to insulin therapy.

MATERIALS AND METHODS

Study population

The Rotterdam Study is a prospective population-based cohort study in Ommoord, a district of Rotterdam, the Netherlands. The design of the Rotterdam Study has been described in more detail elsewhere (18). Briefly, in 1989 all residents within the well-defined study area aged 55 years or older were invited to participate of whom 78% (7983 out of 10275) agreed. There were no other eligibility criteria to enter the Rotterdam Study except minimum age and residency are based on ZIP code. The first examination took place from 1990 to 1993, after which follow-up examinations were conducted every 3-5 years. This study was based on data collected during the third visit (1997-1999). We used data from 971 individuals with available data on inflammatory markers, drawn as a random control sample in a case-cohort study of markers for dementia. 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 Netherlands. All participants in the present analysis provided written informed consent to participate and to obtain information from their treating physicians.

Measurement of inflammatory markers

Fasting blood samples were collected at the research centre. Plasma was isolated and immediately put on ice and stored at -80°C. Citrate plasma (200Ul) was sent in July 2008 to Rules-Based Medicine, Austin, Texas (www.myriadrbm.com). The samples were thawed at room temperature, vortexed, spun at 4000 RPM for 5 minutes for clarification and volume was removed for MAP analysis into a master microtiter plate. Using automated pipetting, an aliquot of each sample was introduced into one of the capture microsphere multiplexes of the Multi Analyte Profile. The mixture of sample and capture microspheres were thoroughly mixed and incubated at room temperature for 1 hour. Multiplexed cocktails of biotinylated, reporter antibodies for each multiplex were then added robotically and after thorough mixing, were incubated for an additional hour at room temperature. Multiplexes were developed using an excess of streptavidin-phycoerythrin solution which was thoroughly mixed into each multiplex and incubated for 1 hour at room temperature. The volume of each multiplexed reaction was reduced by vacuum filtration and the volume increased by dilution into matrix buffer for analysis. Analysis was performed in a Luminex 100 instrument and the resulting data stream was interpreted using proprietary data analysis software developed at Rules-Based Medicine (https://myriadrbm.com/scientific-media/quality-control-systems-white-paper/). For each multiplex, both calibrators and controls were included on each microtiter plate. 8-point calibrators were run in the first and last column of each plate and 3-level controls were included in duplicate. Testing results were determined first for the high, medium and low controls for each multiplex to ensure proper assay performance. Unknown values for each of the analytes localized in a specific multiplex were determined using 4 and 5 parameter, weighted and non-weighted curve fitting algorithms included in the data analysis package.

Fifty inflammatory markers were quantified using multiplex immunoassay on a custom designed human multi-analyte profile. The intra-assay variability was less than 4% and the inter assay variability was less than 13%. Markers with more than 60% completeness of measurements were selected for analysis (26 from 50) (19).

Type 2 diabetes mellitus diagnosis

The participants were followed from the date of baseline center visit onwards. At baseline and during follow-up, cases of pre-diabetes and type 2 DM were ascertained through active follow-up using general practitioners' records, hospital discharge letters and glucose measurements from Rotterdam Study visits which take place approximately every 4 years (20). Diabetes, pre-diabetes and normoglycemia were defined according to the current WHO guidelines. Normoglycemia was defined as a fasting blood glucose level < 6.0 mmol/L; pre-diabetes was defined as a fasting blood glucose between 6.0 mmol/L and 7.0 mmol/L or a non-fasting blood glucose between 7.7 mmol/L and 11.1 mmol/L (when fasting samples were unavailable); type 2 diabetes was defined as a fasting blood glucose ≥ 7.0 mmol/L, a non-fasting blood glucose ≥ 11.1 mmol/L (when fasting samples were unavailable), or the use of blood glucose lowering medication (20). Information regarding the use of blood glucose lowering medication was derived from both structured home interviews and linkage to pharmacy dispensing records. At baseline, more than 95% of the Rotterdam Study population was covered by the pharmacies in the study area. All potential events of

pre-diabetes and type 2 diabetes 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, calculated as a separate variable for every outcome, taking in account the hierarchy of events as follows: pre-diabetes, type 2 diabetes, insulin therapy start (20).

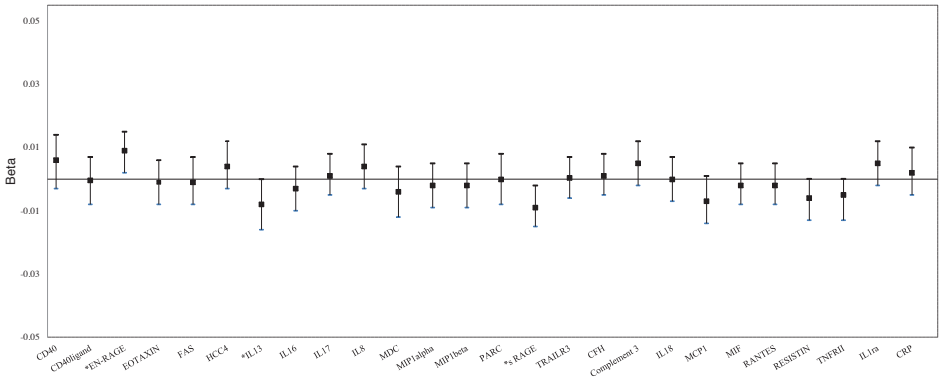
Covariates

Height and weight were measured with the participants standing without shoes and heavy outer garments. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). 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. Blood pressure was measured at the right brachial artery with a random-zero sphygmomanometer with the participant in sitting position, and the mean of 2 consecutive measurements was used. Information on medication use, medical history and smoking behaviour was collected via computerized questionnaires during home visits. Smoking was classified as current versus non-current smokers. Participants were asked whether they were currently smoking cigarettes, cigars, or pipes. 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. Alcohol intake was assessed in grams of ethanol per day. Insulin, glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG) were measured on the COBAS 8000 Modular Analyzer (Roche Diagnostics GmbH). The corresponding interassay coefficients of variations are the following: insulin <8%, glucose <1.4%, lipids <2.1%. HOMA-IR (the homeostatic model assessment to quantify insulin resistance) was calculated dividing the product of fasting glucose (in mmol/L) and fasting insulin (in mU/L) by 22.5. HOMA-B (the homeostatic model assessment of β-cell function) was calculated dividing the product of fasting insulin (in mU/L) and 20 by the difference of glucose (in mmol/L) with 3.5 (21).

Statistical analyses

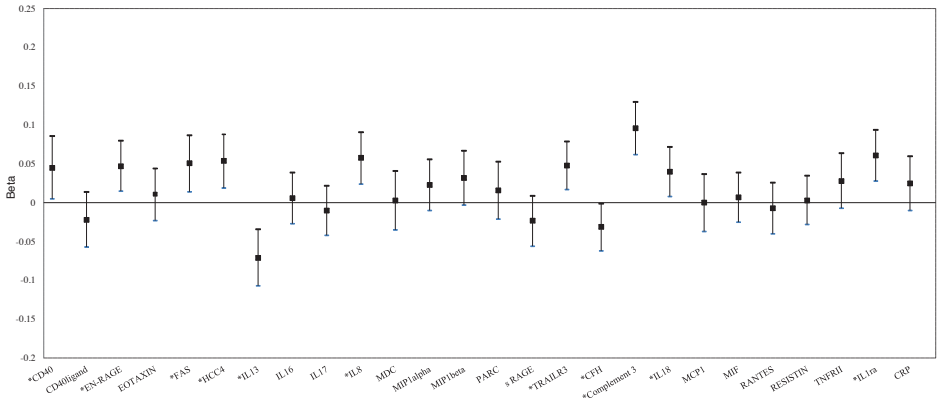
We used linear regression to investigate the association between each inflammatory marker and fasting glucose and fasting insulin in 851 subjects free of diabetes at baseline (excluding 120 prevalent diabetes cases from 971 subjects with available data) as presented at Figure 1.1, Figure 1.2, Supplementary table 2.1, Supplementary table 2.2. Also the associations between markers with HOMA-IR and HOMA-B were investigated using linear regression (Supplementary table 3). Markers with a right-skewed distribution were transformed to the natural logarithmic scale (including fasting glucose and insulin). For a better comparison between the inflammatory markers, all markers were standardized by dividing the measured value by the standard deviation. We defined marker values as an outlier when the value was > 4 standard deviations higher or lower than the mean of the normal variable (not natural log transformed). Participants were excluded from the analyses when the marker value for this person was an outlier. A multiple imputation procedure was used for missing covariates (N= 5 imputations). The analyses with incident pre-diabetes, incident type 2 DM and need for insulin therapy were performed using

Figure 1.1. Associations of inflammatory markers with fasting glucose.



CD40, cluster of differentiation 40; CD40 ligand, cluster of differentiation 40 ligand ; EN-RAGE, Extracellular Newly identified Receptor for Advanced Glycation End-products binding protein; FAS, Fas Cell Surface Death Receptor; HCC4, Human CC chemokine-4; IL13, interleukin 13; IL16, interleukin 16; IL17, interleukin 17; IL8, interleukin 8; MDC, Monocyte Derived Chemokine; MIP1alpha, Macrophage Inflammatory Protein 1 alpha; MIP1beta, Macrophage Inflammatory Protein 1 beta; PARC, Pulmonary and Activation-Regulated Chemokine; sRage, Soluble Receptor of Advanced Glycation End-products; TRAILR3, Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptor 3; CFH, Complement Factor H; IL18, interleukin 18; MCP1, Monocyte Chemotactic Protein 1; RANTES, Regulated Upon Activation, Normally T-Expressed, And Presumably Secreted; TNFR-II, Tumor Necrosis Factor Receptor 2; IL1ra, Interleukin 1 Receptor Antagonist; CRP, C-Reactive Protein. *Significant associations between the marker and fasting glucose. Adjusted for age, sex, BMI, waist circumference (WC), Total Cholesterol, HDL, medication for hypertension, smoking, prevalent CVD, lipid lowering medication.

Figure 1.2. Associations of inflammatory markers with fasting insulin.



CD40, cluster of differentiation 40; CD40 ligand, cluster of differentiation 40 ligand ; EN-RAGE, Extracellular Newly identified Receptor for Advanced Glycation End-products binding protein; FAS, Fas Cell Surface Death Receptor; HCC4, Human CC chemokine-4; IL13, interleukin 13; IL16, interleukin 16; IL17, interleukin 17; IL8, interleukin 8; MDC, Monocyte Derived Chemokine; MIP1alpha, Macrophage Inflammatory Protein 1 alpha; MIP1beta, Macrophage Inflammatory Protein 1 beta; PARC, Pulmonary and Activation-Regulated Chemokine; sRage, Soluble Receptor of Advanced Glycation End-products; TRAILR3, Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptor 3; CFH, Complement Factor H; IL18, interleukin 18; MCP1, Monocyte Chemotactic Protein 1; RANTES, Regulated Upon Activation, Normally T-Expressed, And Presumably Secreted; TNFR-II, Tumor Necrosis Factor Receptor 2; IL1ra, Interleukin 1 Receptor Antagonist; CRP, C-Reactive Protein. *Significant associations between the marker and fasting insulin. Adjusted for age, sex, BMI, waist circumference (WC), Total Cholesterol, HDL, medication for hypertension, smoking, prevalent CVD, lipid lowering medication.

Cox proportional hazard models to calculate hazard ratios (HRs) and 95% confidence intervals (CI). The first model with incident pre-diabetes and diabetes was adjusted for age and sex (table 2). Significant markers were further investigated in multivariable models (table 3). In the second model, we additionally adjusted for body mass index, waist circumference, total cholesterol, HDL-cholesterol, medication for hypertension, smoking, prevalent cardiovascular disease and lipid lowering medication. In the third model we additionally adjusted for C-reaction protein (CRP) levels (except for CRP marker). We sought to investigate the associations between the inflammatory markers and the need for insulin therapy in 115 prevalent diabetes cases with no prevalent use of insulin at baseline (from 120 prevalent cases in total). The inflammatory markers were not correlated to each other, representing 26 independent variables. As a sensitivity analysis, to identify the most robust findings in every analysis, we applied a Bonferroni corrected p-value of 1.9×10^{-3} (0.05/26 markers).The analyses were performed using IBM SPSS Statistics for Windows (IBM SPSS Statistics for Windows, Armonk, New York: IBM Corp) and R V.3.0.1 (R Foundation for Statistical Computing, Vienna, Austria).

Table 1. Baseline characteristics of the study participants.

Characteristic	Value *
Total population number	971
Age, years	73.0 ± 7.5
Men, n (%)	435.0 (44.8)
Waist Circumference, m	0.9 ± 0.1
Body mass index, kg/m^2	26.7 ± 3.9
Systolic blood pressure, mmHg	144.0 ± 21.7
Diastolic blood pressure, mmHg	75.0 ± 11.0
Hypertension medication with indication, n (%)	744.0 (76.6)
Total cholesterol, mmol/L	5.8 ± 1.0
HDL cholesterol, mmol/L	1.4 ± 0.4
Fasting glucose, mmol/L	5.6 (3.54)
Fasting insulin, uIU/L	9.4 (19.87)
Current smokers, n (%)	137.0 (14.1)
Former smokers, n (%)	483.0 (49.7)
Prevalent CVD, n (%)	201.0 (20.7)
Alcohol intake in drinkers (76%), g/day	5.71 (42.73)
Lipid lowering medication, n (%)	122.0 (12.6)

Abbreviations: HDL, high density lipoproteins; CVD, cardiovascular disease.
* Plus-minus values are means ± standard deviation or median (inter-quartile range).

RESULTS

Table 1 summarizes the baseline characteristics of 971 participants, including 120 prevalent diabetes cases. The mean (SD) age at baseline was 73.0 (7.5) years and 44.8% of our population sample were males. The mean BMI (SD) was 26.7 (3.9) kg/m^2 and 12.6% of the study population used statin.

Baseline levels of inflammation markers are presented in Supplementary table 1.4.

Cross-sectional analysis

Figure 1.1 and 1.2 present the multivariable adjusted associations between the inflammatory markers and fasting glucose, fasting insulin in 851 subjects free of diabetes at baseline. Three markers, EN-RAGE, IL13 and sRAGE were significantly associated with fasting glucose. CD40, EN-RAGE, FAS, HCC4, IL13, IL18, TRAILR3, CFH, complement 3, IL18 and IL1ra were significantly associated with fasting insulin.

Prospective analyses

During a median follow-up of 9.5 years in 698 subjects free of pre-diabetes at baseline, 139 cases of pre-diabetes were identified (21 pre-diabetes cases per 1000 person-years). Supplementary table 1.1 presents baseline characteristics among pre-diabetes cases and non-cases.
In age and sex adjusted model, EN-RAGE, IL13, CFH, IL18 and CRP were associated with incident pre-diabetes (table 2). In multivariate models, IL13 (HR = 0.77), EN-RAGE (HR = 1.23) and CRP (HR = 1.26) remained associated with incident pre-diabetes (table 3).
During a median follow-up of 12.1 years in 851 subjects free of diabetes at baseline, 110 cases of incident type 2 diabetes were identified (11 diabetes cases per 1000 person-years). Supplementary table 1.2 presents baseline characteristics among diabetes cases and non-cases.
In age and sex adjusted model, EN-RAGE, IL13, IL17, complement 3, IL18, TNFRII, IL1ra and CRP were associated with incident type 2 diabetes (table 2).
In multivariate models, IL13 (HR = 0.67), IL17 (HR = 0.76) and CRP (HR = 1.32) remained associated with incident type 2 diabetes (table 3).
During a median follow-up of 7.5 years in 115 prevalent diabetics free of insulin at baseline, 26 started insulin therapy (30 insulin starters per 1000 person-years). Supplementary table 1.3 presents baseline characteristics among insulin starters and non-starters.
The only marker associated with need for insulin therapy was IL13. In age and sex adjusted model, the risk for insulin therapy start was 45% lower per standard deviation increase in the natural log-transformed IL13 (HR=0.55, 95% CI: 0.34, 0.90), (Supplementary Table 4). The association between 1L13 and initiation of insulin therapy remained significant after further adjustment for BMI, waist circumference, total cholesterol, HDL, medication for hypertension, smoking, prevalent CVD, lipid lowering medication (HR = 0.49, 95% CI: 0.28, 0.91).

DISCUSSION

Although a sizable number of studies have documented the association of inflammatory markers with type 2 DM, most of them investigated the risk to become diabetic, but not the risk of pre-diabetes and insulin therapy start (8). In this study we investigated a wide range of inflammatory markers for phase-specific prediction of progression to type 2 DM and identified EN-RAGE, IL13 and IL17 as novel inflammatory markers. Higher EN-RAGE levels were associated with an increased risk of incident pre-diabetes, whereas higher IL13 levels were associated with a decreased

Table 2. Age and sex-adjusted associations between markers and incident pre-diabetes, incident type 2 diabetes mellitus.

	Incident pre-diabetes		Incident diabetes	
Marker	**HR(95%CI)**	**P-value**	**HR(95%CI)**	**P-value**
CD40, ng/mL	0.93 (0.72, 1.19)	0.5	1.18 (0.91, 1.52)	0.2
CD40 ligand *, ng/mL	0.95 (0.79, 1.16)	0.6	1.06 (0.85, 1.32)	0.6
EN-RAGE *, ng/mL	1.30 (1.08, 1.56)	**5.0×10^{-3}**	1.25 (1.01, 1.54)	**4.0×10^{-2}**
Eotaxin *, pg/mL	0.95 (0.79, 1.15)	0.6	0.98 (0.80, 1.21)	0.8
FAS *, ng/mL	1.09 (0.88, 1.35)	0.4	1.09 (0.87, 1.38)	0.4
HCC4, ng/mL	1.11 (0.90, 1.35)	0.3	1.24 (0.99, 1.53)	5.4×10^{-2}
IL13 *, pg/mL	0.78 (0.64, 0.94)	**8.0×10^{-3}**	0.62 (0.50, 0.76)	[b]**5.0×10^{-6}**
IL16, pg/mL	1.07 (0.88, 1.29)	0.4	1.17 (0.94, 1.45)	0.1
IL17 *, pg/mL	0.97 (0.81, 1.16)	0.7	0.75 (0.62, 0.91)	**3.0×10^{-3}**
IL8 *, pg/mL	1.05 (0.87, 1.27)	0.5	1.19 (0.97, 1.47)	0.1
MDC, pg/mL	0.94 (0.75, 1.17)	0.5	1.19 (0.94, 1.50)	0.1
MIP1 alpha *, pg/mL	1.09 (0.90, 1.32)	0.3	1.08 (0.87, 1.34)	0.5
MIP1 beta *, pg/mL	1.05 (0.87, 1.26)	0.6	1.00 (0.81, 1.25)	0.9
PARC, ng/mL	1.08 (0.88, 1.32)	0.4	0.94 (0.75, 1.19)	0.6
sRAGE *, ng/mL	0.95 (0.79, 1.14)	0.6	0.91 (0.75, 1.11)	0.3
TRAILR3 *, ng/mL	1.18 (0.98, 1.41)	8.1×10^{-2}	1.19 (0.97, 1.47)	9.1×10^{-2}
CFH *, ug/mL	1.24 (1.02, 1.49)	**2.8×10^{-2}**	1.05 (0.87, 1.28)	0.6
Complement 3*, mg/mL	1.13 (0.94, 1.36)	0.1	1.44 (1.17, 1.77)	[b]**1.0×10^{-3}**
IL18*, pg/mL	1.22 (1.02, 1.47)	**3.2×10^{-2}**	1.35 (1.10, 1.65)	**4.0×10^{-3}**
MCP1*, pg/mL	0.93 (0.76, 1.14)	0.4	0.99 (0.79, 1.25)	0.9
MIF*, ng/mL	0.97 (0.82, 1.14)	0.6	1.11 (0.92, 1.35)	0.2
RANTES*, ng/mL	0.89 (0.75, 1.05)	0.1	1.05 (0.87, 1.27)	0.6
Resistin*, ng/mL	1.02 (0.85, 1.24)	0.8	0.96 (0.78, 1.18)	0.7
TNFRII*, ng/mL	0.97 (0.79, 1.18)	0.7	1.27 (1.03, 1.58)	**2.9×10^{-2}**
Il1ra*, pg/mL	1.04 (0.87, 1.25)	0.6	1.24 (1.02, 1.51)	**3.4×10^{-2}**
CRP*, ug/mL	1.32 (1.10, 1.58)	**3.0×10^{-3}**	1.64 (1.33, 2.02)	[b]**4.0×10^{-6}**

* Naturally log-transformed

CD40, cluster of differentiation 40; CD40 ligand, cluster of differentiation 40 ligand ; EN-RAGE, Extracellular Newly identified Receptor for Advanced Glycation End-products binding protein; FAS, Fas Cell Surface Death Receptor; HCC4, Human CC chemokine-4; IL13, interleukin 13; IL16, interleukin 16; IL17, interleukin 17; IL8, interleukin 8; MDC, Monocyte Derived Chemokine; MIP1alpha, Macrophage Inflammatory Protein 1 alpha; MIP1beta, Macrophage Inflammatory Protein 1 beta; PARC, Pulmonary and Activation-Regulated Chemokine; sRage, Soluble Receptor of Advanced Glycation End-products; TRAILR3, Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptor 3; CFH, Complement Factor H; IL18, interleukin 18; MCP1, Monocyte Chemotactic Protein 1; RANTES, Regulated Upon Activation, Normally T-Expressed, And Presumably Secreted; TNFR-II, Tumor Necrosis Factor Receptor 2; IL1ra, Interleukin 1 Receptor Antagonist; CRP, C-Reactive Protein.

[b]Sensitivity analysis: significant after Bonferroni correction ($p = 0.05/26 = 1.9 \times 10^{-3}$)

The p-values are bold when they are less than or equal to the significance level cut-off of 0.05.

risk of pre-diabetes, incident type 2 DM and need for insulin therapy. Higher IL17 levels were associated with a decreased risk of incident type 2 DM. In addition, this study reconfirm the previously found associations between high CRP levels and the increased risk for type 2 diabetes (6-8).

Table 3. Multivariable-adjusted associations between markers and incident pre-diabetes, incident type 2 diabetes mellitus.

	Incident pre-diabetes		Incident type 2 diabetes	
Marker	**HR(95%CI)**	**P-value**	**HR(95%CI)**	**P-value**
IL13				
Model 1	0.78 (0.64, 0.94)	**8.0×10^{-3}**	0.62 (0.50, 0.76)	**5.0×10^{-6}**
Model 2	0.78 (0.63, 0.98)	**2.9×10^{-2}**	0.68 (0.53, 0.88)	**4.0×10^{-3}**
Model 3	0.77 (0.62, 0.96)	**2.2×10^{-2}**	0.67 (0.52, 0.86)	**2.0×10^{-3}**
IL17				
Model 1	0.97 (0.81, 1.16)	0.7	0.75 (0.62, 0.91)	**3.0×10^{-3}**
Model 2	0.97 (0.82, 1.16)	0.7	0.75 (0.62, 0.91)	**4.0×10^{-3}**
Model 3	0.98 (0.82, 1.17)	0.8	0.76 (0.63, 0.93)	**7.0×10^{-3}**
EN-RAGE				
Model 1	1.30 (1.08, 1.56)	**5.0×10^{-3}**	1.25 (1.01, 1.54)	**4.0×10^{-2}**
Model 2	1.28 (1.06, 1.56)	**1.2×10^{-2}**	1.13 (0.89, 1.41)	0.3
Model 3	1.23 (1.01, 1.51)	**4.1×10^{-2}**	1.05 (0.83, 1.32)	0.6
Complement 3				
Model 1	1.13 (0.94, 1.36)	0.1	1.44 (1.17, 1.77)	**1.0×10^{-3}**
Model 2	1.05 (0.86, 1.27)	0.6	1.19 (0.96, 1.49)	0.1
Model 3	0.99 (0.82, 1.21)	0.9	1.10 (0.87, 1.39)	0.4
CFH				
Model 1	1.24 (1.02, 1.49)	**2.8×10^{-2}**	1.05 (0.87, 1.28)	0.6
Model 2	1.19 (0.99, 1.45)	6.5×10^{-2}	0.98 (0.81, 1.18)	0.8
Model 3	1.18 (0.97, 1.42)	9.7×10^{-2}	0.98 (0.81, 1.18)	0.8
IL18				
Model 1	1.22 (1.02, 1.47)	**3.2×10^{-2}**	1.35 (1.10, 1.65)	**4.0×10^{-3}**
Model 2	1.17 (0.97, 1.41)	0.1	1.22 (0.98, 1.50)	6.7×10^{-2}
Model 3	1.13 (0.94, 1.36)	0.1	1.18 (0.96, 1.46)	0.1
TNFRII				
Model 1	0.97 (0.79, 1.18)	0.7	1.27 (1.03, 1.58)	**2.9×10^{-2}**
Model 2	0.89 (0.73, 1.09)	0.2	1.08 (0.86, 1.37)	0.5
Model 3	0.81 (0.66, 1.01)	6.1×10^{-2}	0.99 (0.78, 1.28)	0.9
IL1ra				
Model 1	1.04 (0.87, 1.25)	0.6	1.24 (1.02, 1.51)	**3.4×10^{-2}**
Model 2	0.97 (0.80, 1.17)	0.7	1.03 (0.83, 1.27)	0.8
Model 3	0.94 (0.78, 1.14)	0.5	0.98 (0.79, 1.22)	0.8
CRP				
Model 1	1.32 (1.10, 1.58)	**3.0×10^{-3}**	1.64 (1.33, 2.02)	**4.0×10^{-6}**
Model 2	1.26 (1.04, 1.53)	**1.8×10^{-2}**	1.32 (1.05, 1.67)	**1.7×10^{-2}**
Model 3	NA	NA	NA	NA

Model 1: age and sex adjusted

Model 2: additionally adjusted for BMI, waist circumference (WC), Total Cholesterol, HDL, medication for hypertension, smoking, prevalent CVD, lipid lowering medication

Model 3: additionally adjusted for CRP

The p-values are bold when they are less than or equal to the significance level cut-off of 0.05.

EN-RAGE, also known as S100A12 or Calgranulin C, is a calcium-binding pro inflammatory protein mainly secreted by granulocytes. The best known target protein of EN-RAGE are RAGE (Receptor for Advanced Glycation Endproducts) (22) and TLR4 (Toll-like receptor 4) (23). Ligation of EN-RAGE with RAGE or TLR4, which are both gatekeepers of the innate immune system, activates inflammatory cascades, including the NF-κB pathway and JNK (c-Jun NH_2 –terminal kinase) (24). NF-κB and JNK are both signaling pathways involved in the pathogenesis of insulin resistance and type 2 DM (25). EN-RAGE is positively associated with chronic inflammatory disorders such as inflammatory bowel disease, chronic kidney disease, subclinical atherosclerosis and coronary artery disease. A cross-sectional study in Italian population found that prediabetic patients exhibited lower RAGE plasma levels as well as increased levels of proinflammatory S100A12 in both prediabetic and diabetic patients (26). In addition, we have previously reported the positive association between EN-RAGE and incident CHD in the Rotterdam Study (19). Kosaki et al observed increased plasma EN-RAGE levels in patients with type 2 DM (27). EN-RAGE was significantly associated with both HOMA-IR and HOMA-B, suggesting proinflammatory EN-RAGE leads to incident type 2 DM via inflammation –induced insulin resistance as well as via B-cell dysfunction (Supplementary table 3).

Interleukin 13 (IL13) is a cytokine mainly produced by the T-helper (Th)-2 subset of lymphocytes, but also from non-T-cell populations such as mast cells, basophils, dendritic cells, keratinocytes and eosinophils (28-30). IL13 is a regulator of inflammation and immune responses (31). IL13 has a common receptor unit (α-chain) with interleukin 4 (IL4), which explains the similarities between IL13 and IL4 (32). Previous research has reported a preventive effect of IL4 on the onset of diabetes in non-obese diabetic mice (NOD mice) (33). Furthermore, Zaccone et al found that IL13 prevents autoimmune diabetes in NOD mice, providing evidence that IL13 down-regulates the immune-inflammatory diabetogenic pathways (34), which is in agreement with our findings. Wong et al suggested the stimulation of IL13 receptors on T-cells, as a new pathway for tolerance induction in NOD mice (35). In addition, IL13 is a B cell stimulating factor, which further supports our observation (36). Stanya et al conclude that IL13 mitigates proinflammatory response in mice and regulates glucose homeostasis via the IL-13rα1–STAT3 signaling pathway in the liver, and that this pathway might provide a target for glycemic control in type 2 DM (37).

We also investigated the associations of IL13 with HOMA-IR and HOMA-B. IL13 was associated with both of them, suggesting a protective role against insulin resistance and B-cell dysfunction (Supplementary table 3).

There are six members in the interleukin 17 (IL17) cytokine family, including IL17A, IL17B, IL17C, IL17D, IL17E (also known as IL25) and IL17F. Among all the members, the biological function and regulation of IL17A and IL17F are best understood. IL17A was produced mainly in T cells, whereas IL-17F was produced in T cells, innate immune cells, and epithelial cells. Functionally, both IL17A and IL17F mediate pro-inflammatory responses (38; 39). IL17 family cytokines have been linked to many autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease and psoriasis (40). The role of IL17 on the risk for type 2 DM remains unclear. Roohi A et al. (41) reported no association between serum IL17 levels and type 1 and 2 diabetes. Another study found that therapeutic improvement of glucoregulation in newly diagnosed type 2 DM patients is associated with a reduction of IL-17 levels (42). Our study suggest a

protective IL17 cytokine against the risk for type 2 DM (HR = 0.76), which is controversial and novel to the already known pro-inflammatory role of IL17 family cytokines. However, a cross-sectional case-control study has reported inverse associations of serum levels of IL17 with type 2 DM as well as with retinopathy, which is in line with our findings (43).

This study has certain strengths and limitations. To our knowledge, this is the first prospective population-based cohort study to investigate the association between a large set of novel inflammatory markers and the progression of type 2 DM with long-term follow-up. Furthermore, we performed sensitivity analysis, adjusting the type I error for multiple testing in order to highlight the most robust associations in every analyses. However, given the novelty of the markers, we reported significant findings at the level of 0.05 to avoid missing possibly important findings (44). Beyond the identification of new novel inflammatory markers for type 2 diabetes, our findings relate them specifically to different stages of the disease. We are also aware of some limitations of the study. First, we had to exclude inflammatory markers with very low serum concentrations. However, the selected markers have > 60% completeness of measurements, indicating acceptable quality of quantification. Second, our population is 55 years and older, thus generalization of the results to a younger age should be done with caution. Also, the Rotterdam Study mainly includes individuals from European Ancestry (98%). The effect estimates might differ between ethnicities. A better prevention of type 2 DM requires the targeting of subjects at high risk in very early phases, such as pre-diabetes (12). In this context, it is worth to investigate novel inflammatory markers that might be detectors of different stages of type 2 DM development (17).

In conclusion, our results show various inflammatory markers are associated with the progression from normoglycemia to type 2 DM and need for insulin therapy in a phase-specific manner. Among them, EN-RAGE is a novel inflammatory marker for pre-diabetes, IL17 for incident type 2 DM and IL13 for pre-diabetes, incident type 2 DM and insulin therapy start. This study only indicates new associations, emphasizing the need for further studies to establish the role of EN-RAGE, IL13 and IL17 in the development of type 2 diabetes.

DECLARATIONS

Author contributions

A.B. ran the analysis and wrote the manuscript. S.L. and M.G. contributed to the analysis. M.A.I. and A.H. designed the study. O.H.F. designed the study and provided resources. M.K. and A.D. designed the study and critically revised the manuscript. All authors have read and approved the manuscript.

Acknowledgments

The dedication, commitment, and contribution of inhabitants, general practitioners, and pharmacists of the Ommoord district to the Rotterdam Study are gratefully acknowledged. We thank Layal Chaker, Jolande Verkroost-van Heemst, and Ke-Xin Wen of the Epidemiology Department of Erasmus Medical Center for their invaluable contribution to the collection of the diabetes data.

Funding

The Rotterdam Study is supported by the Erasmus MC and Erasmus University Rotterdam; the Netherlands Organisation for Scientific Research (NWO); the Netherlands Organisation for Health Research and Development (ZonMw); the Research Institute for Diseases in the Elderly (RIDE); the Netherlands Genomics Initiative (NGI); the Ministry of Education, Culture and Science, the Ministry of Health, Welfare and Sports; the European Commission (DG XII); and the Municipality of Rotterdam.
None of the funders had any role in design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript.
Abbas Dehghan is supported by NWO grant (veni, 916.12.154) and the EUR Fellowship. Maryam Kavousi is supported by the ZonMw grant (VENI 91616079).

Duality of interest

OHF works in ErasmusAGE, a center for aging research across the life course funded by Nestlé Nutrition (Nestec Ltd.); Metagenics Inc.; and AXA. The other authors report no potential conflicts of interest.

Disclosure Statement: The authors have nothing to disclose.

Supplementary Table 1.1. Baseline characteristics among non- pre-diabetes cases/ pre-diabetes cases.

Characteristic	Non- pre-diabetes cases	Incident pre-diabetes cases	p-value
Total population number	559	139	
Age, years	73 ± 7.6	71 ± 6.1	< **0.001**
Men, n (%)	238 (43)	60 (43.2)	0.9
Waist Circumference, m	0.9 ± 0.1	0.9 ± 0.1	0.3
Body mass index, kg/m^2	25.9 ± 3.6	27.0 ± 3.8	**0.003**
Systolic blood pressure, mmHg	143 ± 21.7	142 ± 20.1	0.4
Diastolic blood pressure, mmHg	74.6 ± 10.9	74.9 ± 10.9	0.7
Hypertension medication with indication, n (%)	107 (19.3)	30 (21.6)	**0.052**
Total cholesterol, mmol/L	5.8 ± 1.0	5.8 ± 0.9	0.9
HDL cholesterol, mmol/L	1.5 ± 0.4	1.4 ± 0.4	**0.016**
Fasting glucose, mmol/L	5.3 (4.7 – 5.9)	5.6 (4.9 – 6.0)	< **0.001**
Fasting insulin, uIU/L	8.2 (3.7 – 18.4)	9.6 (3.7 – 22.4)	**0.002**
Current smokers, n (%)	85 (15.4)	19 (13.7)	0.6
Former smokers, n (%)	255 (46.1)	77 (55.4)	0.1
Prevalent CVD, n (%)	94 (17)	15 (10.8)	0.07
Alcohol intake in drinkers (76%), g/day	2.9 (0.0 – 40.2)	2.9 (0.0 – 40.0)	0.6
Lipid lowering medication, n (%)	57 (10.3)	26 (18.7)	**0.007**

Abbreviations: HDL, high density lipoproteins; CVD, cardiovascular disease.
Plus-minus values are means ± standard deviation or median (inter-quartile range).

Supplementary Table 1.2. Baseline characteristics among non- diabetes cases/ diabetes cases.

Characteristic	Non- diabetes cases	Incident diabetes cases	p-value
Total population number	741	110	
Age, years	73 ± 7.5	70 ± 5.9	< **0.001**
Men, n (%)	330 (44.9)	46 (41.8)	0.5
Waist Circumference, m	0.9 ± 0.1	0.9 ± 0.1	< **0.001**
Body mass index, kg/m^2	26.2 ± 3.7	28.4 ± 4.2	< **0.001**
Systolic blood pressure, mmHg	143.4 ± 21.7	144.9 ± 19.6	0.4
Diastolic blood pressure, mmHg	75.0 ± 11.2	75.7 ± 11.2	0.5
Hypertension medication with indication, n (%)	154 (21)	36 (32.7)	**0.02**
Total cholesterol, mmol/L	5.8 ± 1.0	5.9 ± 0.9	0.4
HDL cholesterol, mmol/L	1.4 ± 0.4	1.3 ± 0.4	< **0.001**
Fasting glucose, mmol/L	5.4 (4.7 – 6.3)	6.1 (5.1 – 6.8)	< **0.001**
Fasting insulin, uIU/L	8.5 (3.7 – 19.3)	12.9 (5.3 – 27.3)	< **0.001**
Current smokers, n (%)	107 (14.6)	18 (16.4)	0.6
Former smokers, n (%)	355 (48.3)	57 (51.8)	0.6
Prevalent CVD, n (%)	124 (16.9)	9 (8.2)	**0.02**
Alcohol intake in drinkers (76%), g/day	2.9 (0.0 – 41.9)	2.9 (0.0 – 40.1)	0.5
Lipid lowering medication, n (%)	81 (11)	14 (12.7)	0.8

Abbreviations: HDL, high density lipoproteins; CVD, cardiovascular disease.
Plus-minus values are means ± standard deviation or median (inter-quartile range).

Supplementary Table 1.3. Baseline characteristics among non-insulin starters/ insulin starters.

Characteristic	Non-insulin starters	Insulin starters	p-value
Total population number	89	26	
Age, years	74.9 ± 8.3	73.6 ± 6.5	**0.001**
Men, n (%)	45 (50.6)	12 (46.2)	0.3
Waist Circumference, m	0.9 ± 0.1	0.9 ± 0.1	0.6
Body mass index, kg/m^2	28.3 ± 4.4	28.7 ± 4.7	0.09
Systolic blood pressure, mmHg	145.8 ± 21.0	149.7 ± 28.5	0.7
Diastolic blood pressure, mmHg	75.3 ± 10.1	75.4 ± 9.5	0.7
Hypertension medication with indication, n (%)	39 (43.8)	8 (30.8)	0.5
Total cholesterol, mmol/L	5.7 ± 0.9	5.8 ± 0.8	0.5
HDL cholesterol, mmol/L	1.2 ± 0.4	1.1 ± 0.2	0.2
Fasting glucose, mmol/L	7.5 (5.4 – 12.5)	8.8 (6.6 – 17.2)	**0.02**
Fasting insulin, uIU/L	12.2 (4.6 – 41.1)	12.7 (5.9 – 52.9)	0.7
Current smokers, n (%)	11 (12.4)	0 (0)	**0.03**
Former smokers, n (%)	49 (55.1)	17 (65.4)	0.1
Prevalent CVD, n (%)	22 (24.7)	8 (30.8)	**0.005**
Alcohol intake in drinkers (76%), g/day	1.4 (0.0 – 21.1)	1.4 (0.0 – 18.1)	0.4
Lipid lowering medication, n (%)	15 (16.9)	8 (30.8)	0.6

Abbreviations: HDL, high density lipoproteins; CVD, cardiovascular disease.
Plus-minus values are means ± standard deviation or median (inter-quartile range).

Supplementary Table 1.4. Baseline characteristics of the inflammatory markers.

Marker	Value
Total population free of diabetes	851
CD40, ng/mL	0.73 ± 0.27
CD40 ligand *, ng/mL	0.03 (0.01 – 0.06)
EN-RAGE *, ng/mL	10.70 (4.82 – 24.45)
Eotaxin *, pg/mL	161.00 (65.40 – 330.65)
FAS *, ng/mL	4.65 (2.94 – 8.18)
HCC4, ng/mL	4.87 ± 1.95
IL13 *, pg/mL	76.20 (48.70 - -123.00)
IL16, pg/mL	381.98 ± 103.89
IL17 *, pg/mL	12.90 (6.22 – 23.30)
IL8 *, pg/mL	9.24 (4.25 – 20.92)
MDC, pg/mL	365.61 ± 124.01
MIP1 alpha *, pg/mL	46.20 (27.22 – 73.46)
MIP1 beta *, pg/mL	122.00 (66.06 – 323.20)
PARC, ng/mL	29.93 ± 11.12
sRAGE *, ng/mL	2.67 (1.27 – 5.66)
TRAILR3 *, ng/mL	6.55 (3.48 – 12.48)
CFH *, ug/mL	2520 (890.95 – 3700)
Complement 3*, mg/mL	0.82 (0.62 – 1.06)
IL18*, pg/mL	187 (100 – 381.40)
MCP1*, pg/mL	184 (113 – 309)
MIF*, ng/mL	0.06 (0.01 – 0.15)
RANTES*, ng/mL	0.51 (0.18 – 1.79)
Resistin*, ng/mL	0.42 (0.17 – 0.99)
TNFRII*, ng/mL	3.51 (2.25 – 6.21)
Il1ra*, pg/mL	66.80 (25.99 – 191)
CRP*, ug/mL	1.37 (0.23 – 8.90)

Plus-minus values are means ± standard deviation or median (inter-quartile range). * Naturally log-transformed markers. CD40, cluster of differentiation 40; CD40 ligand, cluster of differentiation 40 ligand ; EN-RAGE, Extracellular Newly identified Receptor for Advanced Glycation End-products binding protein; FAS, Fas Cell Surface Death Receptor; HCC4, Human CC chemokine-4; IL13, interleukin 13; IL16, interleukin 16; IL17, interleukin 17; IL8, interleukin 8; MDC, Monocyte Derived Chemokine; MIP1alpha, Macrophage Inflammatory Protein 1 alpha; MIP1beta, Macrophage Inflammatory Protein 1 beta; PARC, Pulmonary and Activation-Regulated Chemokine; sRage, Soluble Receptor of Advanced Glycation End-products; TRAILR3, Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptor 3; CFH, Complement Factor H; IL18, interleukin 18; MCP1, Monocyte Chemotactic Protein 1; RANTES, Regulated Upon Activation, Normally T-Expressed, And Presumably Secreted; TNFR-II, Tumor Necrosis Factor Receptor 2; IL1ra, Interleukin 1 Receptor Antagonist; CRP, C-Reactive Protein.

Supplementary Table 2.1. Age and sex adjusted associations between markers of inflammation and fasting glucose and insulin.

		Fasting glucose		Fasting insulin	
Marker	**N**	**Beta (95%CI)**	**P-value**	**Beta (95%CI)**	**P-value**
CD40, ng/mL	848	0.009 (0.001, 0.018)	**3.2×10^{-2}**	0.097 (0.050, 0.143)	[b]**4.4×10^{-5}**
CD40 ligand [*], ng/mL	780	-0.001 (-0.008, 0.007)	0.8	-0.018 (-0.060, 0.024)	0.3
EN-RAGE [*], ng/mL	843	0.012 (0.005, 0.019)	[b]**1×10^{-3}**	0.078 (0.041, 0.12)	[b]**3.6×10^{-5}**
Eotaxin [*], pg/mL	841	-0.005 (-0.012, 0.002)	0.1	-0.032 (-0.070, 0.007)	0.1
FAS [*], ng/mL	837	0.004 (-0.003, 0.012)	0.2	0.113 (0.072, 0.153)	[b]**7.0×10^{-8}**
HCC4, ng/mL	850	0.008 (0.001, 0.016)	**2.6×10^{-2}**	0.098 (0.058, 0.138)	[b]**1.0×10^{-6}**
IL13 [*], pg/mL	814	-0.014 (-0.021, -0.007)	[b]**7.7×10^{-5}**	-0.155 (-0.190, -0.119)	[b]**9.6×10^{-17}**
IL16, pg/mL	849	0.001 (-0.006, 0.008)	0.7	0.052 (0.015, 0.090)	**7.0×10^{-3}**
IL17 [*], pg/mL	805	-1×10^{-4} (-0.007, 0.007)	0.9	-0.026 (-0.063, 0.011)	0.1
IL8 [*], pg/mL	824	0.003 (-0.004, 0.011)	0.3	0.057 (0.019, 0.096)	**4.0×10^{-3}**
MDC, pg/mL	846	2×10^{-4} (-0.008, 0.008)	0.9	0.049 (0.006, 0.091)	**2.7×10^{-2}**
MIP1 alpha [*], pg/mL	846	-1.1×10^{-4} (-0.007, 0.007)	0.9	0.052 (0.014, 0.090)	**8.0×10^{-3}**
MIP1 beta [*], pg/mL	844	0.001 (-0.007, 0.008)	0.8	0.063 (0.023, 0.103)	**2.0×10^{-3}**
PARC, ng/mL	845	0.002 (-0.005, 0.010)	0.5	0.039 (-0.003, 0.082)	7.0×10^{-2}
sRAGE [*], ng/mL	847	-0.012 (-0.019, -0.005)	[b]**1×10^{-3}**	-0.045 (-0.082, -0.008)	**1.8×10^{-2}**
TRAILR3 [*], ng/mL	844	0.003 (-0.003, 0.010)	0.3	0.082 (0.046, 0.118)	[b]**7.0×10^{-6}**
CFH [*], ug/mL	840	0.004 (-0.002, 0.011)	0.1	-0.001 (-0.037, 0.034)	0.9
Complement 3[*], mg/mL	851	0.014 (0.008, 0.021)	[b]**2.3×10^{-5}**	0.179 (0.144, 0.213)	[b]**6.0×10^{-23}**
IL18[*], pg/mL	844	0.003 (-0.004, 0.010)	0.3	0.083 (0.046, 0.119)	[b]**9.0×10^{-6}**
MCP1[*], pg/mL	846	-0.008 (-0.016, $-8 \times 10^{-0.005}$)	**4.7×10^{-2}**	-0.015 (-0.058, 0.027)	0.4
MIF[*], ng/mL	831	4×10^{-4} (-0.006, 0.007)	0.8	0.040 (0.004, 0.076)	**3.1×10^{-2}**
RANTES[*], ng/mL	849	-0.003 (-0.010, 0.004)	0.3	-0.023 (-0.059, 0.013)	0.2
Resistin[*], ng/mL	844	-0.005 (-0.012, 0.002)	0.1	0.022 (-0.015, 0.058)	0.2
TNFRII[*], ng/mL	846	2×10^{-4} (-0.007, 0.007)	0.9	0.105 (0.066, 0.144)	[b]**1.0×10^{-7}**
Il1ra[*], pg/mL	821	0.011 (0.004, 0.018)	[b]**1×10^{-3}**	0.139 (0.104, 0.175)	[b]**1.9×10^{-14}**
CRP[*], ug/mL	837	0.012 (0.005, 0.019)	[b]**4.8×10^{-4}**	0.119 (0.083, 0.156)	[b]**1.1×10^{-10}**

[*] Naturally log-transformed

CD40, cluster of differentiation 40; CD40 ligand, cluster of differentiation 40 ligand ; EN-RAGE, Extracellular Newly identified Receptor for Advanced Glycation End-products binding protein; FAS, Fas Cell Surface Death Receptor; HCC4, Human CC chemokine-4; IL13, interleukin 13; IL16, interleukin 16; IL17, interleukin 17; IL8, interleukin 8; MDC, Monocyte Derived Chemokine; MIP1alpha, Macrophage Inflammatory Protein 1 alpha; MIP1beta, Macrophage Inflammatory Protein 1 beta; PARC, Pulmonary and Activation-Regulated Chemokine; sRage, Soluble Receptor of Advanced Glycation End-products; TRAILR3, Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptor 3; CFH, Complement Factor H; IL18, interleukin 18; MCP1, Monocyte Chemotactic Protein 1; RANTES, Regulated Upon Activation, Normally T-Expressed, And Presumably Secreted; TNFR-II, Tumor Necrosis Factor Receptor 2; IL1ra, Interleukin 1 Receptor Antagonist; CRP, C-Reactive Protein.
[b]Sensitivity analysis: significant after Bonferroni correction ($p = 0.05/26 = 1.9 \times 10^{-3}$)

Supplementary Table 2.2. Multivariable adjusted associations between markers of inflammation and fasting glucose and insulin.

		Fasting glucose		Fasting insulin	
Marker	**N**	**Beta (95%CI)**	**P-value**	**Beta (95%CI)**	**P-value**
CD40, ng/mL	848	0.006 (-0.003, 0.014)	0.1	0.045 (0.005, 0.086)	$\mathbf{2.9 \times 10^{-2}}$
CD40 ligand *, ng/mL	780	-4 x 10^{-4} (-0.008, 0.007)	0.9	-0.022 (-0.057, 0.014)	0.2
EN-RAGE *, ng/mL	843	0.009 (0.002, 0.015)	$\mathbf{1.2 \times 10^{-2}}$	0.047 (0.015, 0.080)	$\mathbf{5 \times 10^{-3}}$
Eotaxin *, pg/mL	841	-0.001 (-0.008, 0.006)	0.6	0.011 (-0.023, 0.044)	0.5
FAS *, ng/mL	837	-0.001 (-0.008, 0.007)	0.8	0.051 (0.014, 0.087)	$\mathbf{6 \times 10^{-3}}$
HCC4, ng/mL	850	0.004 (-0.003, 0.012)	0.2	0.054 (0.019, 0.088)	$\mathbf{3 \times 10^{-3}}$
IL13 *, pg/mL	814	-0.008 (-0.016, 0.000)	$\mathbf{4.7 \times 10^{-2}}$	-0.071 (-0.107, -0.034)	[b]$\mathbf{1.6 \times 10^{-4}}$
IL16, pg/mL	849	-0.003 (-0.010, 0.004)	0.3	0.006 (-0.027, 0.039)	0.7
IL17 *, pg/mL	805	0.001 (-0.005, 0.008)	0.7	-0.010 (-0.042, 0.022)	0.5
IL8 *, pg/mL	824	0.004 (-0.003, 0.011)	0.2	0.058 (0.024, 0.091)	[b]$\mathbf{1.0 \times 10^{-3}}$
MDC, pg/mL	846	-0.004 (-0.012, 0.004)	0.3	0.003 (-0.035, 0.041)	0.8
MIP1 alpha *, pg/mL	846	-0.002 (-0.009, 0.005)	0.5	0.023 (-0.010, 0.056)	0.1
MIP1 beta *, pg/mL	844	-0.002 (-0.009, 0.005)	0.5	0.032 (-0.003, 0.067)	6.9×10^{-2}
PARC, ng/mL	845	-1 x 10^{-4} (-0.008, 0.008)	0.9	0.016 (-0.021, 0.053)	0.3
sRAGE *, ng/mL	847	-0.009 (-0.015, -0.002)	$\mathbf{1.4 \times 10^{-2}}$	-0.023 (-0.056, 0.009)	0.1
TRAILR3 *, ng/mL	844	4 x 10^{-4} (-0.006, 0.007)	0.8	0.048 (0.017, 0.079)	$\mathbf{3.0 \times 10^{-3}}$
CFH *, ug/mL	840	0.001 (-0.005, 0.008)	0.6	-0.031 (-0.062, -0.001)	$\mathbf{4.6 \times 10^{-2}}$
Complement 3*, mg/mL	851	0.005 (-0.002, 0.012)	0.1	0.096 (0.062, 0.130)	[b]$\mathbf{2.3 \times 10^{-8}}$
IL18*, pg/mL	844	-1 x 10^{-4} (-0.007, 0.007)	0.9	0.040 (0.008, 0.072)	$\mathbf{1.5 \times 10^{-2}}$
MCP1*, pg/mL	846	-0.007 (-0.014, 0.001)	7.5×10^{-2}	3 x 10^{-4} (-0.037, 0.037)	0.9
MIF*, ng/mL	831	-0.002 (-0.008, 0.005)	0.5	0.007 (-0.025, 0.039)	0.6
RANTES*, ng/mL	849	-0.002 (-0.008, 0.005)	0.6	-0.007 (-0.040, 0.026)	0.6
Resistin*, ng/mL	844	-0.006 (-0.013, 1 x 10^{-4})	5.6×10^{-2}	0.003 (-0.028, 0.035)	0.8
TNFRII*, ng/mL	846	-0.005 (-0.013, 0.002)	0.1	0.028 (-0.007, 0.064)	0.1
Il1ra*, pg/mL	821	0.005 (-0.002, 0.012)	0.2	0.061 (0.028, 0.094)	[b]$\mathbf{2.7 \times 10^{-4}}$
CRP*, ug/mL	837	0.002 (-0.005, 0.010)	0.4	0.025 (-0.010, 0.060)	0.1

* Naturally log-transformed. CD40, cluster of differentiation 40; CD40 ligand, cluster of differentiation 40 ligand ; EN-RAGE, Extracellular Newly identified Receptor for Advanced Glycation End-products binding protein; FAS, Fas Cell Surface Death Receptor; HCC4, Human CC chemokine-4; IL13, interleukin 13; IL16, interleukin 16; IL17, interleukin 17; IL8, interleukin 8; MDC, Monocyte Derived Chemokine; MIP1alpha, Macrophage Inflammatory Protein 1 alpha; MIP1beta, Macrophage Inflammatory Protein 1 beta; PARC, Pulmonary and Activation-Regulated Chemokine; sRage, Soluble Receptor of Advanced Glycation End-products; TRAILR3, Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptor 3; CFH, Complement Factor H; IL18, interleukin 18; MCP1, Monocyte Chemotactic Protein 1; RANTES, Regulated Upon Activation, Normally T-Expressed, And Presumably Secreted; TNFR-II, Tumor Necrosis Factor Receptor 2; IL1ra, Interleukin 1 Receptor Antagonist; CRP, C-Reactive Protein. Adjusted for age, sex, BMI, waist circumference (WC), Total Cholesterol, HDL, medication for hypertension, smoking, prevalent CVD, lipid lowering medication. [b]Sensitivity analysis: significant after Bonferroni correction ($p = 0.05/26 = 1.9 \times 10^{-3}$).

Supplementary Table 3. Associations between markers of inflammation and HOMA indices.

		HOMA-IR		HOMA-B	
Marker	**N**	**Beta (95%CI)**	**P-value**	**Beta (95%CI)**	**P-value**
CD40, ng/mL	848	0.105 (0.056, 0.155)	[b]**3.4×10^{-5}**	0.077 (0.033, 0.122)	[b]**1.0×10^{-3}**
CD40 ligand [*], ng/mL	780	-0.020 (-0.065, 0.025)	0.3	-0.012 (-0.052, 0.029)	0.5
EN-RAGE [*], ng/mL	843	0.090 (0.050, 0.130)	[b]**1.0×10^{-5}**	0.045 (0.009, 0.081)	**1.4×10^{-2}**
Eotaxin [*], pg/mL	841	-0.036 (-0.077, 0.005)	8.8×10^{-2}	-0.019 (-0.056, 0.018)	0.3
FAS [*], ng/mL	837	0.117 (0.073, 0.162)	[b]**1.7×10^{-7}**	0.101 (0.062, 0.141)	[b]**5.5×10^{-7}**
HCC4, ng/mL	850	0.107 (0.064, 0.150)	[b]**9.5×10^{-7}**	0.076 (0.037, 0.114)	[b]**1.1×10^{-4}**
IL13 [*], pg/mL	814	-0.168 (-0.206, -0.130)	[b]**5.0×10^{-17}**	-0.120 (-0.155, -0.085)	[b]**2.0×10^{-11}**
IL16, pg/mL	849	0.054 (0.013, 0.095)	**1.0×10^{-2}**	0.051 (0.014, 0.087)	**7.0×10^{-3}**
IL17 [*], pg/mL	805	-0.027 (-0.067, 0.013)	0.1	-0.024 (-0.060, 0.011)	0.1
IL8 [*], pg/mL	824	0.060 (0.019, 0.102)	**4.0×10^{-3}**	0.051 (0.014, 0.088)	**7.0×10^{-3}**
MDC, pg/mL	846	0.049 (0.003, 0.095)	**3.8×10^{-2}**	0.048 (0.006, 0.089)	**2.4×10^{-2}**
MIP1 alpha [*], pg/mL	846	0.051 (0.010, 0.092)	**1.4×10^{-2}**	0.054 (0.018, 0.091)	**4.0×10^{-3}**
MIP1 beta [*], pg/mL	844	0.063 (0.020, 0.106)	**4.0×10^{-3}**	0.063 (0.025, 0.102)	[b]**1.0×10^{-3}**
PARC, ng/mL	845	0.040 (-0.005, 0.086)	8.4×10^{-2}	0.039 (-0.002, 0.080)	5.9×10^{-2}
sRAGE [*], ng/mL	847	-0.057 (-0.097, -0.017)	**6.0×10^{-3}**	-0.009 (-0.045, 0.027)	0.6
TRAILR3 [*], ng/mL	844	0.085 (0.047, 0.124)	[b]**1.5×10^{-5}**	0.073 (0.039, 0.108)	[b]**3.0×10^{-5}**
CFH [*], ug/mL	840	0.003 (-0.036, 0.041)	0.8	-0.013 (-0.047, 0.022)	0.4
Complement 3[*], mg/mL	851	0.193 (0.156, 0.230)	[b]**6.3×10^{-23}**	0.140 (0.106, 0.174)	[b]**8.8×10^{-16}**
IL18[*], pg/mL	844	0.085 (0.046, 0.125)	[b]**2.2×10^{-5}**	0.077 (0.042, 0.112)	[b]**1.8×10^{-5}**
MCP1[*], pg/mL	846	-0.023 (-0.069, 0.023)	0.3	0.007 (-0.034, 0.048)	0.7
MIF[*], ng/mL	831	0.040 (0.001, 0.079)	**4.2×10^{-2}**	0.040 (0.005, 0.075)	**2.6×10^{-2}**
RANTES[*], ng/mL	849	-0.026 (-0.065, 0.013)	0.1	-0.012 (-0.047, 0.022)	0.4
Resistin[*], ng/mL	844	0.017 (-0.023, 0.057)	0.4	0.037 (0.002, 0.073)	**3.9×10^{-2}**
TNFRII[*], ng/mL	846	0.106 (0.064, 0.148)	[b]**6.9×10^{-7}**	0.103 (0.066, 0.141)	[b]**5.0×10^{-8}**
Il1ra[*], pg/mL	821	0.150 (0.112, 0.189)	[b]**1.9×10^{-14}**	0.110 (0.076, 0.145)	[b]**4.4×10^{-10}**
CRP[*], ug/mL	837	0.132 (0.093, 0.170)	[b]**3.6×10^{-11}**	0.086 (0.051, 0.122)	[b]**2.0×10^{-6}**

Age and sex adjusted. The number of subjects differs for each marker, after outliers exclusion. [*] Naturally log-transformed. CD40, cluster of differentiation 40; CD40 ligand, cluster of differentiation 40 ligand ; EN-RAGE, Extracellular Newly identified Receptor for Advanced Glycation End-products binding protein; FAS, Fas Cell Surface Death Receptor; HCC4, Human CC chemokine-4; IL13, interleukin 13; IL16, interleukin 16; IL17, interleukin 17; IL8, interleukin 8; MDC, Monocyte Derived Chemokine; MIP1alpha, Macrophage Inflammatory Protein 1 alpha; MIP1beta, Macrophage Inflammatory Protein 1 beta; PARC, Pulmonary and Activation-Regulated Chemokine; sRage, Soluble Receptor of Advanced Glycation End-products; TRAILR3, Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptor 3; CFH, Complement Factor H; IL18, interleukin 18; MCP1, Monocyte Chemotactic Protein 1; RANTES, Regulated Upon Activation, Normally T-Expressed, And Presumably Secreted; TNFR-II, Tumor Necrosis Factor Receptor 2; IL1ra, Interleukin 1 Receptor Antagonist; CRP, C-Reactive Protein.
[b]Sensitivity analysis: significant after Bonferroni correction ($p = 0.05/26 = 1.9 \times 10^{-3}$)

Supplementary Table 4. Associations between markers of inflammation and insulin therapy start in diabetics.

		Insulin therapy start	
Marker	**N**	**HR(95%CI)**	**P-value**
CD40, ng/mL	113	1.12 (0.69, 1.83)	0.6
CD40 ligand [*], ng/mL	107	1.10 (0.69, 1.75)	0.6
EN-RAGE [*], ng/mL	115	0.97 (0.62, 1.54)	0.9
Eotaxin [*], pg/mL	113	1.39 (0.91, 2.12)	0.1
FAS [*], ng/mL	114	0.76 (0.48, 1.22)	0.2
HCC4, ng/mL	115	0.93 (0.61, 1.41)	0.7
IL13 [*], pg/mL	109	0.55 (0.34, 0.90)	$\mathbf{1.7 \times 10^{-2}}$
IL16, pg/mL	115	0.99 (0.67, 1.47)	0.9
IL17 [*], pg/mL	106	0.89 (0.62, 1.30)	0.5
IL8 [*], pg/mL	111	0.79 (0.51, 1.24)	0.3
MDC, pg/mL	114	1.45 (0.97, 2.16)	6.8×10^{-2}
MIP1 alpha [*], pg/mL	114	1.02 (0.69, 1.49)	0.9
MIP1 beta [*], pg/mL	114	0.66 (0.35, 1.24)	0.1
PARC, ng/mL	115	0.82 (0.53, 1.27)	0.3
sRAGE [*], ng/mL	112	0.92 (0.63, 1.34)	0.6
TRAILR3 [*], ng/mL	114	0.69 (0.43, 1.10)	0.1
CFH [*], ug/mL	109	1.00 (0.69, 1.44)	0.9
Complement 3[*], mg/mL	115	1.07 (0.73, 1.58)	0.7
IL18[*], pg/mL	114	1.11 (0.71, 1.76)	0.6
MCP1[*], pg/mL	115	0.92 (0.62, 1.35)	0.6
MIF[*], ng/mL	115	1.09 (0.68, 1.73)	0.7
RANTES[*], ng/mL	115	0.89 (0.59, 1.34)	0.5
Resistin[*], ng/mL	113	0.89 (0.59, 1.32)	0.5
TNFRII[*], ng/mL	115	0.77 (0.49, 1.21)	0.2
Il1ra[*], pg/mL	114	1.32 (0.86, 2.03)	0.2
CRP[*], ug/mL	110	1.05 (0.71, 1.54)	0.8

Age and sex adjusted. The number of diabetics at baseline differs for each marker, after outliers exclusion. [*] Naturally log-transformed. CD40, cluster of differentiation 40; CD40 ligand, cluster of differentiation 40 ligand ; EN-RAGE, Extracellular Newly identified Receptor for Advanced Glycation End-products binding protein; FAS, Fas Cell Surface Death Receptor; HCC4, Human CC chemokine-4; IL13, interleukin 13; IL16, interleukin 16; IL17, interleukin 17; IL8, interleukin 8; MDC, Monocyte Derived Chemokine; MIP1alpha, Macrophage Inflammatory Protein 1 alpha; MIP1beta, Macrophage Inflammatory Protein 1 beta; PARC, Pulmonary and Activation-Regulated Chemokine; sRage, Soluble Receptor of Advanced Glycation End-products; TRAILR3, Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptor 3; CFH, Complement Factor H; IL18, interleukin 18; MCP1, Monocyte Chemotactic Protein 1; RANTES, Regulated Upon Activation, Normally T-Expressed, And Presumably Secreted; TNFR-II, Tumor Necrosis Factor Receptor 2; IL1ra, Interleukin 1 Receptor Antagonist; CRP, C-Reactive Protein. [b]Sensitivity analysis: significant after Bonferroni correction ($p = 0.05/26 = 1.9 \times 10^{-3}$)

REFERENCES

1. Hotamisligil GS: Inflammation and metabolic disorders. Nature 2006;444:860-867
2. Bertoni AG, Burke GL, Owusu JA, Carnethon MR, Vaidya D, Barr RG, Jenny NS, Ouyang P, Rotter JI: Inflammation and the incidence of type 2 diabetes: the Multi-Ethnic Study of Atherosclerosis (MESA). Diabetes Care 2010;33:804-810
3. Barzilay JI, Abraham L, Heckbert SR, Cushman M, Kuller LH, Resnick HE, Tracy RP: The relation of markers of inflammation to the development of glucose disorders in the elderly: the Cardiovascular Health Study. Diabetes 2001;50:2384-2389
4. Galle J, Quaschning T, Seibold S, Wanner C: Endothelial dysfunction and inflammation: what is the link? Kidney Int Suppl 2003:S45-49
5. Meigs JB, Hu FB, Rifai N, Manson JE: Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. Jama 2004;291:1978-1986
6. Dehghan A, Kardys I, de Maat MP, Uitterlinden AG, Sijbrands EJ, Bootsma AH, Stijnen T, Hofman A, Schram MT, Witteman JC: Genetic variation, C-reactive protein levels, and incidence of diabetes. Diabetes 2007;56:872-878
7. Wang X, Bao W, Liu J, Ouyang YY, Wang D, Rong S, Xiao X, Shan ZL, Zhang Y, Yao P, Liu LG: Inflammatory markers and risk of type 2 diabetes: a systematic review and meta-analysis. Diabetes Care 2013;36:166-175
8. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM: C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. JAMA 2001;286:327-334
9. Dandona P, Aljada A, Bandyopadhyay A: Inflammation: the link between insulin resistance, obesity and diabetes. Trends Immunol 2004;25:4-7
10. Krakoff J, Funahashi T, Stehouwer CD, Schalkwijk CG, Tanaka S, Matsuzawa Y, Kobes S, Tataranni PA, Hanson RL, Knowler WC, Lindsay RS: Inflammatory markers, adiponectin, and risk of type 2 diabetes in the Pima Indian. Diabetes Care 2003;26:1745-1751
11. Li S, Shin HJ, Ding EL, van Dam RM: Adiponectin levels and risk of type 2 diabetes: a systematic review and meta-analysis. Jama 2009;302:179-188
12. Tabak AG, Herder C, Rathmann W, Brunner EJ, Kivimaki M: Prediabetes: a high-risk state for diabetes development. Lancet 2012;379:2279-2290
13. Fonseca VA: Defining and characterizing the progression of type 2 diabetes. Diabetes Care 2009;32 Suppl 2:S151-156
14. Tabak AG, Jokela M, Akbaraly TN, Brunner EJ, Kivimaki M, Witte DR: Trajectories of glycaemia, insulin sensitivity, and insulin secretion before diagnosis of type 2 diabetes: an analysis from the Whitehall II study. Lancet 2009;373:2215-2221
15. Abdul-Ghani MA, Tripathy D, DeFronzo RA: Contributions of beta-cell dysfunction and insulin resistance to the pathogenesis of impaired glucose tolerance and impaired fasting glucose. Diabetes Care 2006;29:1130-1139
16. Gastaldelli A, Ferrannini E, Miyazaki Y, Matsuda M, DeFronzo RA, San Antonio metabolism s: Beta-cell dysfunction and glucose intolerance: results from the San Antonio metabolism (SAM) study. Diabetologia 2004;47:31-39
17. Grossmann V, Schmitt VH, Zeller T, Panova-Noeva M, Schulz A, Laubert-Reh D, Juenger C, Schnabel RB, Abt TG, Laskowski R, Wiltink J, Schulz E, Blankenberg S, Lackner KJ, Munzel T, Wild PS: Profile of the Immune and Inflammatory Response in Individuals With Prediabetes and Type 2 Diabetes. Diabetes Care 2015;38:1356-1364
18. Hofman A, van Duijn CM, Franco OH, Ikram MA, Janssen HL, Klaver CC, Kuipers EJ, Nijsten TE, Stricker BH, Tiemeier H, Uitterlinden AG, Vernooij MW, Witteman JC: The Rotterdam Study: 2012 objectives and design update. Eur J Epidemiol 2011;26:657-686
19. Ligthart S, Sedaghat S, Ikram MA, Hofman A, Franco OH, Dehghan A: EN-RAGE: a novel inflammatory marker for incident coronary heart disease. Arterioscler Thromb Vasc Biol 2014;34:2695-2699
20. Ligthart S, van Herpt TT, Leening MJ, Kavousi M, Hofman A, Stricker BH, van Hoek M, Sijbrands EJ, Franco OH, Dehghan A: Lifetime risk of developing impaired glucose metabolism and eventual progression from prediabetes to type 2 diabetes: a prospective cohort study. Lancet Diabetes Endocrinol 2016;4:44-51
21. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412-419

22. Brett J, Schmidt AM, Yan SD, Zou YS, Weidman E, Pinsky D, Nowygrod R, Neeper M, Przysiecki C, Shaw A, et al.: Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues. Am J Pathol 1993;143:1699-1712
23. Foell D, Wittkowski H, Kessel C, Luken A, Weinhage T, Varga G, Vogl T, Wirth T, Viemann D, Bjork P, van Zoelen MA, Gohar F, Srikrishna G, Kraft M, Roth J: Proinflammatory S100A12 can activate human monocytes via Toll-like receptor 4. Am J Respir Crit Care Med 2013;187:1324-1334
24. Yang Z, Yan WX, Cai H, Tedla N, Armishaw C, Di Girolamo N, Wang HW, Hampartzoumian T, Simpson JL, Gibson PG, Hunt J, Hart P, Hughes JM, Perry MA, Alewood PF, Geczy CL: S100A12 provokes mast cell activation: a potential amplification pathway in asthma and innate immunity. J Allergy Clin Immunol 2007;119:106-114
25. Andreasen AS, Kelly M, Berg RM, Moller K, Pedersen BK: Type 2 diabetes is associated with altered NF-kappaB DNA binding activity, JNK phosphorylation, and AMPK phosphorylation in skeletal muscle after LPS. PLoS One 2011;6:e23999
26. Di Pino A, Currenti W, Urbano F, Mantegna C, Purrazzo G, Piro S, Purrello F, Rabuazzo AM: Low advanced glycation end product diet improves the lipid and inflammatory profiles of prediabetic subjects. J Clin Lipidol 2016;10:1098-1108
27. Kosaki A, Hasegawa T, Kimura T, Iida K, Hitomi J, Matsubara H, Mori Y, Okigaki M, Toyoda N, Masaki H, Inoue-Shibata M, Nishikawa M, Iwasaka T: Increased plasma S100A12 (EN-RAGE) levels in patients with type 2 diabetes. J Clin Endocrinol Metab 2004;89:5423-5428
28. Schmid-Grendelmeier P, Altznauer F, Fischer B, Bizer C, Straumann A, Menz G, Blaser K, Wuthrich B, Simon HU: Eosinophils express functional IL-13 in eosinophilic inflammatory diseases. J Immunol 2002;169:1021-1027
29. Kim J, Woods A, Becker-Dunn E, Bottomly K: Distinct functional phenotypes of cloned Ia-restricted helper T cells. J Exp Med 1985;162:188-201
30. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL: Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986;136:2348-2357
31. Minty A, Chalon P, Derocq JM, Dumont X, Guillemot JC, Kaghad M, Labit C, Leplatois P, Liauzun P, Miloux B, et al.: Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. Nature 1993;362:248-250
32. Hilton DJ, Zhang JG, Metcalf D, Alexander WS, Nicola NA, Willson TA: Cloning and characterization of a binding subunit of the interleukin 13 receptor that is also a component of the interleukin 4 receptor. Proc Natl Acad Sci U S A 1996;93:497-501
33. Rapoport MJ, Jaramillo A, Zipris D, Lazarus AH, Serreze DV, Leiter EH, Cyopick P, Danska JS, Delovitch TL: Interleukin 4 reverses T cell proliferative unresponsiveness and prevents the onset of diabetes in non-obese diabetic mice. J Exp Med 1993;178:87-99
34. Zaccone P, Phillips J, Conget I, Gomis R, Haskins K, Minty A, Bendtzen K, Cooke A, Nicoletti F: Interleukin-13 prevents autoimmune diabetes in NOD mice. Diabetes 1999;48:1522-1528
35. Wong FS: Stimulating IL-13 receptors on T cells: a new pathway for tolerance induction in diabetes? Diabetes 2011;60:1657-1659
36. Defrance T, Carayon P, Billian G, Guillemot JC, Minty A, Caput D, Ferrara P: Interleukin 13 is a B cell stimulating factor. J Exp Med 1994;179:135-143
37. Stanya KJ, Jacobi D, Liu S, Bhargava P, Dai L, Gangl MR, Inouye K, Barlow JL, Ji Y, Mizgerd JP, Qi L, Shi H, McKenzie AN, Lee CH: Direct control of hepatic glucose production by interleukin-13 in mice. J Clin Invest 2013;123:261-271
38. Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, Fujikado N, Tanahashi Y, Akitsu A, Kotaki H, Sudo K, Nakae S, Sasakawa C, Iwakura Y: Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. Immunity 2009;30:108-119
39. Yang XO, Chang SH, Park H, Nurieva R, Shah B, Acero L, Wang YH, Schluns KS, Broaddus RR, Zhu Z, Dong C: Regulation of inflammatory responses by IL-17F. J Exp Med 2008;205:1063-1075
40. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C: A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005;6:1133-1141
41. Roohi A, Tabrizi M, Abbasi F, Ataie-Jafari A, Nikbin B, Larijani B, Qorbani M, Meysamie A, Asgarian-Omran H, Nikmanesh B, Bajouri A, Shafiey N, Maleki A: Serum IL-17, IL-23, and TGF-beta levels in type 1 and type 2 diabetic patients and age-matched healthy controls. Biomed Res Int 2014;2014:718946

42. Sumarac-Dumanovic M, Jeremic D, Pantovic A, Janjetovic K, Stamenkovic-Pejkovic D, Cvijovic G, Stevanovic D, Micic D, Trajkovic V: Therapeutic improvement of glucoregulation in newly diagnosed type 2 diabetes patients is associated with a reduction of IL-17 levels. Immunobiology 2013;218:1113-1118
43. Nadeem A, Javaid K, Sami W, Zafar A, Jahan S, Zaman S, Nagi A: Inverse relationship of serum IL-17 with type-II diabetes retinopathy. Clin Lab 2013;59:1311-1317
44. Rothman KJ: No adjustments are needed for multiple comparisons. Epidemiology 1990;1:43-46

Chapter 2.2

Relation of antioxidant capacity of diet and markers of oxidative status with C-reactive protein and adipocytokines: a prospective study.

Najada Stringa
Adela Brahimaj
Asija Zaciragic
Abbas Dehghan
M.Arfan Ikram
Albert Hofman
Taulant Muka
Jessica C. Kiefte-de Jong
Oscar H. Franco

Metabolism. 2017

ABSTRACT

Background

The role of dietary antioxidants and plasma oxidant-antioxidant status in low-grade chronic inflammation and adipocytokine levels is not established yet.

Objectives

We aimed to evaluate whether total dietary antioxidant capacity (assessed by dietary ferric reducing antioxidant potential (FRAP)), serum uric acid (UA) and gamma glutamyltransferase (GGT) were associated with low-grade chronic inflammation and circulating adipocytokines.

Methods

Data of 4,506 participants aged ≥55 years from the Rotterdam Study were analyzed. Baseline (1990-1993) FRAP score was assessed by a food frequency questionnaire. Baseline UA and GGT levels were assessed in non-fasting serum samples. Serum high sensitivity C-reactive protein (hs-CRP) was measured at baseline and 10 years later. Plasma leptin, adiponectin, plasminogen activator inhibitor-1 (PAI-1) and resistin levels were assessed 10 years later.

Results

A high FRAP score was associated with lower levels of UA and GGT. Overall, no association was found between FRAP and hs-CRP levels. FRAP score was associated with lower levels of leptin and PAI-1, higher levels of adiponectin, and no difference in resistin levels. Increased levels of UA were associated with higher levels of hs-CRP, PAI-1 and leptin; lower levels of adiponectin and no difference in resistin levels. Similarly, GGT was associated with higher levels of hs-CRP whereas no association was observed between GGT and adipocytokines.

Conclusion

These findings suggest that overall antioxidant capacity of diet and low levels of UA are associated with circulating adipocytokines whereas no consistent association was found with hs-CRP.

Key words

total antioxidant capacity of diet, uric acid, gamma- glutamyltransferase, C- reactive protein, adipocytokines, low-grade inflammation.

1. INTRODUCTION

Low-grade chronic inflammation has been involved in the pathogenesis of atherosclerosis and development of coronary heart disease (CHD) (1; 2). C-reactive protein (CRP), an acute phase reactant, is a general marker of low-grade chronic inflammation and has been associated with markers of atherosclerosis and CHD (2-4). Plasma sensitive CRP (hs-CRP) correlates with obesity and obesity-related disorders, including insulin resistance and type 2 diabetes (5). Adipose tissue synthesizes and releases many inflammatory mediators into the systemic circulation termed adipocytokines, and include leptin, adiponectin, plasminogen activator inhibitor-1 (PAI-1) and resistin, all of which can initiate the development of chronic inflammation and may directly contribute to metabolic and vascular diseases (6-15).

An imbalance between plasma oxidants-antioxidants (oxidative stress) as well as dietary antioxidants have been suggested to play a role in systemic low-grade chronic inflammation (16). Oxidative stress, defined as an increased load of free radicals, induces the activation of NF-κB, a transcription factor involved in cell survival, differentiation, and inflammation (17). Antioxidant molecules neutralize such free radicals and therefore diminish low-grade inflammation. Dietary antioxidants, including vitamin A, E and C, can counteract oxidative stress and therefore its adverse effect on inflammation (18). However, studies evaluating the role of individual antioxidants on inflammation have shown contradictory results, which can be due to not taking into account the interactive effect among nutrients (19). Hence, assessing the overall effects of antioxidants in the diet instead of the individual effects can provide further information regarding the association between diet and inflammation (19). The ferric reducing antioxidant potential (FRAP) measures the overall antioxidant capacity of diet by measuring the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+})(20) and, has been used as a marker of the overall effects of antioxidants in many studies. FRAP has been associated with inflammatory related diseases, including cardiovascular disease and cancer (21; 22). However, only a few studies have assessed its role on inflammation and adipocytokine levels (22-24). Furthermore, serum levels of uric acid (UA) and gamma-glutamyl transferase (GGT) are considered endogenous markers of oxidative stress (25). Both levels of UA and GGT positively correlate with markers of low-grade inflammation including hs-CRP, but how UA and GGT levels relate longitudinally with hs-CRP and adipocytokine levels remains unclear (26-29).

Therefore, we aimed to assess whether FRAP and endogenous markers of oxidative stress, UA and GGT, were associated with low-grade chronic inflammation and circulating adipocytokine concentrations in a prospective cohort of middle aged and elderly men and women.

2. MATERIALS AND METHODS

The study was performed within the Rotterdam Study (RS), a population-based cohort among individuals 55 years and over in the Ommoord district of Rotterdam, the Netherlands. The rationale and design of the RS is described elsewhere (30). The baseline examination (RS-I) took place in 1990-1993. Trained research assistants collected data on medical history, current health status,

use of medication, lifestyle and risk indicators for chronic diseases during an extensive home interview. Subsequently the participants visited the study center for detailed clinical examinations and assessment of diet. Follow up visits were held every 3-4 years.

2.1 MEASUREMENTS

2.1.1 Assessment of ferric reducing antioxidant potential (FRAP)

Dietary antioxidant capacity was assessed from the FFQ (Online Supplemental Material) the participants filled in during the interview. We used the Antioxidant Food Table published by the Institute of Nutrition Research, University of Oslo, which includes measurements of >3,000 foods (31), to calculate each food's contribution to ferric reducing antioxidant potential. The FRAP assay assesses the antioxidant capacity of individual food items to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) (20). Since the food table consisted of foods from several manufacturers, we consulted nutritional experts at Wageningen University (the Netherlands) to determine the linkage of foods from several manufacturers that were closest to the Dutch food products. For each participant, we multiplied the consumption frequency of each food by the corresponding FRAP value (in mmol/100g), and summed these values across all dietary sources. Vitamin supplementation was not included in the FRAP assessment because there were no detailed data available. Most variation in dietary FRAP score was explained by intakes of coffee (65%) and tea (21%) as described previously(21).

2.1.2 Assessment of Uric Acid and Gamma–glutamyltransferase (GGT)

Values of serum UA and GGT were obtained from baseline (1990-1993) non-fasting blood samples, which were centrifuged and the serum was subsequently frozen (−20°C) for 1 week. UA was determined with a Kone Diagnostica reagent kit and a Kone autoanalyzer. In order to check the calibration, 3 control samples were included every 10 samples. If the average values of the control samples of each run (100 samples) were not within 2.5% of the true value, the run was repeated. Day-by-day variation had to be within 5% (32). Serum GGT levels were determined within two weeks using a Merck Diagnostica kit (Merck, Whitehouse Station, NJ, USA) on an Elan Autoanalyzer (Merck).

2.1.3 Assessment of hs-CRP and adipocytokines

hs-CRP was measured in non-fasting frozen serum of study participants at baseline (1990-1993) and at the third center visit (1997-1999). A rate near-infrared particle immunoassay (Immage Immunochemistry System, Beckman Coulter, Fullerton, CA, USA) was used. This system measures concentrations from 0.2 to 1440mg/l, with a within-run precision of 0.5%, a total precision <7.5% and a reliability coefficient of 0.995. Undetectable CRP was scored as 0.2 (n=72).

For assessment of adipocytokines, fasting blood samples were collected at the research center, in the third center visit (1997-1999). Plasma was isolated and immediately put on ice and stored at -80°C. Citrate plasma (200 Ul) was sent in July 2008 to Rules-Based Medicine, Austin, Texas (www.myriadrbm.com). Fifty inflammatory biomarkers were quantified using multiplex immunoassay on a custom designed human multianalyte profile. The intra-assay variability was

less than 4% and the inter assay variability was less than 13%. Biomarkers with more than 60% completeness of measurements were selected for imputation and further analysis. Data on leptin, adiponectin, plasminogen activator inhibitor 1 (PAI-1) and resistin, major inflammatory markers released by adipose tissue (7), were available. The inflammatory markers investigated in the current study have no standard international calibration reference therefore, interpretation of the absolute values should be with caution. Since the current study is conducted within one set of individuals, the use of relative measures should not affect the effect estimates.

2.2 POPULATION FOR ANALYSIS

2.2.1 FRAP and inflammation

In the baseline examination (1990-1993) of the first cohort of the Rotterdam Study, 7,983 participants were included. Of out 7,983 participants, 6,521 participants were invited for dietary intake interview, out of which only 5,435 (83%) participants completed food frequency questionnaire and therefore had complete information on dietary intake. Moreover, out of 7,983 participants, randomly we invited 7,129 participants to assess cardiovascular risk factors, including CRP. However, only 6658 (93.3%) had C-reactive protein assessed. Participants with available information on both dietary and C-reactive protein levels were 5104. Further, we excluded 598 participants who reported use of anti-inflammatory drugs at baseline and/or during the follow-up (n=598), leaving 4,506 participants for the analysis of FRAP with CRP (Figure 1). In addition, leptin, adiponectin, PAI-1 and resistin were measured in a random subsample of 971 participants, hence only 798 participants were included in the analysis of FRAP with adipocytokines (Figure 1).

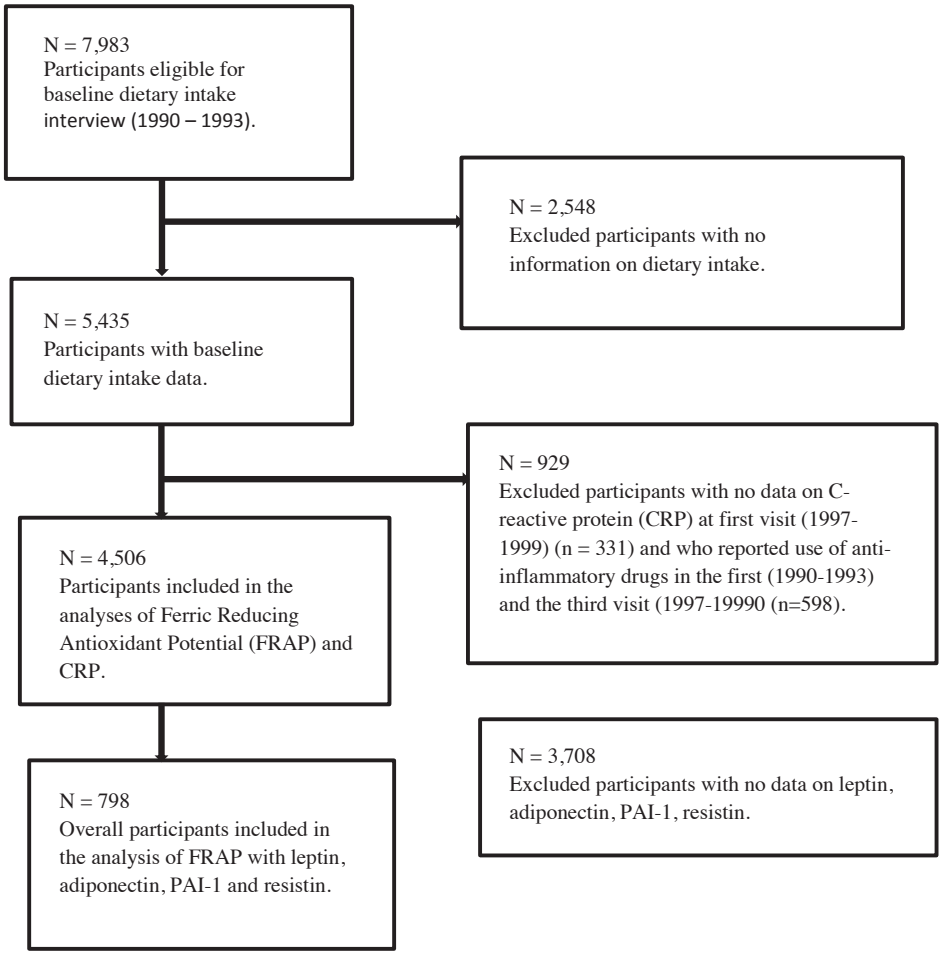
2.2.2 Uric acid, gamma-glutamyltransferase and inflammation

In the baseline examination (1990-1993) of the first cohort of the Rotterdam Study, 7,983 participants were included. Uric acid and GGT data were available for 5,047 subjects (Figure 2). Out of these, 893 participants were excluded either because they did not have CRP measured at the first visit or because they reported use of anti-inflammatory drugs at baseline and/or during the follow-up, leaving 4,154 participants for the analysis of uric acid with CRP and GGT with CRP. 3,447 participants were further excluded because they did not have measures of other inflammatory markers, hence 707 participants were included in the analysis of uric acid and GGT with leptin, adiponectin, PAI-1 and resistin (Figure 2).

2.3 STATISTICAL ANALYSES

Data are presented as mean (± standard deviation) for normally distributed continuous variables, median (range) for continuous variables that are not normally distributed, and percentages for categorical variables. We used natural log-transformed values of serum CRP concentrations, GGT, non-fasting serum glucose, leptin, adiponectin, PAI-1 and resistin to better approximate a normal distribution. Pearson correlations were used to assess the correlations between inflammatory markers. To account for systematic measurement error in FRAP, FRAP was adjusted for total energy intake by using the residual method in the analysis(33). FRAP was analyzed continuously. For analyses evaluating CRP as outcome, we fitted linear regression models us-

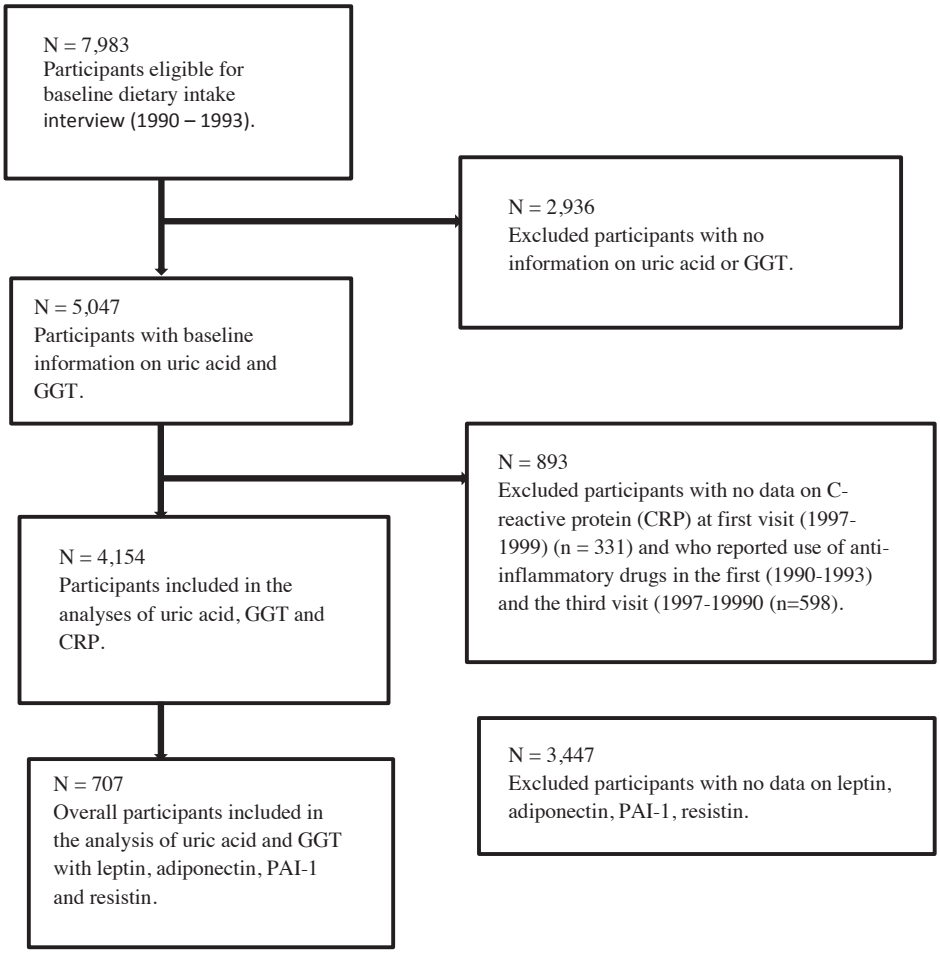
Figure 1. Flow chart of participants included in the analysis of overall antioxidant capacity of diet and inflammation: the Rotterdam Study.



FRAP, ferric reducing antioxidant potential; PAI-1, Plasminogen activator inhibitor-1;

ing generalized estimating equations with exchangeable correlation structure adjusting for the within-subject correlations due to the repeated measurements of CRP in the same individual (inter-class correlation coefficient = 0.682 for natural log-transformed CRP) (34). Multivariable linear regression was used to examine whether FRAP, GGT and UA were independently associated with blood levels of adiponectin, leptin, resistin and PAI-1. Regression coefficients (βs) and 95% confidence intervals were obtained on the basis of robust standard errors (95% CI). First, we calculated age and gender adjusted coefficients (Model 1) for the following exposure: FRAP, GGT and UA. Subsequently in Model 2, we adjusted for potential confounders when the covariates changed the effect estimate by more than 10% in univariate models of each exposure with any of the outcomes assessed. The following potential confounding factors, were evaluated:

Figure 2. Flow Chart of participants included in the analysis of uric acid and gamma-glutamyltransferase (GGT) with inflammatory markers: the Rotterdam Study.



PAI-1, Plasminogen activator inhibitor-1;

body mass index (BMI) (continuous), energy intake (continuous), physical activity(continuous), smoking status (never or former, current), lipid lowering medication use (Yes, No), systolic blood pressure(continuous), total cholesterol(continuous), vitamin supplementation (Yes, No), hormone replacement therapy (HRT) (Yes, No), prevalent chronic diseases (CVD or T2D) (yes, no), non-fasting blood glucose(continuous), education (low, intermediate, high), income (low, intermediate, high), alcohol, energy-adjusted processed meat intake (continuous), energy-adjusted unprocessed meat intake (continuous), Dutch Healthy Diet index (DHDI) (continuous). For the analysis on leptin, adiponectin, PAI-1 and resistin as outcomes, we also adjusted for CRP in the first visit (1990-1993) as a proxy of chronic inflammation at baseline as adipocytokines were measured only at the third round visit (1997-1999). To check for non-linear relation, a quadratic

term was tested in multivariable model 2. Since there is evidence that the association between diet antioxidants and inflammatory biomarkers differs by sex (35), we tested for statistical interaction by adding a product term in model 2. Furthermore, stratified analysis was performed and the results were presented for model 2. We further checked the association of FRAP with uric acid, and FRAP with GGT using multivariable linear regression models. We also performed sensitivity analyses (i) restricting all main analyses to participants with available information on all exposures and outcomes investigated (N=633), (ii) excluding participants with chronic diseases (CVD or T2D) and (iii) further adjusted for BMI change from first to the third visit. A P-value lower than 0.05 was considered as statistically significant, but to account for multiple testing, we adjusted the p-value from 0.05 to 0.0166 by applying the Bonferroni correction for the number of exposures studied (N=3).
To adjust for potential bias associated with missing data we used multiple imputation procedure (N= 5 imputations). All analyses were done using SPSS statistical software (SPSS, version 21.0; SPSS Inc., Chicago, Illinois).

3. RESULTS

The main characteristics of the study population are shown by gender in Table 1. FRAP score and GGT levels were lower in women compare to men (FRAP: 20.02 ± 5.07 mmol/day vs. 20.83 ± 5.95 mmol/day; GGT: median 21 U/l, range 351U/l vs median 27U/l, range 576 U/l) whereas UA levels were higher in women (296.62 ± 71.44 μmol/l vs. 352.88 ± 74.40 μmol/l) (**Table 1**). CRP levels at baseline were slightly lower in women whereas no significant difference was observed in the CRP levels at the third visit (**Table 1**). Also, women had slightly higher BMI (26.55 vs. 25.68 kg/m^2) and leptin levels (median: 14.0 vs 4.02 ng/mL) than men. Although the energy intake was lower in women (1796 vs. 2246.2 kcal/day), they had higher physical activity (89.45 vs 69.15 MET) as well as a healthier diet (DHDI: 31.95 vs. 27.95) than men. Among the adipocytokines, PAI-1 and leptin (r = 0.466, p = 0.01), PAI-1 and CRP (r = 0.325, p = 0.01), PAI-1 and adiponectin (r = -0.270, p = 0.01), leptin and CRP (r = 0.254, p = 0.01) showed the highest correlation (**Supplementary Table S1**). Compared to subjects who did not have information on leptin, adiponectin, PAI-1 and resistin, subjects who had information on these inflammatory markers did not differ with respect to FRAP, but had higher levels of CRP, BMI, systolic blood pressure and higher prevalence of chronic disease (**Supplementary Table S2**).

3.1 The association between FRAP score and inflammatory markers

There was no association between FRAP and hs-CRP levels in the age and gender-adjusted model or multivariable model **(Table 2).** In the multivariable models, FRAP score was associated with lower levels of leptin (β = -0.01, 95% CI = -0.02; -0.001), PAI-1 (β = -0.02, 95% CI= -0.03; -0.01) and higher levels of adiponectin (β = 0.01, 95% CI = 0.002; 0.015). No association was observed between FRAP and resistin. **(Table 2).**

Table 1. Baseline characteristics of study participants (N=4506): the Rotterdam Study.

	Total (N=4506)	Women (N=2571)	Men (N=1935)	P-value[b]
FRAP (mmol/day)	20.37±5.48	20.02±5.07	20.83±5.95	<0.001
Age (years)	67.64±7.74	67.93±8.01	67.26±7.36	0.004
Energy intake (kcal/day)	1989.51±504.48	1796.30±405.97	2246.17±508.24	<0.001
Physical activity (MET hours/week)	78.30±44.28	89.45±43.90	69.15±41.56	<0.001
BMI (kg/m^2)	26.18±3.57	26.55±3.99	25.68±2.85	<0.001
CRP first round[a] (mg/ml)	1.78 (0.86-3.39)	1.74 (0.85-3.13)	1.85 (0.87-3.79)	<0.001
CRP third round[a] (mg/ml)	2.34 (1.16-4.34)	2.36 (1.15-4.26)	2.30 (1.16-4.46)	0.583
Non-fasting serum glucose[a] (mml/l)	6.20 (5.45-7.40)	6.10 (5.40-7.10)	6.40 (5.60-7.70)	<0.001
SBP (mmHg)	183.84±22.05	139.10±22.22	138.50±21.83	0.363
DBP (mmHg)	78.80±11.26	73.29±11.14	74.47±11.39	<0.001
Total Cholesterol (mmol/l)	6.68±1.19	6.92±1.18	6.35±1.12	<0.001
Hormone replacement therapy, n (%)	65 (1.4%)	63 (2.5%)	2 (0.1%)	<0.001
Uric Acid (μmol/l)	320.16±77.76	296.62±71.44	352.88±74.40	<0.001
Vitamin supplement use, n (%)	329 (7.3%)	245 (9.5%)	84 (4.3%)	<0.001
GGT[a] (U/l)	23.00 (18.00-32.00)	21.00 (16.00-28.00)	27.00 (21.00-38.00)	<0.001
Lipid reducing agents, n (%)	119 (2.6%)	66 (2.6%)	53 (2.7%)	0.395
DHDI	30.23±9.20	31.95±9.11	27.95±8.83	<0.001
Prevalent diseases*, n (%)	1490 (33.1%)	712 (27.7%)	778 (40.2%)	<0.001
Smoking: Never or former, n (%)	3440 (76.3%)	2079 (80.9%)	1361 (70.3%)	<0.001
Current, n (%)	1066 (23.7%)	492 (19.1%)	574 (29.7%)	
Income: Low, n (%)	1014 (22.5%)	829 (32.2%)	185 (9.6%)	<0.001
Middle, n (%)	2002 (44.4%)	1062 (41.3%)	940 (48.6%)	
High, n (%)	1490 (33.1%)	680 (26.4%)	810 (41.9%)	
Education: Low, n (%)	2321 (51.5%)	1597 (62.1%)	724 (37.4%)	<0.001
Middle, n (%)	1781 (39.5%)	862 (33.5%)	919(47.5%)	
High, n (%)	404 (9.0%)	112 (4.4%)	292 (15.1%)	
Processed meat intake (servings/day)	1.47±1.24	1.19±1.05	1.84±1.37	<0.001
Unprocessed meat intake (servings/day)	0.74±0.47	0.69±0.42	0.82±0.53	0.048
Alcohol[#]:				<0.001
Quartile I (<0.1886g), n (%)	1126 (25.0%)	847 (32.9%)	279 (14.4%)	
Quartile II (0.1886-3.6813g), n (%)	1127 (25.0%)	798 (31.0%)	329 (17.0%)	
Quartile III (3.6813-15.1401g), n (%)	1127 (25.0%)	572 (22.2%)	555 (28.7%)	
Quartile IV (>15.1401g), n (%)	1126 (25.0%)	354 (13.8%)	772 (39.9%)	
Leptin[c] (ng/mL)	7.63 (3.82-16.20)	14.00 (7.85-22.00)	4.02 (2.44-6.64)	<0.001
Adiponectin[c] (μg/mL)	3.42 (2.25-5.00)	4.34 (3.17-5.89)	2.7 (1.94-3.63)	<0.001
PAI-1[c] (ng/mL)	17.15 (9.98-28.63)	17.90 (10.30-33.20)	16.10 (9.66-26.15)	0.009
Resistin[c] (ng/mL)	0.42 (0.31-0.58)	0.42 (0.31-0.58)	0.43 (0.31-0.59)	0.951

FRAP, ferric reducing antioxidant potential; BMI, Body mass index; CRP, C-reactive protein; DHDI, Dutch healthy diet index (excluding fruits and vegetables); DBP, diastolic blood pressure; GGT, Gamma glutamyltransferase; PAI-1, Plasminogen activator inhibitor - 1; SBP, systolic blood pressure

[a] Median (Range between 25th percentile and 75th percentile)

[b] Comparison between men and women. For continuous variables = Independent sample T-Test; For categorical variables = Chi^2 (χ^2)

[c] N=798 included in the analyses of FRAP and adipocytokines.

*Prevalent disease include cardiovascular disease and type 2 diabetes.

[#] Quartile I refers to values < 25th percentile; Quartile II refers to values between 25th and 50th percentile; Quartile III refers to values between 50th and 75th percentile; Quartile IV refers to values >75th percentile.

Table 2. Association of ferric reducing antioxidant potential with C-reactive protein and adipocytokines: the Rotterdam Study.

	Model 1 β (95% CI)	Model 2 β (95%CI)
CRP[§] (N=4507)	0.001 (-0.004,0.007)	-0.002(-0.007,0.003)a
Leptin[§] (N=798)	-0.012 (-0.023, -0.001)	-0.009(-0.017, -0.00005)b
Adiponectin[§] (N=798)	0.009 (0.002,0.015)*	0.009(0.003,0.016)*b
PAI-1[§] (N=798)	-0.018(-0.028, -0.008)*	-0.018(-0.027, -0.008)*b
Resistin[§] (N= 798)	0.002 (-0.006,0.009)	0.001(-0.006,0.009)b

CI, confidence interval; FRAP, ferric reducing antioxidant potential; CRP, C-reactive protein; PAI-I, Plasminogen Activator Inhibitor-1.

§ Variables were log transformed to better approximate normal distribution.

*remains significant after Bonferroni correction (p=0.0166)

βs and 95% confidence intervals were estimated using generalized estimated equations (for C-reactive protein as outcome) and linear regression models (for leptin, adiponectin, PAI-1 and resistin as outcomes) adjusted for age and gender (Model 1), and additionally adjusted for body mass index, smoking status, prevalent diseases, systolic blood pressure, non-fasting glucose, total cholesterol, index1(time), energy intake, income, alcohol, statin use (Model 2a). For adipocytokines, model 2 was further adjusted for C-reactive protein (Model 2b). Additional adjustment for other covariates did not change the effect estimate with >10%.

3.2 The association between UA, GGT and inflammatory markers

After multivariable adjustment, increased levels of UA were associated with higher levels of hs-CRP (β = 0.12, 95% CI = 0.09; 0.16), leptin (β = 0.10, 95% CI = 0.05; 0.15) PAI-1 (β = 0.15, 95% CI = 0.09; 0.20), and lower levels of adiponectin (β = -0.07, 95% CI = -0.10; -0.03) **(Table 3).** No association was observed between UA and resistin (**Table 3**). Similarly, after correcting for confounding factors, GGT was associated with higher levels of hs-CRP (β = 0.06, 95% CI = 0.13; 0.19) whereas no association was observed between GGT and adipocytokines (**Table 3**).

Table 3. Association of uric acid and gamma glutamyltransferase with C-reactive protein and adipocytokines: the Rotterdam study.

	Uric acid (per SD)		GGT (per SD)[§]	
	Model 1 β (95% CI)	Model 2 β (95%CI)	Model 1 β (95% CI)	Model 2 β (95% CI)
CRP[§](N=4154)	0.198 (0.167,0.228)*	0.123 (0.091,0.155)*[a]	0.213 (0.181,0.245)*	0.160 (0.128,0.191)* [a]
Leptin[§](N=707)	0.257 (0.197,0.316)*	0.100 (0.048,0.152)*[b]	0.101 (0.040,0.161)*	-0.020 (-0.070,0.030)[b]
Adiponectin[§](N=707)	-0.099 (-0.135,-0.064)*	-0.066 (-0.103,-0.028)*[b]	-0.041 (-0.075,-0.006)*	-0.005 (-0.041,0.032)[b]
PAI-1[§](N=707)	0.246 (0.193,0.300)*	0.147 (0.091,0.203)*[b]	0.148 (0.094,0.202)	0.047 (-0.007,0.100)[b]
Resistin[§](N=707)	0.014 (-0.028,0.056)	0.026 (-0.020,0.072)[b]	0.006 (-0.035,0.046)	0.012 (-0.032,0.055)[b]

CI, confidence interval; CRP, C-reactive protein; GGT, gamma glutamyltransferase; PAI-1, Plasminogen Activator Inhibitor-1; SD, standard deviation.

[§] Variables were log transformed to better approximate normal distribution.

* remains significant after Bonferroni correction (p=0.0166)

βs and 95% confidence intervals were estimated using generalized estimated equations (for C-reactive protein as outcome) and linear regression models (for leptin, adiponectin, PAI-1 and resistin as outcomes) adjusted for age and sex (Model 1) and additionally adjusted for baseline body mass time, time of measurement, non-fasting glucose, energy intake, total cholesterol, hormone replacement therapy, systolic blood pressure, diastolic blood pressure, statin use, income, alcohol+ GGT/uric acid (adjustment for GGT when uric acid was the exposure and vice versa) (Model 2a). For adipocytokines as outcomes, model 2 was further adjusted for baseline C-reactive protein (Model 2b). Results are presented per standard deviation uric acid (for CRP as outcome: 1SD= 80.5611 μmol/L; for adipocytokines as outcome: 1SD=73,1832 μmol/L) and GGT levels (for CRP as outcome: 1SD=29,9731 U/L ; for adipocytokines as outcome: 1SD=22,4034 U/L).

3.3 Effect modification by gender

A significant effect modification by sex was found for the association between FRAP score and hs-CRP (*P*-interaction = 0.009). After stratification, a high dietary FRAP score was associated with lower levels of hs-CRP in women (β = -0.01, 95% CI = -0.02; -0.003), whereas no association was observed in men (**Supplementary Table S3**). No effect modification by sex was observed for the association between FRAP score with the adipocytokine levels (All *P*-interaction > 0.05). Similarly, the analyses were not different between strata of sex (**Supplementary Table S3**). Also, no sex differences were observed for the association of UA and GGT with CRP and adipocytokines (All *P*-intercation > 0.05) (**Supplementary Table S4**).

3.4 Sensitivity analyses

Higher levels of FRAP score were associated with lower levels of both UA (β = -0.003, 95% CI = -0.005; -0.002) and GGT (β = -0.006, 95% CI = -0.009; -0.003), after correcting for confounders **(Supplementary Figure S1 and Supplementary Table S5)**. There was no evidence against a linear relation in all the main analyses (all *P*-values for quadratic term > 0.05, data not shown). Also, all associations that were statistically significant in the main analyses remained unchanged in terms of statistical significance when the analyses were restricted to (i) participants with available measures of FRAP, UA, GGT, CRP, leptin, adiponectin, PAI-1 and resistin (n = 633) (data not shown), (ii) to subjects without chronic diseases (**Supplementary Table S6 and S7**) or (iii) when we further adjusted for changes in BMI between the first and third visit (data not shown). The associations of FRAP with adiponectin and PAI-1, of UA with hs-CRP, leptin, adiponectin, and PAI-1, and the association of GGT with hs-CRP, remained significant after we applied the Bonferroni correction (all p < 0.0166).

4. DISCUSSION

Overall a higher FRAP score was associated with leptin, adiponectin, and PAI-1 but not with CRP levels. Furthermore, increased levels of both GGT and UA levels were associated with higher levels of pro-inflammatory markers and lower levels of anti-inflammatory markers.

In the current investigation, no association was found between FRAP and CRP levels in the overall population, however, in women, a higher FRAP score was associated with diminished chronic inflammation. Similar to our findings, Detopoulou et al in a cross-sectional study of 532 men and women found no association between FRAP and CRP levels in the total population (36). In contrast, a cross-sectional study from Brighenti et al (23) , which used the TAC assay to measure antioxidant capacity, showed an association with lower levels of CRP in an adult Italian population including both men and women. We did find an interaction with gender, suggesting that the association between FRAP and CRP levels is present only in women, which is in line with the results of previous studies conducted in women. For example, the study from Kobayashi at al. (24) showed that dietary total antioxidant capacity was associated with lower serum CRP concentrations in young Japanese women (474 women, aged 18-22 years) regardless of assay used to measure it. Also, in a 9-month observational study among postmenopausal women, Wang and

his colleagues showed that consumption of diets rich in total antioxidants was associated with lower plasma CRP levels (37).
Several studies show a stronger defense against oxidative damage in the female liver tissue, which is the major determinant of CRP levels (38). Animal studies have shown that, compared to males, antioxidant capacity of diet assessed by FRAP and other methods is higher in liver tissue (38). Also, females have greater mean hepatic alpha-tocopherol levels, total capacity of the cellular systems that detoxify reactive oxygen species or free radical-drug metabolites seems to be higher in the female rat liver(39). These evidence may account for the sex differences observed in the association between FRAP and CRP levels in our study, which merits further investigation.
Similar to our findings, previous studies (27; 40) have shown that increased UA levels are significantly associated with increased hs-CRP levels. Also in a study of Park et al. (41) in post-menopausal women uric acid was associated with lower adiponectin levels. Another study from Ali et al. (42) found that high GGT levels are associated with high hs-CRP levels implicating that elevated GGT levels are associated with burden of subclinical vascular inflammation.
To our knowledge, this is the first study to show that the FRAP score was a determinant of leptin and PAI-I concentrations. In line with our findings, a previous study has shown an association between FRAP score and higher adiponectin levels (36). Previous studies (43) have indicated that total antioxidant capacity of diet is associated with less central adiposity, as well as to metabolic (e.g. insulin resistance index) and oxidative stress markers in healthy young adults (e.g. oxidized-LDL, malondialdehyde). Central adiposity, mainly abdominal adiposity is the main producer of anti-inflammatory (adiponectin) and pro-inflammatory markers (leptin, resistin and PAI-1) (12; 44; 45). Leptin is an adipocyte-derived hormone that reduces food intake and increases energy expenditure by acting in the hypothalamus (46; 47) and has also pro-inflammatory effects (7; 8). Leptin levels correlate with higher indices of adiposity, however, individuals with similar degrees of adiposity have variations in serum leptin levels (46; 48). Adiponectin is one of the most abundant adipocyte-derived hormones and appears to improve insulin sensitivity and vascular inflammation through its actions in liver and muscle (7). Several studies have demonstrated that adiponectin is a marker and a mediator of metabolic risk, including the risk for conversion to diabetes and risk of myocardial infarction (49). PAI-1, is another hormone secreted from fat cells, and is suggested to be a possible contributor to obesity-induced diabetes and atherosclerosis (50). Resistin, on the other hand, is almost an exclusively white adipose tissue-expressed polypeptide, and has also been linked to energy homeostasis and diet-induced obesity, insulin resistance and diabetes (51). Other factors, including hormonal and nutritional factors have been suggested to influence concentrations of these inflammatory markers(52). Our study also indicates that the antioxidant diet, GGT and UA may affect the levels of leptin, adiponectin, and PAI-I but not resistin independent of obesity. It was reported that uric acid induces CRP expression by implication on cell proliferation and nitric oxide production of human vascular cells (53). Elevation of serum GGT is involved in the inflammatory response. It is plausible that elevation in GGT might occur before elevation in CRP, if oxidative stress leads to an inflammatory response (54). These data imply that inflammation may be one of the underlying mechanisms linking an antioxidant diet, GGT and UA with cardiometabolic outcomes, which needs to be elucidated by

future studies. However future studies are needed to clarify specific inflammatory markers that may be involved in the pathway.

Probably oxidative stress is the pathway that links antioxidants with a low inflammatory profile. The human body has a number of defense mechanisms against oxidative stress including antioxidants, preventive and repair mechanism and physical defense (17). Antioxidants themselves can be divided into enzymatic antioxidants (glutathione peroxidase, peroxide dismutase and catalase) and non-enzymatic antioxidants like ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), carotenoids, flavonoids. Coffee and tea are the main contributors of FRAP in Rotterdam Study and in other studies as well (21; 55). The anti-inflammatory effects of both coffee and tea have been previously reported (56). On the other hand, the anti-inflammatory effect of fruits and vegetables is supposed to come from vitamins and flavonoids they contain (19). Antioxidants act scavenging ROS and inhibit NF-κβ, even though not all at the same level. This may lead to decreased oxidative stress, and therefore in diminished low-grade chronic inflammation.

Our study is unique among previous investigations because of its prospective design, large population-based study group and adjustment for a broad range of confounders. Also, to our knowledge, this is one of the first prospective studies to use measures of CRP in two time points. Also, in our study, we could assess the association between FRAP and markers of oxidative stress, such as GGT and UA, showing a strong association, and therefore supporting internal validity. Nevertheless, it has some limitations. First, assessment of diet was done at baseline and there may have been changes in antioxidant consumption over time. However, it has been shown that dietary habits change very little over time in middle-aged adults (57). Second, the FFQ can be limited by errors in reporting and recall and by incomplete assessment of all sources of antioxidant intake, which may introduce misclassification in dietary intake and would bias results toward the null. Third, we did not have repeated measures for leptin, adiponectin, PAI-1 and resistin. Also, these markers were assessed 10 years later from FRAP, UA and GGT measurements. Moreover, we had no measurements of other adipocyte-derived inflammatory markers like interleukin-6 or tumor necrosis factor-α or more accurate measures of oxidative stress such as ROS, that could have strengthened the results. Furthermore, we used a subpopulation for the analysis regarding adiponectin, resistin, leptin and PAI-1 as outcome, which may have introduced selection bias since this population was different with respect to some health characteristics. However, it has been shown that using a restricted source population for a cohort study usually leads to bias towards the null which may have led to an underestimation of the observed associations in our study of the exposure (58). Moreover, it has been shown that using a selected source population for a cohort study usually leads to bias towards the null. Furthermore, the restriction of the main analysis in the participants with available information on all exposures and outcomes investigated in this study provided similar results, and therefore, selection bias is less likely to have happened. Finally, physical activity was measured at the third round of the Rotterdam Study. Therefore, we cannot fully exclude residual confounding by physical activity levels.

5. CONCLUSIONS

In conclusion, we found no consistent association between FRAP and CRP levels, while both UA and GGT were associated with low CRP. Furthermore, high overall dietary antioxidant capacity of diet and lower levels of UA were associated with lower levels of pro-inflammatory adipocytokines and higher levels of anti-inflammatory adipocytokines.

ACKNOWLEDGEMENTS

The authors thank the study participants, the supporting staff of the Rotterdam Study and the participating general practitioners.

FUNDING SOURCES

The Rotterdam Study is funded by Erasmus MC and Erasmus University, Rotterdam, the Netherlands; the Netherlands Organization for Scientific Research (NWO); the Netherlands Organization for the Health Research and Development (ZonMw); the Research Institute for Diseases in the Elderly (RIDE); the Ministry of Education, Culture and Science; the Ministry for Health, Welfare and Sports; the European Commission (DG XII); and the Municipality of Rotterdam. TM, JCK and OHF work in ErasmusAGE, a center for aging research across the life course funded by Nestlé Nutrition (Nestec Ltd.); Metagenics Inc.; and AXA. NS, AB and AZ have been financially supported by Erasmus Mundus Western Balkans (ERAWEB), a project funded by the European Commission. AD is supported by NWO grant (Veni, 916.12.154) and the EUR Fellowship. These funding sources had no role in design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review or approval of the manuscript.

DISCLOSURE

The authors declare no conflict of interest.

CONTRIBUTORS/AUTHORSHIP

TM and OHF conceived and designed the study. NS, AB, TM and OHF participated in the statistical analyses, data interpretation, manuscript writing and revising and had primary responsibility for the final content of the manuscript. JCK participated in data synthesis/analysis and interpretation of the data. NS, AB, TM, JCK and OHF drafted the final manuscript. AH designed the Rotterdam Study and participated in data interpretation, manuscript writing and revising. All authors contributed to the critical revision of the manuscript and approved the final version.

REFERENCES

1. Libby P, Ridker PM: Inflammation and atherosclerosis: role of C-reactive protein in risk assessment. Am J Med 2004;116 Suppl 6A:9S-16S
2. Libby P, Ridker PM, Maseri A: Inflammation and atherosclerosis. Circulation 2002;105:1135-1143
3. Rifai N, Ridker PM: Inflammatory markers and coronary heart disease. Curr Opin Lipidol 2002;13:383-389
4. Tremblay J, Hamet P: Biomarkers of vascular complications in type 2 diabetes. Metabolism: Clinical & Experimental 2015;64:S28-32
5. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM: C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. Jama 2001;286:327-334
6. Van Gaal LF, Mertens IL, De Block CE: Mechanisms linking obesity with cardiovascular disease. 2006;444:875-880
7. Ouchi N, Parker JL, Lugus JJ, Walsh K: Adipokines in inflammation and metabolic disease. Nat Rev Immunol 2011;11:85-97
8. Sainz N, Barrenetxe J, Moreno-Aliaga MJ, Martinez JA: Leptin resistance and diet-induced obesity: central and peripheral actions of leptin. Metabolism: Clinical & Experimental 2015;64:35-46
9. Nakanishi S, Yamane K, Kamei N, Nojima H, Okubo M, Kohno N: A protective effect of adiponectin against oxidative stress in Japanese Americans: the association between adiponectin or leptin and urinary isoprostane. Metabolism: Clinical & Experimental 2005;54:194-199
10. Park HK, Ahima RS: Physiology of leptin: energy homeostasis, neuroendocrine function and metabolism. Metabolism: Clinical & Experimental 2015;64:24-34
11. Gasbarrino K, Gorgui J, Nauche B, Cote R, Daskalopoulou SS: Corrigendum to "circulating adiponectin and carotid intima-media thickness: a systematic review and meta-analysis" [Metabolism 65 (2016) 968-986]. Metabolism: Clinical & Experimental 2016;65:1434-1442
12. Kizer JR: Adiponectin, cardiovascular disease, and mortality: parsing the dual prognostic implications of a complex adipokine. Metabolism: Clinical & Experimental 2014;63:1079-1083
13. Potter van Loon BJ, Kluft C, Radder JK, Blankenstein MA, Meinders AE: The cardiovascular risk factor plasminogen activator inhibitor type 1 is related to insulin resistance. Metabolism: Clinical & Experimental 1993;42:945-949
14. Nakamura T, Adachi H, Hirai Y, Satoh A, Ohuchida M, Imaizumi T: Association of plasminogen activator inhibitor-1 with insulin resistance in Japan where obesity is rare. Metabolism: Clinical & Experimental 2003;52:226-229
15. Putz DM, Goldner WS, Bar RS, Haynes WG, Sivitz WI: Adiponectin and C-reactive protein in obesity, type 2 diabetes, and monodrug therapy. Metabolism: Clinical & Experimental 2004;53:1454-1461
16. Savini I, Catani MV, Evangelista D, Gasperi V, Avigliano L: Obesity-Associated Oxidative Stress: Strategies Finalized to Improve Redox State. International Journal of Molecular Sciences 2013;14:10497-10538
17. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J: Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007;39:44-84
18. Willcox JK, Ash SL, Catignani GL: Antioxidants and prevention of chronic disease. Crit Rev Food Sci Nutr 2004;44:275-295
19. Alissa EM, Ferns GA: Dietary Fruits and Vegetables and Cardiovascular Diseases Risk. Crit Rev Food Sci Nutr 2015:0
20. Benzie IFF, Strain JJ: The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. Analytical Biochemistry 1996;239:70-76
21. Pantavos A, Ruiter R, Feskens EF, de Keyser CE, Hofman A, Stricker BH, Franco OH, Kiefte-de Jong JC: Total dietary antioxidant capacity, individual antioxidant intake and breast cancer risk: the Rotterdam Study. Int J Cancer 2015;136:2178-2186
22. Korkmaz GG, Altinoglu E, Civelek S, Sozer V, Erdenen F, Tabak O, Uzun H: The association of oxidative stress markers with conventional risk factors in the metabolic syndrome. Metabolism: Clinical & Experimental 2013;62:828-835
23. Brighenti F, Valtuena S, Pellegrini N, Ardigo D, Del Rio D, Salvatore S, Piatti P, Serafini M, Zavaroni I: Total antioxidant capacity of the diet is inversely and independently related to plasma concentration of high-sensitivity C-reactive protein in adult Italian subjects. Br J Nutr 2005;93:619-625
24. Kobayashi S, Murakami K, Sasaki S, Uenishi K, Yamasaki M, Hayabuchi H, Goda T, Oka J, Baba K, Ohki K, Watanabe R, Sugiyamama Y: Dietary total antioxidant capacity from different assays in relation to serum C-reactive protein among young Japanese women. Nutr J 2012;11:91

25. Lee DH, Blomhoff R, Jacobs DR, Jr.: Is serum gamma glutamyltransferase a marker of oxidative stress? Free Radic Res 2004;38:535-539
26. Wijnands JMA, Boonen A, Dagnelie PC, van Greevenbroek MMJ, van der Kallen CJH, Ferreira I, Schalkwijk CG, Feskens EJM, Stehouwer CDA, van der Linden S, Arts ICW: The cross-sectional association between uric acid and atherosclerosis and the role of low-grade inflammation: the CODAM study. Rheumatology 2014;53:2053-2062
27. Ruggiero C, Cherubini A, Ble A, Bos AJ, Maggio M, Dixit VD, Lauretani F, Bandinelli S, Senin U, Ferrucci L: Uric acid and inflammatory markers. Eur Heart J 2006;27:1174-1181
28. Liu X, Hamnvik OP, Chamberland JP, Petrou M, Gong H, Christophi CA, Christiani DC, Kales SN, Mantzoros CS: Circulating alanine transaminase (ALT) and gamma-glutamyl transferase (GGT), but not fetuin-A, are associated with metabolic risk factors, at baseline and at two-year follow-up: the prospective Cyprus Metabolism Study. Metabolism 2014;63:773-782
29. Fukui M, Tanaka M, Toda H, Asano M, Yamazaki M, Hasegawa G, Imai S, Fujinami A, Ohta M, Nakamura N: The serum concentration of allograft inflammatory factor-1 is correlated with metabolic parameters in healthy subjects. Metabolism: Clinical & Experimental 2012;61:1021-1025
30. Hofman A, Brusselle GG, Darwish Murad S, van Duijn CM, Franco OH, Goedegebure A, Ikram MA, Klaver CC, Nijsten TE, Peeters RP, Stricker BH, Tiemeier HW, Uitterlinden AG, Vernooij MW: The Rotterdam Study: 2016 objectives and design update. Eur J Epidemiol 2015;30:661-708
31. Carlsen MH, Halvorsen BL, Holte K, Bøhn SK, Dragland S, Sampson L, Willey C, Senoo H, Umezono Y, Sanada C, Barikmo I, Berhe N, Willett WC, Phillips KM, Jacobs DR, Blomhoff R: The total antioxidant content of more than 3100 foods, beverages, spices, herbs and supplements used worldwide. Nutrition Journal 2010;9:3-3
32. Bos MJ, Koudstaal PJ, Hofman A, Witteman JCM, Breteler MMB: Uric Acid Is a Risk Factor for Myocardial Infarction and Stroke: The Rotterdam Study. Stroke 2006;37:1503-1507
33. Willett WC, Howe GR, Kushi LH: Adjustment for total energy intake in epidemiologic studies. The American Journal of Clinical Nutrition 1997;65:1220S-1228S
34. Zeger SL, Liang KY, Albert PS: Models for longitudinal data: a generalized estimating equation approach. Biometrics 1988;44:1049-1060
35. Oliveira A, Rodriguez-Artalejo F, Lopes C: The association of fruits, vegetables, antioxidant vitamins and fibre intake with high-sensitivity C-reactive protein: sex and body mass index interactions. Eur J Clin Nutr 2009;63:1345-1352
36. Detopoulou P, Panagiotakos DB, Chrysohoou C, Fragopoulou E, Nomikos T, Antonopoulou S, Pitsavos C, Stefanadis C: Dietary antioxidant capacity and concentration of adiponectin in apparently healthy adults: the ATTICA study. Eur J Clin Nutr 2010;64:161-168
37. Wang Y, Yang M, Lee SG, Davis CG, Koo SI, Fernandez ML, Volek JS, Chun OK: Diets high in total antioxidant capacity improve risk biomarkers of cardiovascular disease: a 9-month observational study among overweight/obese postmenopausal women. Eur J Nutr 2014;53:1363-1369
38. Katalinic V, Modun D, Music I, Boban M: Gender differences in antioxidant capacity of rat tissues determined by 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonate; ABTS) and ferric reducing antioxidant power (FRAP) assays. Comp Biochem Physiol C Toxicol Pharmacol 2005;140:47-52
39. Julicher RH, Sterrenberg L, Haenen GR, Bast A, Noordhoek J: Sex differences in the cellular defence system against free radicals from oxygen or drug metabolites in rat. Arch Toxicol 1984;56:83-86
40. Keenan T, Blaha MJ, Nasir K, Silverman MG, Tota-Maharaj R, Carvalho JAM, Conceição RD, Blumenthal RS, Santos RD: Relation of Uric Acid to Serum Levels of High-Sensitivity C-Reactive Protein, Triglycerides, and High-Density Lipoprotein Cholesterol and to Hepatic Steatosis. The American Journal of Cardiology 2012;110:1787-1792
41. Park JS, Kang S, Ahn CW, Cha BS, Kim KR, Lee HC: Relationships between serum uric acid, adiponectin and arterial stiffness in postmenopausal women. Maturitas 2012;73:344-348
42. Ali SS, Shaharyar S, Blaha MJ, Jamal O, Agatston AS, Blumenthal RS, Conceicao RD, Carvalho JAM, Santos RD, Nasir K: ELEVATED GAMMA-GLUTAMYL TRANSFERASE IS INDEPENDENTLY ASSOCIATED WITH BURDEN OF SUBCLINICAL VASCULAR INFLAMMATION. Journal of the American College of Cardiology 2013;61
43. Hermsdorff HH, Puchau B, Volp AC, Barbosa KB, Bressan J, Zulet MA, Martinez JA: Dietary total antioxidant capacity is inversely related to central adiposity as well as to metabolic and oxidative stress markers in healthy young adults. Nutr Metab (Lond) 2011;8:59

44. Nakatsuji H, Kishida K, Sekimoto R, Komura N, Kihara S, Funahashi T, Shimomura I: Accumulation of adiponectin in inflamed adipose tissues of obese mice. Metabolism 2014;63:542-553
45. Bluher M, Mantzoros CS: From leptin to other adipokines in health and disease: facts and expectations at the beginning of the 21st century. Metabolism 2015;64:131-145
46. Brydon L: Adiposity, leptin and stress reactivity in humans. Biol Psychol 2011;86:114-120
47. Friedman JM, Mantzoros CS: 20 years of leptin: from the discovery of the leptin gene to leptin in our therapeutic armamentarium. Metabolism 2015;64:1-4
48. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, et al.: Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 1996;334:292-295
49. Snijder MB, Heine RJ, Seidell JC, Bouter LM, Stehouwer CD, Nijpels G, Funahashi T, Matsuzawa Y, Shimomura I, Dekker JM: Associations of adiponectin levels with incident impaired glucose metabolism and type 2 diabetes in older men and women: the hoorn study. Diabetes Care 2006;29:2498-2503
50. Lyon CJ, Hsueh WA: Effect of plasminogen activator inhibitor-1 in diabetes mellitus and cardiovascular disease. Am J Med 2003;115 Suppl 8A:62S-68S
51. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA: The hormone resistin links obesity to diabetes. Nature 2001;409:307-312
52. Alexe DM, Syridou G, Petridou ET: Determinants of early life leptin levels and later life degenerative outcomes. Clin Med Res 2006;4:326-335
53. Kang DH, Park SK, Lee IK, Johnson RJ: Uric acid-induced C-reactive protein expression: implication on cell proliferation and nitric oxide production of human vascular cells. J Am Soc Nephrol 2005;16:3553-3562
54. Lee DH, Jacobs DR, Jr.: Association between serum gamma-glutamyltransferase and C-reactive protein. Atherosclerosis 2005;178:327-330
55. Qureshi SA, Lund AC, Veierod MB, Carlsen MH, Blomhoff R, Andersen LF, Ursin G: Food items contributing most to variation in antioxidant intake; a cross-sectional study among Norwegian women. BMC Public Health 2014;14:45
56. Tangney CC, Rasmussen HE: Polyphenols, inflammation, and cardiovascular disease. Curr Atheroscler Rep 2013;15:324
57. Goldbohm RA, Vantveer P, Vandenbrandt PA, Vanthof MA, Brants HAM, Sturmans F, Hermus RJJ: Reproducibility of a Food Frequency Questionnaire and Stability of Dietary Habits Determined from 5 Annually Repeated Measurements. European Journal of Clinical Nutrition 1995;49:420-429
58. Nilsen RM, Suren P, Gunnes N, Alsaker ER, Bresnahan M, Hirtz D, Hornig M, Lie KK, Lipkin WI, Reichborn-Kjennerud T, Roth C, Schjolberg S, Smith GD, Susser E, Vollset SE, Oyen AS, Magnus P, Stoltenberg C: Analysis of self-selection bias in a population-based cohort study of autism spectrum disorders. Paediatr Perinat Epidemiol 2013;27:553-563

SUPPLEMENTAL MATERIAL

Included in the Online Supplemental Material:

Assessment of diet
Assessment of covariates
Supplemental Table S1 Pearson correlation coefficient between hs-CRP and adipocytokines.
Supplemental Table S2 Association of FRAP with hs-CRP and adipocytokines by gender, the Rotterdam Study.
Supplemental Table S3 Association of uric acid (uric acid/sd) and GGT (GGT/sd) with hs-CRP and adipocytokines by gender, the Rotterdam Study.
Supplemental Table S4 Association of FRAP with Uric acid and GGT, The Rotterdam Study
Supplemental Table S5 Association of FRAP with hs-CRP and adipocytokines excluding people with CVD or type 2 diabetes, the Rotterdam Study.
Supplemental Table S6 Association of uric acid (uric acid/sd) and GGT (GGT/sd) with hs-CRP and adipocytokines excluding people with CVD or diabetes, the Rotterdam Study.
Supplemental Figure S1 Flow Chart of participants included in the analysis of overall antioxidant capacity of diet and Uric acid/ gamma-glutamyltransferse

Assessment of diet

At baseline (1990-1993), participants completed a checklist of all foods and beverages they had consumed at least twice a month during the preceding year. Then based on the completed checklist, they did an interview at the study center with a trained dietician. A computerized validated 170-item semi quantitative food frequency questionnaire (FFQ) was used. A validation study comparing this questionnaire with a 2-week food diary demonstrated reproducible and valid estimates; Pearson correlation after adjustment for age, sex, energy and within-person variation were between 0.44 and 0.85 for macro- and micronutrients as described in detail previously. These dietary data were converted into total energy intake per day using the Dutch Food Composition Table of 1993.

Assessment of covariates

The information on current health status, medical history, medication use, smoking and socioeconomic status were assessed at the home interview at baseline. Education was defined as low (primary education), intermediate (secondary general or vocational education), or high (higher vocational education or university). Household income was categorized in low (< 1700euro/month), middle (1700-3000euro/month) or high (≥ 3000euro/month). Participants were asked whether they were currently smoking cigarettes, cigars or pipe. Blood pressure was measured in the sitting position at the right upper arm with a random-zero-sphygmomanometer. Cardiovascular diseases were defined as a history of myocardial infarction, coronary artery bypass or percutaneous transluminal coronary angioplasty. Type 2 diabetes mellitus was diagnosed if a random serum glucose level was ≥11 mmol/L or if a person used glucose lowering drugs. Development of chronic diseases until the third round of study was defined as the presence of

cardiovascular diseases or diabetes mellitus at baseline and until the end of the third round visits (December 1999). Total cholesterol was determined by an automated enzymatic procedure in a non-fasting blood sample. The Dutch Healthy Diet (DHD)-index was used to take into account overall dietary quality. The DHD represents compliance to the Dutch Guidelines for a Healthy Diet as assessed from the FFQ at baseline. To avoid over adjustment, components used to calculate FRAP as fruits and vegetables were removed from the DHD-index. At the third visit to research center, the total weekly duration of physical activity was measured by an adapted version of the Zutphen Physical Activity Questionnaire and the LASA Physical Activity Questionnaire. Physical height and body 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).

Supplemental Table 1. Pearson correlation coefficient between hs-CRP and adipocytokines: the Rotterdam Study.

	C-reactive protein	Leptin	Adiponectin	Plasminogen Activator Inhibitor-1	Resistin
C-reactive protein	1				
Leptin	0.254**	1			
Adiponectin	-0.166**	0.048	1		
Plasminogen Activator Inhibitor-1	0.325**	0.466**	-0.270**	1	
Resistin	0.171**	0.018	0.029	-0.068	1

**Correlation is significant at the 0.01 level (2-tailed)

All variables were log transformed to better approximate normal distribution.

Supplemental Table 2. Association of FRAP with hs-CRP and adipocytokines by gender: the Rotterdam Study.

	Women	Men
	Model 2 β (95%CI)	**Model 2 β (95%CI)**
CRP[$]	-0.010(-0.017, -0.003)[a]	0.006(-0.002,0.013)[a]
Leptin[$]	-0.009(-0.021,0.003)[b]	-0.009(-0.022,0.003)[b]
Adiponectin[$]	0.010(0.002,0.019)[d]	0.007(-0.002,0.016)[d]
PAI-1[$]	-0.013(-0.027,0.000)[c]	-0.020(-0.033, -0.007)[c]
Resistin[$]	-0.004(-0.014,0.006)[e]	0.004(-0.007,0.015)[e]

FRAP, ferric reducing antioxidant potential; CRP, C-reactive protein; PAI-1, Plasminogen Activator Inhibitor-1.

[$] Variables were log transformed to better approximate normal distribution.

All associations remain significant after Bonferroni correction (p=0.0166)

Model 1: Adjusted for age

Model 2[a]: Model 1 + BMI, Smoking status, prevalent diseases, systolic blood pressure (SBP), non-fasting glucose, total cholesterol, index1(time). Additional adjustment for physical activity, energy intake, DHDI, education, income, alcohol, Statin use, hormone replacement therapy (HRT), vitamin intake, processed meat intake, unprocessed meat intake did not change the effect estimate with >10%.

Model 2[b]: Model 1 + BMI, Smoking status, energy intake, baseline CRP, non-fasting glucose, total cholesterol. Additional adjustment for physical activity, DHDI, prevalent disease, SBP, education, income, alcohol, Statin use, HRT, vitamin intake, processed meat intake, unprocessed meat intake did not change the effect estimate with >10%.

Model 2[c]: Model 1 + BMI, energy intake, baseline CRP, non-fasting glucose, total cholesterol. Additional adjustment for physical activity, DHDI, prevalent disease, SBP, smoking status, education, income, alcohol, Statin use, HRT, vitamin intake, processed meat intake, unprocessed meat intake did not change the effect estimate with >10%.

Model 2[d]: Model 1 + BMI, energy intake, baseline CRP, prevalent diseases, income, alcohol, statin use, non-fasting glucose, total cholesterol. Additional adjustment for physical activity, DHDI, SBP, smoking status, education, HRT, vitamin intake, processed meat intake, unprocessed meat intake did not change the effect estimate with >10%.

Model 2[e]: Model 1 + BMI, energy intake, baseline CRP, non-fasting glucose, total cholesterol. Additional adjustment for physical activity, DHDI, prevalent disease, SBP, smoking status, education, income, alcohol, Statin use, hormone replacement therapy, vitamin intake, processed meat intake, unprocessed meat intake did not change the effect estimate with >10%.

Outcome: hsCRP Male N= 1935 Female N=2571

Outcome: adipocytokines Male N= 365 Female N=433

Supplemental Table 3. Association of uric acid (uric acid/sd) and GGT (GGT/sd) with hs-CRP and adipocytokines by gender: the Rotterdam Study.

	Uric Acid Model 2 β (95%CI)		GGT[$] Model 2 β (95%CI)	
	Women	Men	Women	Men
CRP[$]	0.135 (0.098,0.173)	0.105 (0.046,0.163)	0.184 (0.144,0.223)[a]	0.158 (0.108,0.208)[a]
Leptin[$]	0.110 (0.044,0.176)	0.128 (0.042,0.214)	-0.030 (-0.099,0.039)[b]	-0.007 (-0.080,0.067)[b]
Adiponectin[$]	-0.075 (-0.122,-0.028)	-0.033 (-0.095,0.028)	-0.032 (-0.080,0.017)[c]	0.031 (-0.020,0.083)[c]
PAI-1[$]	0.162 (0.086,0.237)	0.138 (0.055,0.220)	0.027 (-0.052,0.105)[d]	0.098 (0.029,0.168)[d]
Resistin[$]	0.054 (-0.003,0.111)	-0.024 (-0.100,0.052)	-0.013 (-0.073,0.047)[e]	0.032 (-0.032,0.096)[e]

CRP, C-reactive protein; GGT, gamma glutamyltransferase; PAI-1, Plasminogen Activator Inhibitor-1.

[$] Variables were log transformed to better approximate normal distribution.

All associations remain significant after Bonferroni correction (p=0.0166)

Model 1: Adjusted for Age

Model 2[a]: Model 1 + BMI, Index 1(time) + GGT/uric acid

Model2[b]: Model1 +BMI, baseline CRP+GGT/uric acid (+glucose + Total cholesterol in GGT model)

Model2[c]: Model1+BMI, baseline CRP, non-fasting glucose +GGT/uric acid (+Energy intake in GGT model)

Model2[d]: Model1+BMI, baseline CRP, non-fasting glucose +GGT/uric acid (+Energy intake in GGT model)

Model2[e]: Model1+BMI, baseline CRP, non-fasting glucose, cholesterol, income, alcohol, statin use, diastolic blood pressure +GGT (for uric acid as outcome)

Model2[e]: Model1+BMI, baseline CRP, non-fasting glucose, cholesterol, income, alcohol, statin use, systolic blood pressure, energy intake, hormone replacement therapy + uric acid (for GGT as outcome)

Outcome: hsCRP Men N= 1668 Women N=2486 Outcome: adipocytokines Men N=306 Women N=401

Supplemental Table 4. Association of FRAP with uric acid and GGT, The Rotterdam Study.

FRAP	Model 1 β (95% CI)	Model 2 β (95% CI)
Uric acid (N=3505)	-0.002(-0.004-0.001)	-0.003(-0.005-0.002)[a]
GGT[$] (N=3505)	-0.008(-0.011-0,004)	-0.006(-0.009-0.003)[b]

FRAP, ferric reducing antioxidant potential; GGT, gamma glutamyltransferase;

[$] Variables were log transformed to better approximate normal distribution.

Model 1: Age and gender

Model 2[a]: Model 1+ BMI + baseline CRP. Additional adjustment for smoking status, physical activity, energy intake, DHDI, prevalent diseases, systolic blood pressure(SBP), total cholesterol, education, income, alcohol, Statin use, hormone replacement therapy (HRT), vitamin intake, processed meat intake, unprocessed meat intake, glomerular filtration rate(GFR), waist-to-hip ratio did not change the effect estimate with >10%.

Model 2[b]: Model 1+ SBP + diastolic blood pressure+ uric acid + total cholesterol.

Additional adjustment for smoking status, physical activity, energy intake, DHDI, prevalent diseases, BMI, baseline CRP, education, income, alcohol, Statin use, HRT, vitamin intake, processed meat intake, unprocessed meat intake, GFR, waist-to-hip ratio did not change the effect estimate with >10%.

Supplemental Table 5. Association of FRAP with hs-CRP and adipocytokines excluding people with CVD or type 2 diabetes: the Rotterdam Study.

	Model 1 β (95% CI)	Model 2 β (95%CI)
CRP[$] (N=4507)	0.003(-0.004,0.009)	-0.003(-0.009,0.003)[a]
Leptin[$] (N=798)	-0.010(-0.022,0.003)	-0.008(-0.018,0.002)[b]
Adiponectin[$] (N=798)	0.007(0.000,0.014)	0.009(0.002,0.016)[c]
PAI-1[$] (N=798)	-0.014(-0.026, -0.003)	-0.015(-0.026, -0.004)[d]
Resistin[$] (N= 798)	-0.00008(-0.008,0.008)	-0.001(-0.009,0.008)[e]

FRAP, ferric reducing antioxidant potential; CRP, C-reactive protein; PAI-1, Plasminogen Activator Inhibitor-1.

[$] Variables were log transformed to better approximate normal distribution.

Model 1: Adjusted for age and gender

Model 2[a]: Model 1 + BMI, smoking status, prevalent diseases, systolic blood pressure (SBP), non-fasting glucose, total cholesterol, index1(time). Additional adjustment for physical activity, energy intake, DHDI, education, income, alcohol, Statin use, hormone replacement therapy(HRT), vitamin intake, processed meat intake, unprocessed meat intake did not change the effect estimate with >10%.

Model 2[b]: Model 1 + BMI, Smoking status, energy intake, baseline CRP, non-fasting glucose, total cholesterol. Additional adjustment for physical activity, DHDI, prevalent disease, SBP, education, income, alcohol, Statin use, HRT, vitamin intake, processed meat intake, unprocessed meat intake did not change the effect estimate with >10%.

Model 2[c]: Model 1 + BMI, energy intake, baseline CRP, prevalent diseases, income, alcohol, statin use, non-fasting glucose, total cholesterol.

Additional adjustment for physical activity, DHDI, SBP, smoking status, education, HRT, vitamin intake, processed meat intake, unprocessed meat intake did not change the effect estimate with >10%.

Model 2[d]: Model 1 + BMI, energy intake, baseline CRP, non-fasting glucose, total cholesterol. Additional adjustment for physical activity, DHDI, prevalent disease, SBP, smoking status, education, income, alcohol, Statin use, HRT, vitamin intake, processed meat intake, unprocessed meat intake did not change the effect estimate with >10%.

Model 2[e]: Model 1 + BMI, energy intake, baseline CRP, non-fasting glucose, total cholesterol.

Additional adjustment for physical activity, DHDI, prevalent disease, SBP, smoking status, education, income, alcohol, Statin use, HRT, vitamin intake, processed meat intake, unprocessed meat intake did not change the effect estimate with >10%.

Supplemental Table 6. Association of uric acid (uric acid/sd) and GGT (GGT/sd) with hs-CRP and adipocytokines excluding people with CVD or type 2 diabetes: the Rotterdam Study.

	Uric acid		GGT[$]	
	Model 1 β (95% CI)	Model 2 β (95%CI)	Model 1 β (95% CI)	Model 2 β (95% CI)
CRP[$](N=4154)	0.195 (0.160,0.230)	0.111 (0.074,0.148)[a]	0.214 (0.178,0.249)	0.164 (0.128,0.199)[a]
Leptin[$](N=707)	0.270 (0.203,0.337)	0.127 (0.069,0.185)[b]	0.094 (0.027,0.161)	-0.042 (-0.097,0.013)[b]
Adiponectin[$](N=707)	-0.092 (-0.130, -0.054)	-0.055 (-0.096, -0.015)[c]	-0.040 (-0.078, -0.003)	-0.001 (-0.039,0.037)[c]
PAI-1[$](N=707)	0.257 (0.196,0.317)	0.178 (0.115,0.241)[d]	0.124 (0.063,0.185)	0.022 (-0.037,0.081)[d]
Resistin[$](N=707)	0.035 (-0.012,0.082)	0.049 (-0.002,0.101)[e]	0.013 (-0.032,0.058)	0.013 (-0.035,0.062)[e]

CRP, C-reactive protein; GGT, gamma glutamyltransferase; PAI-1, Plasminogen Activator Inhibitor

[$] Variables were log transformed to better approximate normal distribution.

Model 1: Age and gender

Model 2[a]: Model 1 + BMI, Index 1(time)+GGT/uric acid

Model 2[b]: Model1 +BMI, baseline CRP+GGT/uric acid (+glucose + total cholesterol in GGT model)

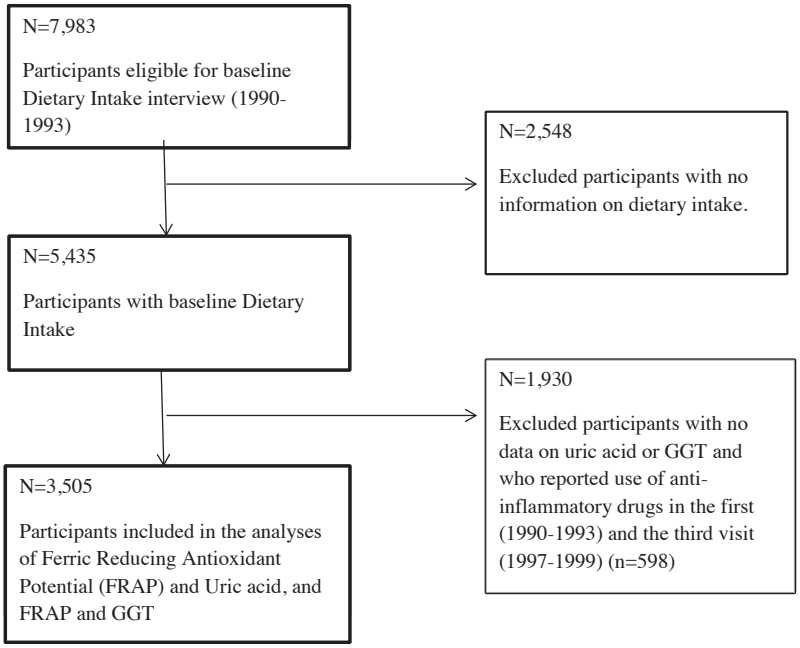
Model 2[c]: Model1+BMI, baseline CRP, non-fasting glucose +GGT/uric acid (+Energy intake in GGT model)

Model 2[d]: Model1+BMI, baseline CRP, non-fasting glucose +GGT/uric acid (+Energy intake in GGT model)

Model 2[e]: Model1+BMI, baseline CRP, non-fasting glucose, total cholesterol, income, alcohol, statin use, diastolic blood pressure +GGT (for uric acid as outcome)

Model 2[e]: Model1+BMI, baseline CRP, non-fasting glucose, energy intake, total cholesterol, hormone replacement therapy, systolic blood pressure, Statin use, alcohol, income +uric acid (for GGT as outcome)

Supplemental Figure S1. Flow Chart of participants included in the analysis of overall antioxidant capacity of diet and Uric acid/ gamma-glutamyltransferase (GGT): the Rotterdam Study.



Chapter 2.3

The association between serum uric acid and the incidence of prediabetes and type 2 diabetes mellitus: The Rotterdam Study.

Niels van der Schaft
Adela Brahimaj
Ke-xin Wen
Oscar H. Franco
Abbas Dehghan

Plos One 2017

ABSTRACT

Background

Limited evidence is available about the association between serum uric acid and sub-stages of the spectrum from normoglycaemia to type 2 diabetes mellitus. We aimed to investigate the association between serum uric acid and risk of prediabetes and type 2 diabetes mellitus.

Methods

Eligible participants of the Rotterdam Study (n = 8,367) were classified into mutually exclusive subgroups of normoglycaemia (n = 7,030) and prediabetes (n = 1,337) at baseline. These subgroups were followed up for incident prediabetes (n = 1,071) and incident type 2 diabetes mellitus (n = 407), respectively. We used Cox proportional hazard models to determine hazard ratios (HRs) for incident prediabetes among individuals with normoglycaemia and incident type 2 diabetes mellitus among individuals with prediabetes.

Results

The mean duration of follow-up was 7.5 years for incident prediabetes and 7.2 years for incident type 2 diabetes mellitus. A standard deviation increment in serum uric acid was significantly associated with incident prediabetes among individuals with normoglycaemia (HR 1.10, 95% confidence interval (CI) 1.01; 1.18), but not with incident type 2 diabetes mellitus among individuals with prediabetes (HR 1.07, 95% CI 0.94; 1.21). Exclusion of individuals who used diuretics or individuals with hypertension did not change our results. Serum uric acid was significantly associated with incident prediabetes among normoglycaemic women (HR 1.13, 95% CI 1.02; 1.25) but not among normoglycaemic men (HR 1.08, 95% CI 0.96; 1.21). In contrast, serum uric acid was significantly associated with incident type 2 diabetes mellitus among prediabetic men (HR 1.23, 95% CI 1.01; 1.48) but not among prediabetic women (HR 1.00, 95% CI 0.84; 1.19).

Conclusions

Our findings agree with the notion that serum uric acid is more closely related to early-phase mechanisms in the development of type 2 diabetes mellitus than late-phase mechanisms.

INTRODUCTION

Uric acid is generated during nucleotide and adenosine triphosphate (ATP) metabolism and comprises the end product of human purine metabolism.(1) We have previously demonstrated in a large population-based cohort study that elevated serum levels of uric acid are associated with increased risk of type 2 diabetes mellitus (DM) independently of other risk factors.(2) This association has since then been replicated in many other prospective studies and subsequent meta-analyses.(3-6) In addition, serum uric acid has been associated with various cardiovascular and metabolic conditions such as hypertension, obesity, heart failure and atrial fibrillation in large population-based studies.(7)

Prediabetes is a disorder of glucose homeostasis characterized by impaired glucose tolerance or impaired fasting glucose. These are both reversible stages of intermediate hyperglycaemia that provide an increased risk of type 2 DM.(8) Prediabetes can therefore be regarded as an important reversible stage that could lead to type 2 DM, and early identification of prediabetes might contribute to the prevention of type 2 DM. Despite its established association with incident type 2 DM, serum uric acid has not been studied extensively in relation to incident prediabetes in individuals with normoglycaemia or incident type 2 DM in individuals with established prediabetes. Therefore, the objective of the present study is to determine whether serum uric acid is associated with incident prediabetes among normoglycaemic individuals and type 2 DM among prediabetic individuals. This study is performed within the framework of the Rotterdam Study, a large population-based prospective cohort study of participants aged 45 years and older.(9)

MATERIALS AND METHODS

The Rotterdam Study

The methodology of the Rotterdam Study has been outlined extensively elsewhere.(9) Briefly, the study initially consisted of 7,983 residents of the Ommoord district aged 55 years and over in the city of Rotterdam, the Netherlands (RS-I). Following extension of the cohort in 2000 (RS-II), when individuals who had become 55 years of age or moved into the district since the study start were added to the cohort, and 2006 (RS-III), when individuals aged 45-54 years also became eligible for participation, the total number of subjects was 14,926 by the end of 2008.(9) These participants undergo physical examinations at the Rotterdam Study research facility and home interviews every 3-4 years. Data is collected on health status, risk factors for various diseases common in the elderly, anthropometric characteristics, incident disease and cause-specific mortality. (9) The Medical Ethics Committee of the Erasmus Medical Centre Rotterdam and the review board of the Dutch Ministry of Health, Welfare and Sport have approved this population-based cohort study, and all participants have provided written informed consent. For the purposes of this analysis, we combined data from cohorts RS-I (using the third visit in 1997-1999 as baseline), RS-II (baseline visit 2000-2001) and RS-III (baseline visit 2006-2009) of the Rotterdam Study.

Definition of type 2 diabetes mellitus, prediabetes and normoglycaemia

As per the Rotterdam Study protocol and WHO guidelines, type 2 DM was defined as having a fasting plasma glucose level ≥ 7.0 mmol/L, a non-fasting plasma glucose ≥ 11.1 mmol/L, the use of oral anti-diabetic medication or insulin, treatment by diet with type 2 DM as an indication and/or being registered by a general practitioner as having type 2 DM.(10; 11) Prediabetes was defined as a fasting plasma glucose level 6.0-6.9 mmol/L or a non-fasting plasma glucose level 7.7-11.1 mmol/L, in addition to absence of all type 2 DM criteria. Normoglycaemia was defined as a fasting plasma glucose level ≤ 6.0 mmol/L and absence of any of the above criteria for prediabetes and type 2 DM. Fasting blood samples were obtained by means of venipuncture at the Rotterdam Study research facility. The samples were stored at −80°C in 5 mL aliquots. Within one week of sampling, glucose levels were measured by means of the glucose hexokinase method. (12) All measurements were performed at the clinical chemistry laboratory of Erasmus Medical Center, Rotterdam.

Measurement of serum uric acid

Serum uric acid was determined in non-fasting blood samples, centrifuged for 10 minutes at 3,000 RPM and then stored for one week at -20°C. Uric acid activity was determined using a Kone Diagnostica reagent kit and a Kone auto-analyser. After every 10 samples, 3 control samples were included to check calibration. If the average values of the control samples were not within 2.5% of the true value in each run of 100 samples, this run was repeated. Day-by-day variation had to be within 5% of this average value.

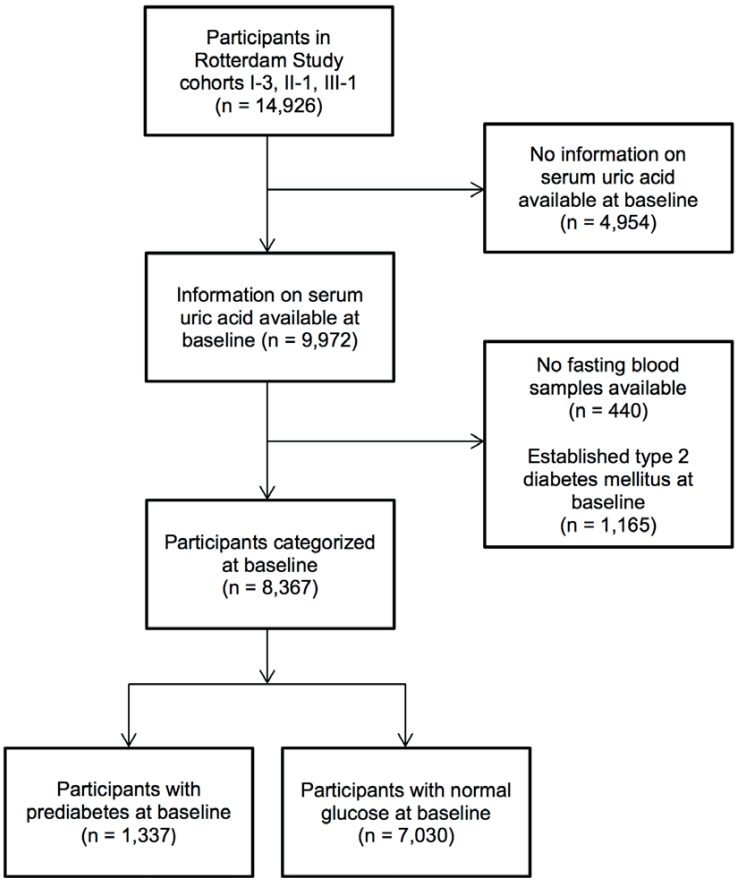
Covariates

In our study, the following covariates are considered: age, sex, body mass index (BMI), smoking status, daily alcohol intake, total serum cholesterol, serum HDL cholesterol, systolic blood pressure, serum insulin, serum glucose, hypertension (defined as having a systolic blood pressure > 140 mmHg, a diastolic blood pressure > 100 mmHg or receiving blood-pressure lowering medication with "hypertension" as an indication), physical activity, use of diuretics and estimated glomerular filtration rate (eGFR). Data on serum glucose, total serum cholesterol, serum HDL-cholesterol, serum insulin, blood pressure and eGFR were obtained at baseline by means of venipuncture, performed during participants' visits to the Rotterdam Study research facility. Anthropometric characteristics were also recorded at the Rotterdam Study research facility. eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.(13) The disease status with respect to type 2 DM and prediabetes was ascertained through follow-up using general practitioners' records and hospital discharge letters, collected as part of the Rotterdam Study. Physical activity was assessed at baseline by means of a modified version of the Zutphen Study Physical Activity Questionnaire and the LASA Physical Activity Questionnaire.(9) Metabolic equivalents of task (MET) hours per week were calculated based on time spent in light, moderate and vigorous activity. Data concerning the use of medication, alcohol consumption and smoking at baseline was obtained through Rotterdam Study home interviews and, for medication, consulting pharmacy dispensing records.

Statistical analysis

To determine the association between serum uric acid and risk of incident prediabetes or incident type 2 DM, Cox proportional hazards regression was performed with serum uric acid as the primary dependent variable and either incident prediabetes or incident type 2 DM as the response variable. The timescale in these models is follow-up time in years from baseline to either of the clinical endpoints, death, loss-to-follow-up or January 1st, 2012. Models adjusted only for age, sex and cohort as well as multivariable-adjusted Cox models were designed. The confounders BMI, smoking status, daily alcohol intake, total serum cholesterol, serum HDL cholesterol, systolic blood pressure, serum insulin, serum glucose, hypertension status, physical activity, use of diuretics and eGFR, selected based on previous literature, were added to the models adjusted for age, sex and cohort incrementally. The covariates serum insulin level, serum glucose level, daily alcohol intake and physical activity were log-transformed in the analyses because they displayed non-normality. Non-linearity was accounted for by inclusion of polynomial terms in the regression models if necessary. Interaction of uric acid with age and sex was investigated by

Figure 1. Selection of the study population.



introducing the product of the variables age and sex with uric acid to the regression models. Five-fold multiple imputation was performed to reduce bias that could arise from missing values. The results of our analyses are presented as hazard ratios (HR) with corresponding 95% confidence intervals. A p-value < 0.05 was considered statistically significant. Analyses were performed using SPSS Statistics version 21 (IBM Corp., Armonk, New York, USA) and R version 3.2.4 (The R foundation for Statistical Computing, Vienna, Austria).

RESULTS

The total study population eligible for analysis (n = 8,367) was divided into two mutually exclusive subgroups: a subgroup with normoglycaemia at baseline (n = 7,030) and a subgroup with prevalent prediabetes at baseline (n = 1,337). The selection procedure of our study population and the subgroups is outlined in Fig 1. Baseline characteristics of the study population are presented in Table 1.

Table 1. Baseline characteristics of the study population.

		Normoglycaemia at baseline n = 7,030	Missing data (%)	Prediabetes at baseline n = 1,337	Missing data (%)
Age (years)		64.2 (9.7)	0.0%	66.6 (9.4)	0.0%
Sex	Male (%) Female (%)	2,890 (41.1%) 4,140 (58.9%)	0.0%	665 (49.7%) 672 (50.3%)	0.0%
Body Mass Index		26.7 (3.9)	0.7%	28.5 (4.4)	0.6%
Serum Total Cholesterol (mmol/L)		5.8 (1.0)	0.0%	5.8 (1.0)	0.2%
Serum HDL (mmol/L)		1.4 (0.4)	0.6%	1.3 (0.4)	0.8%
Systolic Blood Pressure (mmHg)		137.2 (20.5)	0.5%	145.4 (20.8)	0.2%
Serum Insulin (pmol/L)[a]		66.0 (44.0)	0.2%	93.0 (67.0)	0.1%
Alcohol Consumption (g/day)[ab]		10.1 (12.6)	30.6%	13.7 (17.7)	35.2%
	Active (%) Former or Never (%)	6,008 (85.2%) 975 (14.1%)	0.7%	1,154 (86.3%) 176 (13.2%)	0.5%
Smoking	Active (%) Former or Never (%)	1,236 (17.6%) 5,794 (82.4%)	0.7%	237 (17.7%) 1,100 (82.3%)	0.4%
Hypertension[c]	Yes (%) No (%)	3,981 (56.6%) 3.049 (43.4%)	1.3%	1,000 (74.8%) 337 (25.2%)	0.7%
Use of diuretics	Yes (%) No (%)	581 (8.3%) 6,449 (91.7%)	2.9%	199 (14.9%) 1,138 (85.1%)	3.1%
Serum Glucose (mmol/L)[a]		5.3 (0.6)	0.0%	6.3 (0.4)	0.2%
Estimated Glomerular Filtration Rate (mL/min/1.73m^2)		79.9 (15.7)	1.2%	77.4 (16.1)	0.5%
Metabolic Equivalents of Task (hours/week)[a]		71.6 (64.5)	11.6%	68.7 (64.0)	10.2%
Serum Uric Acid (mmol/L)		0.31 (0.07)	n/a	0.35 (0.08)	n/a

Variables are presented as mean (standard deviation) unless otherwise indicated. [a]Variable is presented as median (interquartile range). [b]Median alcohol consumption applies only to active drinkers. [c]Hypertension is defined as having a systolic blood pressure > 140 mmHg, a diastolic blood pressure > 100 mmHg or receiving blood-pressure lowering medication with "hypertension" as an indication.

Table 2. The association between serum uric acid and incidence of prediabetes and type 2 diabetes mellitus.

	Incident prediabetes in normoglycaemic individuals	P-value	Incident type 2 DM in prediabetic individuals	P-value
Model 1[a]	1.31 (1.23 ; 1.40)	< 0.001	1.17 (1.06 ; 1.30)	0.002
Model 2[b]	1.30 (1.21 ; 1.40)	< 0.001	1.21 (1.08 ; 1.35)	0.001
Model 3[c]	1.10 (1.01 ; 1.18)	0.022	1.07 (0.94 ; 1.21)	0.330

Results are presented as Hazard Ratio (95% confidence interval) for a standard deviation increment in serum uric acid. [a]Model 1: adjusted for age, sex and Rotterdam Study cohort. [b]Model 2: model 1 + hypertension status, serum total cholesterol, eGFR, MET-hours per week, systolic blood pressure and use of diuretics. [c]Model 3: model 2 + daily alcohol intake, serum HDL, smoking status, BMI, serum glucose and serum insulin.

Over a mean follow-up time of 7.5 years, 1,071 individuals with normoglycaemia at baseline developed prediabetes (incidence rate 20.2 per 1,000 person-years). In this analysis, the percentage of individuals who were lost to follow up was 0.6% (40 out of 7,030 individuals). The results of our analysis of the association between serum uric acid and incident prediabetes are presented in Table 2. We found a significant association between serum uric acid and incident prediabetes within individuals who were normoglycaemic at baseline in a model adjusted only for age, sex and cohort (HR 1.31 per SD increment, 95% confidence interval (CI) 1.23 ; 1.40). This association was attenuated but remained significant in the multivariable-adjusted model (HR 1.10, 95% CI 1.01; 1.18). Performing separate analyses for men and women, we found that the association between serum uric acid and incident prediabetes was present in both men (HR 1.28, 95% CI 1.16; 1.41) and women (HR 1.34, 95% CI 1.23; 1.45) in models adjusted for age and cohort (Table 3). After multivariable adjustment, serum uric acid was significantly associated with incident prediabetes among women (HR 1.13, 95% CI 1.02; 1.25) but not among men (HR 1.08, 95% CI 0.96; 1.21). Exclusion of individuals who use diuretics or individuals with hypertension did not materially change our findings (Table 4). The association failed to reach statistical significance upon exclusion of individuals with a BMI ≥ 25 (HR 1.14, 95% CI 0.98; 1.33). In the multivariable-adjusted model we also analysed serum uric acid in quartiles, providing quartile-specific HRs relative to the first quartile (Fig 2).

Table 3. The association between serum uric acid and incidence of prediabetes and type 2 diabetes mellitus, stratified by gender.

	Model	Incident prediabetes in normoglycaemic individuals	P-value	Incident type 2 diabetes in prediabetic individuals	P-value
Men	1[a]	1.28 (1.16 ; 1.41)	< 0.001	1.19 (1.01 ; 1.40)	0.038
	2[b]	1.26 (1.13 ; 1.40)	< 0.001	1.30 (1.09 ; 1.56)	0.004
	3[c]	1.08 (0.96 ; 1.21)	0.216	1.23 (1.01 ; 1.48)	0.039
Women	1[a]	1.34 (1.23 ; 1.45)	< 0.001	1.18 (1.03 ; 1.35)	0.015
	2[b]	1.35 (1.23 ; 1.48)	< 0.001	1.19 (1.02 ; 1.38)	0.027
	3[c]	1.13 (1.02 ; 1.25)	0.024	1.00 (0.84 ; 1.19)	0.877

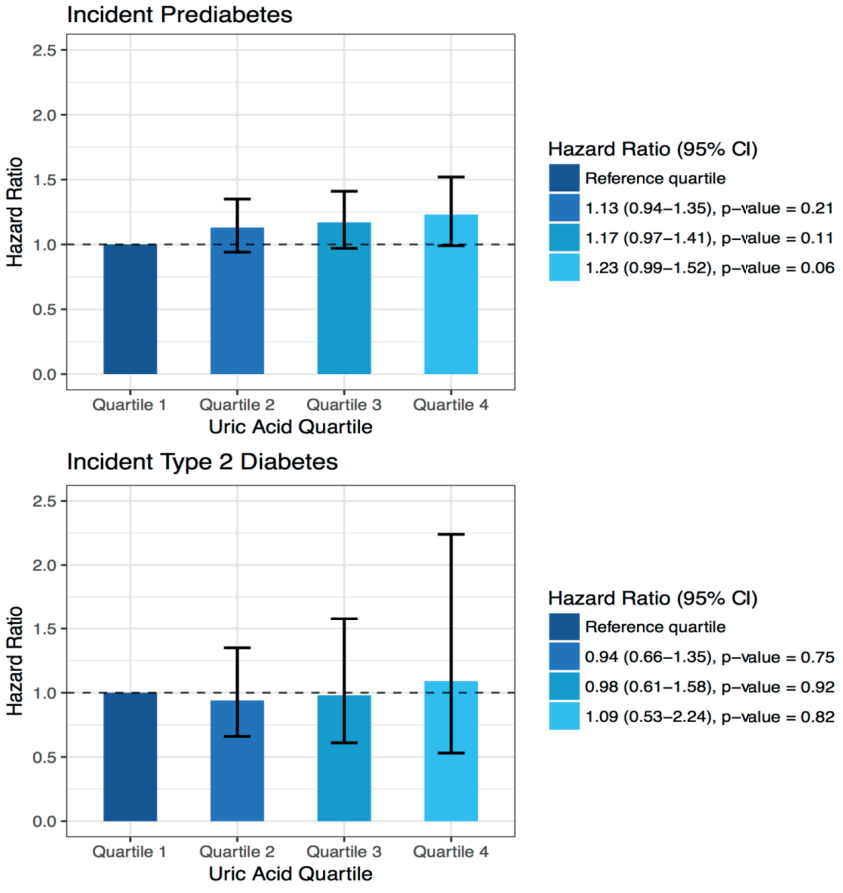
Results are presented as Hazard Ratio (95% confidence interval) for a standard deviation increment in serum uric acid. [a]Model 1: adjusted for age, sex and Rotterdam Study cohort. bModel 2: model 1 + hypertension status, serum total cholesterol, eGFR, MET-hours per week, systolic blood pressure and use of diuretics. [c]Model 3: model 2 + daily alcohol intake, serum HDL, smoking status, BMI, serum glucose and serum insulin.

Table 4. Subgroup analyses for the association between serum uric acid and incident prediabetes and incident type 2 diabetes mellitus.

	Incident prediabetes in normoglycaemic individuals	P-value	Incident type 2 DM in prediabetic individuals	P-value
Exclusion of participants who use diuretics	1.11 (1.02 ; 1.21)	0.016	1.05 (0.92 ; 1.21)	0.497
Exclusion of participants with hypertension	1.16 (1.00 ; 1.34)	0.045	1.14 (0.84 ; 1.56)	0.412
Exclusion of participants with a BMI ≥ 25	1.14 (0.98 ; 1.33)	0.097	1.10 (0.75 ; 1.61)	0.647

Results are presented as multivariable-adjusted Hazard Ratios (95% confidence interval) for a standard deviation increment in serum uric acid, adjusted for age, sex, Rotterdam Study cohort, hypertension status, serum total cholesterol, eGFR, MET-hours per week, systolic blood pressure, use of diuretics, daily alcohol intake, serum HDL, smoking status, BMI, serum glucose and serum insulin.

Figure 2. Quartile-specific hazard ratios for serum uric acid in association with incident prediabetes and incident type 2 diabetes mellitus.



A total of 407 individuals with prediabetes at baseline developed type 2 DM over a mean follow-up time of 7.2 years (incidence rate 42.4 per 1,000 person-years). In this analysis, the percentage of individuals who were lost to follow up was 0.4% (6 out of 1,337 individuals). Serum uric acid was significantly associated with incident type 2 DM in individuals with prediabetes in a model adjusting only for age, sex and cohort (HR 1.17, 95% CI 1.06; 1.30), but this association weakened and was not statistically significant in the multivariable-adjusted model (HR 1.07, 95% CI 0.94; 1.21) (Table 2). In sex-specific analyses, the association was significant among men (HR 1.19, 95% CI 1.01; 1.40), and women (HR 1.18, 95% CI 1.03; 1.35) in models adjusted for age and cohort (Table 3). After multivariable adjustment, serum uric acid was significantly associated with incident type 2 DM among men (HR 1.23, 95% CI 1.01; 1.48) but not among women (HR 1.00, 95% CI 0.84; 1.19). Exclusion of diuretic users, individuals with hypertension or individuals with a BMI ≥ 25 did not change our findings (Table 4). No significant difference was observed in any serum uric acid quartile compared to the first quartile (Fig 2).

DISCUSSION

We have found that higher serum uric acid levels are associated with an increased risk of incident prediabetes in individuals with normoglycaemia aged 45 years or over, independently of confounders. No significant association was observed between serum uric acid and incident type 2 DM in individuals with prediabetes after multivariable adjustment.

The result with relation to incident prediabetes is consistent with previous research on this subject using impaired fasting glucose as an endpoint.(14-17). This could indicate that serum uric acid is more closely associated with early-phase rather than late-phase mechanisms that play a role in the development of type 2 DM. Typically, insulin resistance impairs pancreatic β-cell physiology and compensatory mechanisms, thereby inducing β-cell dysfunction as a consequence.(18) Insulin resistance could therefore be regarded as a reflection of early mechanisms that contribute to the development of type 2 DM, whereas β-cell dysfunction reflects the influence of late-stage mechanisms.(19) Currently, not much evidence is available concerning the relation between serum uric acid and pancreatic β-cell function. Tang and colleagues found an independent positive association between serum uric acid levels and residual pancreatic β-cell function.(20) In their cross-sectional analysis of 1,021 individuals with type 2 DM, they observed that patients with higher serum uric acid had greater insulin secretion ability in early disease stages, but their residual β-cell function decayed more quickly. The authors suggest that this increased insulin secretion might be a compensatory mechanism to overcome initial insulin resistance. In addition, Shimodaira and colleagues observed a significant negative association between serum uric acid and disposition index, a measure of pancreatic β-cell function, in a cross-sectional analysis among non-diabetic Japanese women after adjustment for age, BMI, systolic blood pressure, HbA1c, serum triglyceride level, serum HDL level and use of antihypertensive or antilipidemic drugs.(21) However, no definitive conclusions regarding the association between serum uric acid and pancreatic β-cell function can be drawn at this point. Further population-based, prospective studies investigating this association are warranted. Although the association between serum

uric acid and incident prediabetes was not significant among individuals with BMI < 25, this finding is most likely due to a lack of statistical power, because individuals with a BMI ≥ 25 constitute over half of our sample size in this subgroup.

Serum uric acid has been investigated in relation to incident type 2 DM in individuals with impaired fasting glucose by Kramer and colleagues, who found a significant association (OR 1.75, 95% CI 1.1 ; 2.9) after adjusting for various confounders in study population with characteristics similar to ours.(22) We were not able to replicate this finding in our analysis, in which we had a considerably larger sample available and were able to adjust for a more comprehensive set of confounding variables. It is possible that residual confounding in the previous study could account for this difference, because Kramer and colleagues were unable to adjust for smoking status and serum HDL level. These covariates were particularly impactful in our multivariable-adjusted model. Excluding these covariates from the model yields an increase in the effect estimate (HR 1.13, 95% CI 1.00; 1.27) compared to the model which includes them (HR 1.07, 95% CI 0.94; 1.21). We also observe a steep decrease in the estimated hazard ratio for incident type 2 diabetes between model 2 and 3 in our analysis. The variable that is responsible for most of this decrease is serum HDL. It has been demonstrated that serum HDL is associated with plasma glucose levels and that it is strongly inversely associated with serum uric acid levels.(23; 24) Therefore, serum HDL can be regarded as a particularly strong confounder of this association.

There have been conflicting results reported in the literature about a possible sex-specific nature of the association between serum uric acid and impaired fasting glucose.(16; 17) In our study, we observe that serum uric acid is significantly associated with incident prediabetes among normoglycaemic women, but not among normoglycaemic men. Several studies report that the association between serum uric acid and glucose-related endpoints is especially pronounced among women.(15; 16; 25; 26) The difference between men and women with relation to incident prediabetes in our study can likely be attributed to residual confounding. We also have fewer events among men (n = 439) than among women (n = 632) in this analysis, which might lead to more imprecision in our estimated hazard ratio for men.

In contrast to this finding relating to incident prediabetes, serum uric acid was significantly associated with incident type 2 diabetes among men with prediabetes, but not among women with prediabetes in our study after multivariable adjustment. This observation was despite the fact that the number of events was higher among women (n = 222) than among men (n = 185) in this analysis. No other study has investigated the relation between serum uric acid and type 2 diabetes specifically among men with established glucose intolerance. Our result might suggest that serum uric acid affects women more strongly in the early stages of glucose intolerance development, whereas it affects men more strongly in more advanced stages. Potential biological mechanisms underlying this phenomenon have not yet been investigated in the literature, and further research is warranted.

Our findings build on the conclusion of a report by Kodama and colleagues, who performed a meta-analysis on the association between serum uric acid and incident type 2 diabetes in populations not stratified by glucose tolerance status (normoglycaemia or prediabetes) at baseline.(27) They conclude that serum uric acid is significantly associated with incident type 2 diabetes mellitus across 11 cohort studies, and that their result should encourage other studies to identify

sub-populations for which the association might be especially important. We report that serum uric acid appears to be most strongly associated with the early stages of the development of type 2 diabetes. A similar meta-analysis by Jia and colleagues also found a positive association between serum uric acid and a combined endpoint of incident impaired fasting glucose and incident type 2 diabetes.(5) Our results further characterize the association between serum uric acid and glucose intolerance by treating incident prediabetes and incident type 2 diabetes as separate endpoints.

The strengths of our study include its prospective nature, which minimizes the chance of reverse causation, its long follow-up time and our ability to adjust for a large set of confounders. We provide a comprehensive overview of the relation between serum uric acid and different sub-stages on the spectrum between normoglycaemia and type 2 DM. However, our study population consisted of mainly elderly individuals and roughly 95% of our participants were of Caucasian ethnicity. Therefore, our results cannot be generalized to populations with a different composition without further consideration. Finally, we cannot exclude the possibility of residual confounding, although the fact that we adjusted for many covariates should minimize the chance of this type of bias.

In conclusion, serum uric acid was independently and positively associated with incident prediabetes in individuals with normoglycaemia but not with incident type 2 DM in individuals with prediabetes in a large population-based cohort of individuals aged 45 years and over. Our results indicate that serum uric acid might be more closely associated with early-phase pathogenic mechanisms that contribute to the development of type 2 DM rather than late-phase mechanisms.

ACKNOWLEDGEMENTS

The dedication, commitment, and contribution of inhabitants, general practitioners, and pharmacists of the Ommoord district to the Rotterdam Study are gratefully acknowledged. We thank Symen Ligthart, Layal Chaker and Jolande Verkroost-van Heemst of Erasmus University Medical Center for their invaluable contribution to the collection and organization of the diabetes data.

REFERENCES

1. Kanbay M, Segal M, Afsar B, Kang DH, Rodriguez-Iturbe B, Johnson RJ: The role of uric acid in the pathogenesis of human cardiovascular disease. Heart 2013;99:759-766
2. Dehghan A, van Hoek M, Sijbrands EJ, Hofman A, Witteman JC: High serum uric acid as a novel risk factor for type 2 diabetes. Diabetes Care 2008;31:361-362
3. Wang T, Bi Y, Xu M, Huang Y, Xu Y, Li X, Wang W, Ning G: Serum uric acid associates with the incidence of type 2 diabetes in a prospective cohort of middle-aged and elderly Chinese. Endocrine 2011;40:109-116
4. Bhole V, Choi JW, Kim SW, de Vera M, Choi H: Serum uric acid levels and the risk of type 2 diabetes: a prospective study. Am J Med 2010;123:957-961
5. Jia Z, Zhang X, Kang S, Wu Y: Serum uric acid levels and incidence of impaired fasting glucose and type 2 diabetes mellitus: a meta-analysis of cohort studies. Diabetes Res Clin Pract 2013;101:88-96
6. Lv Q, Meng XF, He FF, Chen S, Su H, Xiong J, Gao P, Tian XJ, Liu JS, Zhu ZH, Huang K, Zhang C: High serum uric acid and increased risk of type 2 diabetes: a systemic review and meta-analysis of prospective cohort studies. PLoS One 2013;8:e56864
7. Wu AH, Gladden JD, Ahmed M, Ahmed A, Filippatos G: Relation of serum uric acid to cardiovascular disease. Int J Cardiol 2016;213:4-7
8. American Diabetes Association: Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 1997;20:1183-1197
9. Hofman A, Brusselle GG, Darwish Murad S, van Duijn CM, Franco OH, Goedegebure A, Ikram MA, Klaver CC, Nijsten TE, Peeters RP, Stricker BH, Tiemeier HW, Uitterlinden AG, Vernooij MW: The Rotterdam Study: 2016 objectives and design update. Eur J Epidemiol 2015;30:661-708
10. World Health Organization: Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia: Report of a WHO/IDF Consultation. Geneva:, 2006, p. 3
11. Ligthart S, van Herpt TT, Leening MJ, Kavousi M, Hofman A, Stricker BH, van Hoek M, Sijbrands EJ, Franco OH, Dehghan A: Lifetime risk of developing impaired glucose metabolism and eventual progression from prediabetes to type 2 diabetes: a prospective cohort study. The lancet Diabetes & endocrinology 2016;4:44-51
12. Neeley WE: Simple automated determination of serum or plasma glucose by a hexokinase-glucose-6 -phosphate dehydrogenase method. Clin Chem 1972;18:509-515
13. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, 3rd, Feldman HI, Kusek JW, Eggers P, Van Lente F, Greene T, Coresh J, Ckd EPI: A new equation to estimate glomerular filtration rate. Ann Intern Med 2009;150:604-612
14. Krishnan E, Pandya BJ, Chung L, Hariri A, Dabbous O: Hyperuricemia in young adults and risk of insulin resistance, prediabetes, and diabetes: a 15-year follow-up study. Am J Epidemiol 2012;176:108-116
15. Meisinger C, Doring A, Stockl D, Thorand B, Kowall B, Rathmann W: Uric acid is more strongly associated with impaired glucose regulation in women than in men from the general population: the KORA F4-Study. PLoS One 2012;7:e37180
16. Kawamoto R, Tabara Y, Kohara K, Kusunoki T, Abe M, Miki T: Serum uric acid is more strongly associated with impaired fasting glucose in women than in men from a community-dwelling population. PLoS One 2013;8:e65886
17. Liu Y, Jin C, Xing A, Liu X, Chen S, Li D, Feng P, Liu J, Li Z, Wu S: Serum uric acid levels and the risk of impaired fasting glucose: a prospective study in adults of north China. PLoS One 2013;8:e84712
18. Cerf ME: Beta cell dysfunction and insulin resistance. Front Endocrinol (Lausanne) 2013;4:37
19. Martin BC, Warram JH, Krolewski AS, Bergman RN, Soeldner JS, Kahn CR: Role of glucose and insulin resistance in development of type 2 diabetes mellitus: results of a 25-year follow-up study. Lancet 1992;340:925-929
20. Tang W, Fu Q, Zhang Q, Sun M, Gao Y, Liu X, Qian L, Shan S, Yang T: The association between serum uric acid and residual beta -cell function in type 2 diabetes. J Diabetes Res 2014;2014:709691
21. Shimodaira M, Niwa T, Nakajima K, Kobayashi M, Hanyu N, Nakayama T: The relationship between serum uric acid levels and beta-cell functions in nondiabetic subjects. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme 2014;46:950-954
22. Kramer CK, von Muhlen D, Jassal SK, Barrett-Connor E: Serum uric acid levels improve prediction of incident type 2 diabetes in individuals with impaired fasting glucose: the Rancho Bernardo Study. Diabetes Care 2009;32:1272-1273

23. Drew BG, Duffy SJ, Formosa MF, Natoli AK, Henstridge DC, Penfold SA, Thomas WG, Mukhamedova N, de Courten B, Forbes JM, Yap FY, Kaye DM, van Hall G, Febbraio MA, Kemp BE, Sviridov D, Steinberg GR, Kingwell BA: High-density lipoprotein modulates glucose metabolism in patients with type 2 diabetes mellitus. Circulation 2009;119:2103-2111
24. Peng TC, Wang CC, Kao TW, Chan JY, Yang YH, Chang YW, Chen WL: Relationship between hyperuricemia and lipid profiles in US adults. Biomed Res Int 2015;2015:127596
25. Yamada T, Fukatsu M, Suzuki S, Wada T, Joh T: Elevated serum uric acid predicts impaired fasting glucose and type 2 diabetes only among Japanese women undergoing health checkups. Diabetes Metab 2011;37:252-258
26. Kivity S, Kopel E, Steinlauf S, Segev S, Sidi Y, Olchovsky D: The association between serum uric acid and diabetes mellitus is stronger in women. J Womens Health (Larchmt) 2013;22:782-789
27. Kodama S, Saito K, Yachi Y, Asumi M, Sugawara A, Totsuka K, Saito A, Sone H: Association between serum uric acid and development of type 2 diabetes. Diabetes Care 2009;32:1737-1742

Chapter 3

Lipids, body fat and type 2 diabetes

Chapter 3.1

Serum Levels of Apolipoproteins and Incident Type 2 Diabetes: A Prospective Cohort Study.

Adela Brahimaj
Symen Ligthart
M. Arfan Ikram
Albert Hofman
Oscar H. Franco
Eric. J. G. Sijbrands
Maryam Kavousi
Abbas Dehghan

Diabetes Care 2016

ABSTRACT

Objective

We aimed to investigate the role of serum levels of different apolipoproteins on the risk for type 2 diabetes (T2D).

Research design and Methods

We used data from 971 individuals from the prospective population-based Rotterdam Study. We studied the association of HDL-C, apoA1, apoCIII, apoD, apoE as well as ratios of apolipoproteins with apoA1 with the risk of T2D. All apolipoproteins, ratios and HDL-C were naturally log-transformed to reach normal distribution.

First, their cross-sectional associations with fasting glucose and insulin were investigated using linear regression. Second, Cox proportional hazard models were used to examine whether apolipoproteins predict the risk for T2D among individuals free of diabetes at baseline. We also studied the apolipoproteins jointly by calculating apolipoproteinic score from the first step and then performing Cox regression with it.

Results

During a median follow-up of 13.3 years, 110 individuals developed diabetes.

After adjustment for age, sex, body mass index, parental history of diabetes, hypertension, alcohol use, smoking, prevalent CVD, serum lipid reducing agents: HDL-C (per 1 SD naturally log-transformed, HR = 0.74; 95% CI: 0.57, 0.97), apoCIII (1.65;1.42, 1.91), apoE (1.36; 1.18, 1.55), apoCIII-to-apoA1 ratio (1.72; 1.51, 1.95), apoE-to-apoA1ratio (1.28; 1.13, 1.45) and apolipoproteinic score (1.60; 1.39, 1.83) remained significant. Only apoCIII (1.42; 1.03, 1.96) and apoCIII-to-apoA-1ratio (1.56; 1.04, 2.36) survived the adjustment for triglycerides in the last model.

Conclusions

Serum apoCIII levels as well as apoCIII-to-apoA1 ratio are associated with incident type 2 diabetes. They are associated independent of known risk factors and stronger than HDL-C levels.

INTRODUCTION

Low levels of high density lipoprotein cholesterol (HDL-C) is a known risk factor for type 2 diabetes (T2D) preceding the onset of the disease (1).
Recently, not only HDL content, but different lipoprotein particles are progressively gaining attention as new markers for T2D risk, independent of established risk factors (2; 3). Furthermore, a novel lipoprotein insulin resistance index (LP-IR), which combines six lipoproteins parameters (higher levels of large VLDL particles and small LDL particles, lower levels of large HDL particles, smaller mean LDL and HDL particle size, and larger mean VLDL particle size) was found to be independently associated with incident diabetes (3; 4).
Given that lipoproteins are biochemical assemblies, their role in T2D etiology is deeper explored by investigating associations between their main components such as apolipoproteins and the risk for T2D. Part of interest in studying apolipoproteins is also the lately reported lack of causality between reduced HDL-C and increased risk of T2D, raising the question whether part of the beneficial effects attributed to HDL-C may be due to the composition of HDL particles, including apolipoproteins (apo) and supporting their role as probable determinants in the association between HDL-C levels and T2D (5).
Few studies have shown that serum levels of apolipoproteins such as apoA1 and apoB are stronger biomarkers of T2D or its complications than traditional lipids (6; 7). Higher HDL-C-to- apoA1 ratio and HDL-C-to-apoAII ratio are strongly and independently related to a lower risk of T2D in different populations (8-10). We have previously found that apoCIII promotor variants increase T2D risk and need for insulin treatment in lean participants (11). Total serum apoCIII, mainly because of the fraction in HDL particle was found to be a key diabetogenic risk factor among Turks (12). However, the prospective study among Turks had a short follow-up of 4.4 ± 1.2 years. A comprehensive investigation of the role of serum apolipoproteinic profile of different apolipoproteins, as biomarkers for incident T2D and possible targets for future therapy is still lacking.
We hypothesized that serum levels of different apolipoproteins, as well as the ratios of those apolipoproteins with apoA1, are stronger biomarkers for incident T2D than HDL-C.
To this end, we studied the associations of serum HDL-C, apoA1, apoCIII, apoD, apoE levels as well as the ratios of ApoCIII-to-apoA1, apoD-to-apoA1 and apoE-to-apoA1 with incident T2D in the Rotterdam Study, a prospective population-based cohort study.

RESEARCH DESIGN AND METHODS

Study population

The Rotterdam Study is a prospective population-based cohort study in Ommoord, a district of Rotterdam, the Netherlands. The design of the Rotterdam Study has been described in more details elsewhere (13). Briefly, in 1989 all residents within the well-defined study area aged 55 years or older were invited to participate of whom 78% (7983 out of 10275) agreed. There were no other eligibility criteria to enter the Rotterdam Study except minimum age and residential area

based on postal code. The first examination took place from 1990 to 1993, after which, follow-up examinations were conducted every 3-5 years. This study was based on data collected during the third visit (1997-1999). The measures were done based on a case-cohort study design. In this analysis, we have only used the random subset of the Rotterdam Study. 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 Netherlands. All participants in the present analysis provided written informed consent to participate and to obtain information from their treating physicians.

Measurement of apolipoproteins

In the third visit, fasting blood samples were collected at the research center. Plasma was isolated and immediately put on ice and stored at -80°C. Citrate plasma (200Ul) was sent in July 2008 to Rules-Based Medicine, Austin, Texas (www.myriadrbm.com). Fifty biomarkers were quantified using multiplex immunoassay on a custom designed human multianalyte profile, including four apolipoproteins. The intra-assay variability was less than 4% and the inter assay variability was less than 13%. The markers investigated in the current study have no standard international calibration reference, therefore interpretation of the absolute values should be with caution. Since all values in our set of individuals have been quantified using the same assay, relative measures like hazard ratios of associations should not have been effected. In this study we included the apolipoproteins that, besides other lipoproteins, are constituents of high density lipoproteins as well: apolipoprotein A1 (apoA1), apolipoprotein CIII (apoCIII), apolipoprotein D (apoD) and apolipoprotein E (apoE). Their measurements were available in g/l. We turned them in mol/l dividing their value in g/l by their molecular weight in g/mol: apoA1 (30778 g/mol), apoCIII (10852 g/mol), apoD (19820 g/mol), apoE (36154 g/mol) (www.myriadrbm.com).

Type 2 diabetes mellitus

Participants were followed from the date of baseline center visit onwards. At baseline and during follow-up, cases of prediabetes and T2D were ascertained through active follow-up using general practitioners' records, hospital discharge letters and glucose measurements from Rotterdam Study visits which take place approximately every 4 years. Diabetes, pre-diabetes and normoglycemia were defined according to the current WHO guidelines. Normoglycemia was defined as a fasting blood glucose level < 6.0 mmol/L; pre-diabetes was defined as a fasting blood glucose between 6.0 mmol/L and 7.0 mmol/L or a non-fasting blood glucose between 7.7 mmol/L and 11.1 mmol/L (when fasting samples were unavailable); type 2 diabetes was defined as a fasting blood glucose ≥ 7.0 mmol/L, a non-fasting blood glucose ≥ 11.1 mmol/L (when fasting samples were unavailable), or the use of blood glucose lowering medication (14). Information regarding the use of blood glucose lowering medication was derived from both structured home interviews and linkage to pharmacy dispensing 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 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.

Covariates

Height and weight were measured with the participants standing without shoes and heavy outer garments. Body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared. 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. Blood pressure was measured at the right brachial artery with a random-zero sphygmomanometer with the participant in sitting position, and the mean of 2 consecutive measurements was used. Insulin, glucose, high-density lipoprotein cholesterol (HDL-C), triglycerides (TG) were measured on the COBAS 8000 Modular Analyzer (Roche Diagnostics GmbH). The corresponding interassay coefficients of variations are the following: insulin < 8%, glucose < 1.4% and lipids < 2.1%. Information on medication use, parental diabetes history and tobacco smoking behaviour was collected by trained research assistants via computerized questionnaires during home visits. 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. Smoking was classified as current versus non-current smokers. Alcohol intake was assessed in grams of ethanol per day from the questionnaires. Participants were asked for the average daily consumption.

Statistical analyses

We used linear regression models to investigate the age, sex and prevalent diabetes adjusted association of serum levels of HDL-C, apoA1, apoCIII, apoD, apoE as well as ratios of apolipoproteins with apoA1with fasting glucose and with fasting insulin in 971 subjects (120 of them with prevalent diabetes). Markers with a right-skewed distribution were transformed to the natural logarithmic scale (all apolipoproteins, their ratios, HDL-C, triglycerides, fasting glucose and insulin). We defined apolipoprotein value as an outlier when the value was four standard deviations higher or lower than the mean of the normal variable. After excluding the outliers, apolipoprotein levels were standardized by dividing the measured value by the standard deviation. To study the apolipoprotein levels jointly, not just individually, we calculated an apolipoproteinic score using beta values from the cross-sectional analysis with glucose levels (table 2). The score was calculated by the formula ($-0.013 \times apoA1 + 0.016 \times apoCIII - 0.009 \times apoD + 0.018 \times apoE$). Among 851 subjects free of diabetes, we assessed the association of serum levels of HDL-C itself, apoA1, apoCIII, apoD, apoE, molar ratios of every apolipoprotein with apoA1 as well as the apolipoproteinic score, using Cox proportional hazard models. The first model was adjusted for age and sex. In the second model we additionally adjusted for body mass index. In the third model we added parental history of diabetes, hypertension, alcohol use, smoking, prevalent CVD and serum lipid reducing agents. In the fourth model we added triglycerides. Multiple imputation procedure was used (N= 5 imputations) to impute the missing data on covariates such as weight (0.82 %), height (0.82 %), smoking (1.03 %), triglycerides (2.37 %), prevalent CVD (0.62 %). We also assessed linearity of the associations by adding quadratic term to regression models. All analyses were conducted in SPSS software version 21 (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp).

RESULTS

Table 1 summarizes the baseline characteristics of 971 participants. The mean (SD) age at baseline was 73 (7.5) years and 44.8% of the population were males. The mean BMI (SD) was 26.7 (3.9) kg/m^2 and 12.6% of the study population used lipid lowering medication.

Table 1. Baseline characteristics of study participants.

Characteristics	Total (n=971)
Age, y	73 ± 7.5
Men, n (%)	435 (44.8)
Parental history of diabetes	59 (6.1)
Waist circumference, m	0.93 ± 0.12
Body mass index, kg/m^2	26.7 ± 3.9
Systolic blood pressure,mmHg	144 ± 21.7
Diastolic blood pressure, mmHg	75 ± 11
Hypertension medication, n (%)	241 (24.8)
Total cholesterol, mmol/L	5.8 ± 0.98
HDL cholesterol, mmol/L	1.4 ± 0.4
*Triglycerides, mmol/L	1.3 (0.62 - 3.40)
Current smokers, n (%)	137 (14.1)
Former smokers, n (%)	483 (49.7)
Never smokers, n (%)	351 (36.2)
Prevalent cardiovascular disease, n (%)	201 (20.7)
*Alcohol intake in drinkers, g/day	5.71 (42.73)
Lipid lowering medication, n (%)	122 (12.6)
*Fasting glucose, mmol/L	5.6 (3.54)
*Fasting insulin, pmol/L	65 (138)
*Apolipoprotein A1, umol/l	7.4 (8.4)
*Apolipoprotein CIII, umol/l	6.3 (8.2)
*Apolipoprotein D, umol/l	3.9 (4.1)
*Apolipoprotein E, umol/l	1.2 (1.7)
*ApoCIII-to-apoA1 ratio	0.8 (1.4)
*ApoD-to-apoA1 ratio	0.5 (0.8)
*ApoE-to-apoA1 ratio	0.2 (0.3)

Plus-minus values are means ± SD or

* median (inter-quartile range). Abbreviations: apoA1, apolipoprotein A1; apoCIII, apolipoprotein CIII; apoD, apolipoprotein D; apoE, apolipoprotein E; HDL-C, high density lipoprotein cholesterol.

Cross-sectional analysis

Table 2 presents the associations between HDL-C, apoA1, apoCIII, apoD, apoE as well as ratios of apolipoproteins with apoA1and fasting glucose and fasting insulin. ApoCIII was positively associated with fasting glucose. ApoE, apoCIII-to-apoA1ratio and apoE-to-apoA1ratio were positively associated with fasting glucose and fasting insulin. HDL-C, apoA1 and apoD were inversely associated with fasting glucose and insulin.

Table 2. Serum levels of HDL-C, apolipoproteins, ratios and fasting glucose and insulin.

		Fasting Glucose		**Fasting Insulin**	
Biomarker	N	Beta(95%CI)	p-value	Beta(95%CI)	p-value
HDL-C	949	-0.018 (-0.027, -0.009)	$\mathbf{8.7 \times 10^{-5}}$	-0.185 (-0.222, -0.147)	$\mathbf{5.1 \times 10^{-24}}$
apoA1	968	-0.013 (-0.022, -0.004)	$\mathbf{4 \times 10^{-3}}$	-0.170 (-0.208, -0.131)	$\mathbf{3.9 \times 10^{-19}}$
apoCIII	968	0.016 (0.008, 0.024)	$\mathbf{1.8 \times 10^{-4}}$	0.036 (-0.001, 0.072)	5.8×10^{-2}
apoD	943	-0.009 (-0.017, -0.001)	$\mathbf{3 \times 10^{-2}}$	-0.120 (-0.157, -0.084)	$\mathbf{1.5 \times 10^{-10}}$
apoE	938	0.018 (0.009, 0.026)	$\mathbf{4.5 \times 10^{-5}}$	0.103 (0.06, 0.146)	$\mathbf{9 \times 10^{-6}}$
ApoCIII-to-apoA1 ratio	965	0.023 (0.014, 0.031)	$\mathbf{8.5 \times 10^{-8}}$	0.141 (0.104, 0.178)	$\mathbf{7.9 \times 10^{-14}}$
ApoD-to-apoA1 ratio	940	0.004 9-0.005, 0.013)	0.3	0.025 (-0.015, 0.066)	0.2
ApoE-to-apoA1 ratio	935	0.02 (0.012, 0.029)	$\mathbf{1 \times 10^{-6}}$	0.158 (0.118, 0.199)	$\mathbf{3.5 \times 10^{-11}}$

Abbreviations: apoA1, apolipoprotein A1; apoCIII, apolipoprotein CIII; apoD, apolipoprotein D; apoE, apolipoprotein E; HDL-C, high density lipoprotein cholesterol; N, number of subjects after the exclusion of the outliers for each apolipoprotein.
Adjusted for age, sex, prevalent diabetes.
Betas are per 1SD naturally log-transformed.
Apolipoproteins are in molar units per liter and their ratios are based on molar weights.

Prospective analyses

Among 851 subjects free of diabetes, 110 incident diabetes cases were identified during a median follow-up of 13.5 years (10 diabetes cases per 1000 person-years).

After adjustment for age, sex, body mass index, parental history of diabetes, hypertension, alcohol use, smoking, prevalent CVD, serum lipid reducing agents (table 3, model 3): high serum HDL-C levels remained associated with lower risk for T2D, whereas high serum levels of apoCIII, apoE as well as apoCIII-to-apoA1 ratio, apoE-to-apoA1ratio and the apolipoproteinic score remained associated with higher risk for T2D. Only apoCIII (per 1 SD naturally log-transformed, HR =

Table 3. Associations between HDL-C, apolipoproteins, their ratios and score and incident type 2 diabetes mellitus.

	Model1		Model2		Model3		Model4	
Biomarker	HR(95%CI)	p-value	HR(95%CI)	p-value	HR(95%CI)	p-value	HR(95%CI)	p-value
HDL-C	0.68 (0.55, 0.84)	$\mathbf{3.6 \times 10^{-4}}$	0.76 (0.61, 0.95)	$\mathbf{1.4 \times 10^{-2}}$	0.74 (0.57, 0.97)	$\mathbf{2.7 \times 10^{-2}}$	0.89 (0.65, 1.21)	0.4
apoA1	0.72 (0.58, 0.89)	$\mathbf{3 \times 10^{-3}}$	0.80 (0.64, 1.00)	$\mathbf{5.3 \times 10^{-2}}$	0.85 (0.65, 1.11)	0.2	1.01 (0.75, 1.35)	0.9
apoCIII	1.49 (1.21, 1.83)	$\mathbf{1.4 \times 10^{-4}}$	1.48 (1.33, 1.64)	$\mathbf{2.6 \times 10^{-4}}$	1.65 (1.42, 1.91)	$\mathbf{1.2 \times 10^{-5}}$	1.42 (1.03, 1.96)	$\mathbf{3.3 \times 10^{-2}}$
apoD	0.77 (0.64, 0.94)	$\mathbf{8 \times 10^{-3}}$	0.85 (0.69, 1.04)	0.1	0.89 (0.71, 1.14)	0.3	0.89 (0.70, 1.14)	0.3
apoE	1.38 (1.11, 1.72)	$\mathbf{4 \times 10^{-3}}$	1.25 (0.99, 1.56)	$\mathbf{5 \times 10^{-2}}$	1.36 (1.18, 1.55)	$\mathbf{1.6 \times 10^{-2}}$	1.10 (0.80, 1.52)	0.5
ApoCIII/apoA1	1.64 (1.35, 2.00)	$\mathbf{8.3 \times 10^{-7}}$	1.55 (1.26, 1.89)	$\mathbf{2.7 \times 10^{-5}}$	1.72 (1.51, 1.95)	$\mathbf{2.4 \times 10^{-4}}$	1.56 (1.04, 2.36)	$\mathbf{3 \times 10^{-2}}$
ApoD/apoA1	1.01 (0.81, 1.25)	0.9	1.02 (0.82, 1.26)	0.8	1.01 (0.89, 1.16)	0.9	0.89 (0.69, 1.15)	0.3
ApoE/apoA1	1.41 (1.16, 1.72)	$\mathbf{1 \times 10^{-3}}$	1.26 (1.02, 1.54)	$\mathbf{2.9 \times 10^{-2}}$	1.28 (1.13, 1.45)	$\mathbf{1.5 \times 10^{-2}}$	1.04 (0.77, 1.40)	0.8
Apolipoproteinic score	1.65 (1.35, 2.02)	$\mathbf{8.6 \times 10^{-7}}$	1.49 (1.21, 1.85)	$\mathbf{1.8 \times 10^{-4}}$	1.60 (1.39, 1.83)	$\mathbf{1 \times 10^{-3}}$	1.44 (0.96, 2.17)	7.9×10^{-2}

Model 1: adjusted for age and sex
Model 2: additionally adjusted for body mass index
Model 3: additionally adjusted for parental history of diabetes, hypertension, alcohol use, smoking, prevalent CVD, serum lipid reducing agents
Model 4: additionally adjusted for triglycerides
HRs are per 1SD naturally log-transformed.
Apolipoproteins are in molar units per liter and their ratios are based on molar weights.

1.42; 95% CI: 1.03, 1.96) and apoCIII-to-apoA1ratio (per 1 SD naturally log-transformed, HR = 1.56; 95% CI: 1.04, 2.36) survived the adjustment for triglycerides in the last model.
All these adjustments slightly changed the effect estimates of the association between serum apoCIII levels as well as apoCIII-to-apoA1 ratio and incident T2D from the age and sex adjusted model (per 1 SD naturally log-transformed, HR = 1.49), (per 1 SD naturally log-transformed, HR = 1.64) to the final model (per 1SD naturally log-transformed, HR= 1.42), (per 1 SD naturally log-transformed, HR = 1.56).
We assessed linearity of the associations by adding quadratic term to regression models. None of the quadratic terms were significant (P value > 0.1).

DISCUSSION

In this prospective population-based cohort study, we investigated the associations of serum levels of apoliporoteins (apoA1, apoCIII, apoD, apoE), HDL-C as well as the ratios of the apolipoproteins with apoA1 and their apolipoproteinic score with the risk of T2D. After adjustment for age, sex, body mass index, parental history of diabetes, hypertension, alcohol use, smoking, prevalent CVD, serum lipid reducing agents high serum HDL-C levels remained associated with lower risk for T2D, whereas high serum levels of apoCIII, apoE as well as apoCIII-to-apoA1 ratio, apoE-to-apoA1ratio and the apolipoproteinic score remained associated with higher risk for T2D. Only apoCIII and apoCIII-to-apoA1ratio survived the adjustment for triglycerides, being identified as stronger T2D risk marker than HDL-C.
ApoA1 is the major proteinic component of the antiatherogenic HDL and its main indictor (15; 16). Thus, we calculated molar ratios of serum apolipoproteins, which have in common the presence in HDL besides other lipoproteins, to investigate how the variability of the serum apoliporoteinic flux could be associated with the risk for T2D and whether this variability could be a better risk marker than HDL-C.
Few previous studies have shown that serum levels of apolipoproteins better estimate the risk of T2D or its complications than traditional lipids (6; 16). Higher HDL-C-to-apoA1 ratio and apoA1 levels have been strongly and independently related to a lower risk of incident T2D in different populations (8-10). In agreement with these studies we report here that serum apoA1 levels are associated with risk of T2D. We, however, extended the list to a larger set of apolipoproteins and included serum levels of apoCIII, apoD and apoE. Our results indicate that apoCIII is a better predictor of T2D compared to apoA1. Associations of serum levels of apoCIII with the risk of T2D have been previously investigated prospectively (12), but in a shorter follow-up of 4.4 ± 1.2 years using logistic regression, whereas we performed survival analysis having available incident diabetes date during a median follow-up of 13.3 years.
The studied apolipoproteins are found in combination with HDL, LDL, VLDL or chylomicrons (17). The variation in apolipoprotein levels, therefore, represents both the variation in apolipoproteins flux and the variation in the levels of lipids they bind. Even interventions that modify apolipoprotein levels affect lipid levels simultaneously. For instance, gemfibrozil increases apoA1 and apoE concentrations as well as HDL-C levels (18). Since dyslipidemia starts before diagnosis

of T2D (19), the association of apolipoproteins and type 2 diabetes could be confounded by lipid levels. To disentangle the effect of apolipoproteins from HDL, LDL, VLDL or chylomicrons we used molar ratios of serum apolipoproteins levels to apoA1 and we adjusted their associations for lipid levels. ApoCIII was the only apolipoprotein that remained significant after adjustments and its ratio with apoA1 strongly predicted T2D as well. This may indicate that total flux of apoCIII and its ratio with the main indictor of HDL (apoA1) has a stronger impact in T2D aetiology than HDL-C itself.

Genetically elevated levels of apoCIII have been related to higher risk of diabetes. We have previously showed the associations of apoCIII promoter variants with the risk of T2D in lean healthy subjects (11). In a paper by Hokanson et al, an apoCIII haplotype was associated with type 1 diabetes (20). These studies may indicate that the association we report in this study between apoCIII and T2D is likely to be causal. The mechanisms through which apoCIII may cause T2D remain unclear, however, Avall et al have recently found that apoCIII links islet insulin resistance to beta cell failure in diabetes (21). This may indicate that apoCIII is mainly working through beta cell dysfunction rather than insulin resistance.

The typical dyslipidemia of T2D is characterized by high triglyceride levels and low HDL-C levels (22). ApoCIII is an atherogenic protein found on HDL, VLDL and LDL. ApoCIII leads to hypertriglyceridemia through three actions that can impair plasma lipoprotein metabolism. First, apoCIII inhibits VLDL clearance which results not only in hypertriglyceridemia, but also increases the formation of small dense LDL, known as LP-IR index components (4; 23). Second, apoCIII at high concentrations inhibits the action of lipoprotein lipase to hydrolyze lipoprotein triglyceride in vitro, however not yet shown in humans (24). Third, it has been shown recently that apoCIII assists in the formation of TG-rich VLDL in liver cells, stimulating the secretion of VLDL (25). Furthermore, antisense inhibition of apoCIII lowers apoCIII and triglycerides in hypertriglyceridemia (26). All these previous findings provide evidence that triglycerides are downstream of apoCIII. This is in line with our results, as when adjusted for triglycerides, the associations between apoCIII and T2D became weaker although it remained significant, suggesting that other pathways might be involved.

Previous studies suggest that apolipoproteins in lipoproteins or plasma may predict CHD better (27-30) and their HDL fraction seems to be the most important (31). This might be true also for T2D. Total serum apoCIII, mainly because of the fraction in HDL particle was found to be a key diabetogenic risk factor in a study among Turks (12). However, further investigation is needed to better explore the role of apoiliporoteins in T2D etiology and to compare the importance of their HDL fraction with the non-HDL fraction.

Our findings have important clinical implications. Our results emphasize the importance of the variability in the total serum apolipoproteinic flux rather than HDL-C levels itself. Patients with type 2 diabetes have defective HDL particles with altered composition, in addition to low HDL-C (32; 33). Thus, HDL components such as apolipoproteins might be good candidate biomarkers to evaluate the risk of T2D, and even potential targets for prevention or treatment of the disease if found to be casual (34; 35).

This study has certain strengths and limitations. To our knowledge, this is the first prospective

population-based cohort study to investigate the association between human serum levels of apoCIII, apoD and apoE as well as their ratios to apoA1, with incident T2D during a long-term follow-up. We use data from a well-characterized prospective cohort study, which allowed us to correct for a wide range of covariates. We are also aware of limitations of the study. Our population is 55 years and older and mainly comprised of individuals of European ancestry. Thus, generalization of the results to younger age groups and other ethnicities should be done with caution. Reference values for apolipoproteins depend on choice of assay techniques, therefore comparison of the values should take in account the chosen technique (36). We do not have available measurements of apolipoproteins amount separately in every lipoprotein particle and specifically in HDL, but only total serum levels of them, so we could not study the diabetes risk apolipoproteinic profile within HDL or other lipoprotein particles that are compound by the studied apolipoproteins. The studied apolipoproteins have all in common their presence in HDL, but serum apoA1 and apoD are totally found in HDL. Chylomicrons, secreted from the intestinal enterocyte, also contain apoA1, but it is quickly transferred to HDL in the bloodstream (15; 16).
In conclusion, serum levels of apoCIII and apoCIII-to-apoA1 ratio, are associated with risk of T2D independent of known risk factors and stronger than HDL-C levels. Aside the observational association between HDL-C and T2D, our findings highlight the role of variation in composition of HDL particles and other lipoproteins in relation to the disease. Therapeutic approaches for T2D should aim the normalization of both quantity and composition of HDL particles and other lipoproteins. More data are needed to determine importance of levels of apoCIII in specific lipoproteins for T2D risk assessment and management and to elucidate the interaction between triglycerides and apoC-III in relation to risk of T2D.

AUTHOR CONTRIBUTION

AB ran the analysis and wrote the manuscript. SL contributed to the analysis. MAI and AH designed the study. OHF designed the study and provided resources. MK, EJGS and AD designed the study and critically revised the manuscript. All authors have read and approved the manuscript. AD is the guarantor of this work.

ACKNOWLEDGEMENTS

The dedication, commitment, and contribution of inhabitants, general practitioners, and pharmacists of the Ommoord district to the Rotterdam Study are gratefully acknowledged. We thank Layal Chaker, Jolande Verkroost-van Heemst, and Ke-Xin Wen of Erasmus MC for their invaluable contribution to the collection of the diabetes data. AD is the guarantor of this work.

FUNDING

The Rotterdam Study is supported by the Erasmus MC and Erasmus University Rotterdam; the Netherlands Organization for Scientific Research (NWO); the Netherlands Organisation for Health Research and Development (ZonMw); the Research Institute for Diseases in the Elderly (RIDE); the Netherlands Genomics Initiative (NGI); the Ministry of Education, Culture and Sci-

ence, the Ministry of Health, Welfare and Sports; the European Commission (DG XII); and the Municipality of Rotterdam.
None of the funders had any role in design and conduct of the study, collection, management, analysis, and interpretation of the data, and preparation, review, or approval of the manuscript.
AD is supported by NWO grant (veni, 916.12.154) and the EUR Fellowship. MK is supported by the NWO VENI grant (VENI, 91616079).

DISCLOSURES

OHF works in ErasmusAGE, a center for aging research across the life course funded by Nestlé Nutrition (Nestec Ltd.); Metagenics Inc.; and AXA. The other authors report no potential conflicts of interest.

REFERENCES

1. Haffner SM, Stern MP, Hazuda HP, Mitchell BD, Patterson JK: Cardiovascular risk factors in confirmed prediabetic individuals. Does the clock for coronary heart disease start ticking before the onset of clinical diabetes? Jama 1990;263:2893-2898
2. Sparks JD, Sparks CE, Adeli K: Selective hepatic insulin resistance, VLDL overproduction, and hypertriglyceridemia. Arterioscler Thromb Vasc Biol 2012;32:2104-2112
3. Mackey RH, Mora S, Bertoni AG, Wassel CL, Carnethon MR, Sibley CT, Goff DC, Jr.: Lipoprotein particles and incident type 2 diabetes in the multi-ethnic study of atherosclerosis. Diabetes Care 2015;38:628-636
4. Shalaurova I, Connelly MA, Garvey WT, Otvos JD: Lipoprotein insulin resistance index: a lipoprotein particle-derived measure of insulin resistance. Metab Syndr Relat Disord 2014;12:422-429
5. Haase CL, Tybjaerg-Hansen A, Nordestgaard BG, Frikke-Schmidt R: High-density lipoprotein cholesterol and risk of type 2 diabetes: a Mendelian randomization study. Diabetes 2015;
6. Onat A, Hergenc G, Bulur S, Ugur M, Kucukdurmaz Z, Can G: The paradox of high apolipoprotein A-I levels independently predicting incident type-2 diabetes among Turks. Int J Cardiol 2010;142:72-79
7. Sasongko MB, Wong TY, Nguyen TT, Kawasaki R, Jenkins A, Shaw J, Wang JJ: Serum apolipoprotein AI and B are stronger biomarkers of diabetic retinopathy than traditional lipids. Diabetes Care 2011;34:474-479
8. Jian ZH, Lung CC, Ko PC, Sun YH, Huang JY, Ho CC, Ho CY, Chiang YC, Chen CJ, Liaw YP: The association between the apolipoprotein A1/ high density lipoprotein -cholesterol and diabetes in Taiwan - a cross-sectional study. BMC Endocr Disord 2013;13:42
9. Fizelova M, Miilunpohja M, Kangas AJ, Soininen P, Kuusisto J, Ala-Korpela M, Laakso M, Stancakova A: Associations of multiple lipoprotein and apolipoprotein measures with worsening of glycemia and incident type 2 diabetes in 6607 non-diabetic Finnish men. Atherosclerosis 2015;240:272-277
10. Abbasi A, Corpeleijn E, Gansevoort RT, Gans RO, Hillege HL, Stolk RP, Navis G, Bakker SJ, Dullaart RP: Role of HDL cholesterol and estimates of HDL particle composition in future development of type 2 diabetes in the general population: the PREVEND study. J Clin Endocrinol Metab 2013;98:E1352-1359
11. van Hoek M, van Herpt TW, Dehghan A, Hofman A, Lieverse AG, van Duijn CM, Witteman JC, Sijbrands EJ: Association of an APOC3 promoter variant with type 2 diabetes risk and need for insulin treatment in lean persons. Diabetologia 2011;54:1360-1367
12. Onat A, Hergenc G, Ayhan E, Ugur M, Kaya H, Tuncer M, Can G: Serum apolipoprotein C-III in high-density lipoprotein: a key diabetogenic risk factor in Turks. Diabet Med 2009;26:981-988
13. Hofman A, Brusselle GG, Darwish Murad S, van Duijn CM, Franco OH, Goedegebure A, Ikram MA, Klaver CC, Nijsten TE, Peeters RP, Stricker BH, Tiemeier HW, Uitterlinden AG, Vernooij MW: The Rotterdam Study: 2016 objectives and design update. Eur J Epidemiol 2015;30:661-708
14. World Health Organization. Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation. Geneva: World Health Organization 2006:1-50.
15. Gordon SM, Deng J, Lu LJ, Davidson WS: Proteomic characterization of human plasma high density lipoprotein fractionated by gel filtration chromatography. J Proteome Res 2010;9:5239-5249
16. Baker HN, Delahunty T, Gotto AM, Jr., Jackson RL: The primary structure of high density apolipoprotein-glutamine-I. Proc Natl Acad Sci U S A 1974;71:3631-3634
17. Gustafson A, Alaupovic P, Furman RH: Studies of the Composition and Structure of Serum Lipoproteins: Isolation, Purification, and Characterization of Very Low Density Lipoproteins of Human Serum. Biochemistry 1965;4:596-605
18. Krause BR, Newton RS: Gemfibrozil increases both apo A-I and apo E concentrations. Comparison to other lipid regulators in cholesterol-fed rats. Atherosclerosis 1986;59:95-98
19. von Eckardstein A, Schulte H, Assmann G: Risk for diabetes mellitus in middle-aged Caucasian male participants of the PROCAM study: implications for the definition of impaired fasting glucose by the American Diabetes Association. Prospective Cardiovascular Munster. J Clin Endocrinol Metab 2000;85:3101-3108
20. Hokanson JE, Kinney GL, Cheng S, Erlich HA, Kretowski A, Rewers M: Susceptibility to type 1 diabetes is associated with ApoCIII gene haplotypes. Diabetes 2006;55:834-838
21. Avall K, Ali Y, Leibiger IB, Leibiger B, Moede T, Paschen M, Dicker A, Dare E, Kohler M, Ilegems E, Abdulreda MH, Graham M, Crooke RM, Tay VS, Refai E, Nilsson SK, Jacob S, Selander L, Berggren PO, Juntti-Berggren L: Apolipoprotein CIII links islet insulin resistance to beta-cell failure in diabetes. Proc Natl Acad Sci U S A 2015;112:E2611-2619
22. Chahil TJ, Ginsberg HN: Diabetic dyslipidemia. Endocrinol Metab Clin North Am 2006;35:491-510, vii-viii

23. Zheng C, Khoo C, Furtado J, Sacks FM: Apolipoprotein C-III and the metabolic basis for hypertriglyceridemia and the dense low-density lipoprotein phenotype. Circulation 2010;121:1722-1734
24. Wang CS, McConathy WJ, Kloer HU, Alaupovic P: Modulation of lipoprotein lipase activity by apolipoproteins. Effect of apolipoprotein C-III. J Clin Invest 1985;75:384-390
25. Sundaram M, Zhong S, Bou Khalil M, Links PH, Zhao Y, Iqbal J, Hussain MM, Parks RJ, Wang Y, Yao Z: Expression of apolipoprotein C-III in McA-RH7777 cells enhances VLDL assembly and secretion under lipid-rich conditions. J Lipid Res 2010;51:150-161
26. Gaudet D, Alexander VJ, Baker BF, Brisson D, Tremblay K, Singleton W, Geary RS, Hughes SG, Viney NJ, Graham MJ, Crooke RM, Witztum JL, Brunzell JD, Kastelein JJ: Antisense Inhibition of Apolipoprotein C-III in Patients with Hypertriglyceridemia. N Engl J Med 2015;373:438-447
27. Emerging Risk Factors C, Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, Wood AM, Lewington S, Sattar N, Packard CJ, Collins R, Thompson SG, Danesh J: Major lipids, apolipoproteins, and risk of vascular disease. JAMA 2009;302:1993-2000
28. Nissen SE, Tardif JC, Nicholls SJ, Revkin JH, Shear CL, Duggan WT, Ruzyllo W, Bachinsky WB, Lasala GP, Tuzcu EM, Investigators I: Effect of torcetrapib on the progression of coronary atherosclerosis. N Engl J Med 2007;356:1304-1316
29. Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJ, Komajda M, Lopez-Sendon J, Mosca L, Tardif JC, Waters DD, Shear CL, Revkin JH, Buhr KA, Fisher MR, Tall AR, Brewer B, Investigators I: Effects of torcetrapib in patients at high risk for coronary events. N Engl J Med 2007;357:2109-2122
30. Sacks FM, Alaupovic P, Moye LA, Cole TG, Sussex B, Stampfer MJ, Pfeffer MA, Braunwald E: VLDL, apolipoproteins B, CIII, and E, and risk of recurrent coronary events in the Cholesterol and Recurrent Events (CARE) trial. Circulation 2000;102:1886-1892
31. Vaisar T: Proteomics investigations of HDL: challenges and promise. Curr Vasc Pharmacol 2012;10:410-421
32. Kontush A, Chapman MJ: Why is HDL functionally deficient in type 2 diabetes? Curr Diab Rep 2008;8:51-59
33. Verges B: Lipid modification in type 2 diabetes: the role of LDL and HDL. Fundam Clin Pharmacol 2009;23:681-685
34. Barter PJ: High density lipoprotein: a therapeutic target in type 2 diabetes. Endocrinol Metab (Seoul) 2013;28:169-177
35. von Eckardstein A, Sibler RA: Possible contributions of lipoproteins and cholesterol to the pathogenesis of diabetes mellitus type 2. Curr Opin Lipidol 2011;22:26-32
36. Boerma GJ, de Bruijn AM, van Teunenbroek A: Reference values for apolipoprotein A-I and apolipoprotein B in serum still depend on choice of assay techniques. Eur J Clin Chem Clin Biochem 1994;32:923-927

Chapter 3.2

Novel metabolic indices and incident type 2 diabetes among women and men: the Rotterdam Study.

Adela Brahimaj
Fernando Rivadeneira
Taulant Muka
Eric. J. G. Sijbrands
Oscar H. Franco
Abbas Dehghan
Maryam Kavousi

ABSTRACT

Background and aims

Both visceral and truncal fat have been associated with metabolic disturbances. We aimed to investigate the associations of several novel metabolic indices, combining anthropometric and lipid measures, and DXA measurements on body fat with incident type 2 diabetes (T2D) among women and men from the large population-based Rotterdam Study.

Materials and methods

We used Cox proportional hazard models to investigate associations between Visceral Adiposity Index (VAI), Lipid Accumulation Product (LAP), the product of Triglycerides and Glucose (TyG), their formula components, and DXA measurements with incident T2D. Associations were adjusted for traditional cardiovascular risk factors.

Results

Among 5576 women and 3988 men free of diabetes, 511 women and 388 men developed T2D during a median follow-up of 6.5 years. In adjusted models, the 3 metabolic indices; VAI (per 1-SD naturally log-transformed HR; 95% CI: 1.49; 1.36, 1.65 in women, 1.37; 1.22, 1.53 in men), LAP (1.35; 1.16, 1.56 in women, 1.19; 1.01, 1.42 in men), and TyG (1.73; 1.52, 1.98 in women, 1.43; 1.26, 1.62 in men), gynoid fat mass (0.64; 0.45,0.89) and android to gynoid ratio (1.48; 1.14, 1.94) in women and total fat mass (1.45; 1.00, 2.11) in men were associated with incident T2D. Body mass index (1.45; 1.28, 1.65) remained the strongest predictor for T2D in men.

Conclusions

Among women, novel combined metabolic indices were stronger risk markers for T2D than the traditional anthropometric and laboratory measures and were comparable to DXA measures. Neither combined metabolic indices nor DXA measures were superior to traditional anthropometric and lipid measures in association with T2D among men.

Abbreviations:

VAI – Visceral Adiposity Index
LAP – Lipid Accumulation Product
TyG – the product of triglycerides and glucose
DXA – Dual energy X-ray Absorptiometry
T2D – Type 2 Diabetes
CVD – Cardiovascular Diseases
HR – Hazard Ratio
CI – Confidence Interval

INTRODUCTION

Body fat distribution, rather than its magnitude, is increasingly linked to the risk for type 2 diabetes (1). Both visceral adipose tissue (VAT) and truncal fat depot have been associated with type 2 diabetes (2-4) and metabolic syndrome (5; 6).
VAT is a hormonally active component of total body fat. The risk of developing diabetes has been shown to be higher in individuals with excess of visceral adiposity, with (3) or without (7) manifestations of obesity. While VAT plays a key role in the association between adiposity and glucose metabolism (4; 8-10), traditional anthropometric measures such as body mass index (BMI) and waist circumference (WC), are not able to distinguish VAT from subcutaneous adipose tissue (11). Furthermore, VAT accounts for an increased cardiometabolic risk regardless of BMI levels (12). Truncal fat depot can be partitioned into upper body (android or central) and lower body (gynoid or peripheral) area. High android-gynoid percent fat ratio has shown a greater correlation with cardiometabolic dysregulation than BMI (13). Among the elderly, android fat depot seems to be more closely associated with metabolic syndrome than abdominal visceral fat (5).
The imaging modalities for assessing adipose tissue distribution are inconvenient and expensive. While Computed Tomography (CT) (2; 12) and Magnetic Resonance Imaging (MRI) (3) are the golden standard measures for quantification of VAT, Dual-energy X-Ray Absorptiometry (DXA) is the well-validated imaging method for precise measurement of body fat mass in various body compartments (i.e. android and gynoid fat) (14). Recently, different metabolic indices combining both anthropometric and lipid measures have been used as indicators of body fat. These novel indices, including Visceral Adiposity Index (VAI), Lipid Accumulation Product (LAP), and the product of Triglycerides and Glucose (TyG), have been suggested as early markers of insulin resistance mainly in cross-sectional studies (15-17). However, the associations of these novel metabolic indices with incident type 2 diabetes remain unclear. Therefore, we studied the associations of different novel metabolic indices and their formula components with incident type 2 diabetes among women and men from the large prospective population-based Rotterdam Study. We further assessed the associations of truncal fat depot measured by DXA with incident type 2 diabetes.

RESEARCH DESIGN AND METHODS

Study population

The study was performed in the framework of the Rotterdam Study (RS). RS is a prospective population-based cohort study in Ommoord, a district of Rotterdam, the Netherlands. The design of the Rotterdam Study has been described in more details elsewhere (18). The original cohort (RSI) started in 1989 when all residents within the well-defined study area aged 55 years or older were invited to participate of whom 78% (7983 out of 10275) agreed. The first examination of the original cohort (RSI-1) took place from 1990 to 1993. The cohort has been extended twice (RSII in 2000 and RSIII in 2006) to include the participants who were 45 years or older or moved to the study research area. For all 3 cohorts of RS, follow-up examinations were conducted every 3-5 years. The study has been approved by the medical ethics committee according to the Popula-

tion Screening Act: Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of Netherlands. All participants provided written informed consent to participate in the study and to obtain information from their treating physicians.

The current study was based on data collected during the third visit of the first cohort (RSI-3; 1997-1999), the first visit of the second cohort (RSII-1; 2000-2001), and the first visit of the third cohort (RSIII-1; 2006-2008). From 11740 subjects in the three visits of the Rotterdam Study, diabetes data were available for 10898 subjects (6241 women and 4657 men). After excluding 1334 prevalent diabetes cases (665 women and 669 men), 9564 subjects (5576 women and 3988 men) were included in the analysis of different metabolic indices and incident type 2 diabetes. DXA measurements on body fat were available in 3518 subjects (2026 women and 1492 men) with available diabetes data at the fourth visit of the first cohort (RSI-4; 2002-2004) and the second visit of the second cohort (RSII-2; 2004-2005). After excluding 556 prevalent diabetes cases (292 women and 264 men) at the time of DXA measurement, 2962 subjects (1734 women and 1228 men) were included in the analysis between DXA measures of body fat and incident type 2 diabetes.

Combined metabolic indices

Novel metabolic indices combine anthropometric measures such as BMI and WC with lipid measures; triglyceride (TG) or high-density lipoprotein (HDL) cholesterol, or fasting plasma glucose (FPG).

LAP, VAI, and TyG were calculated using the published formulas. LAP was calculated as LAP = $(WC - 65) \times TG$ for men and LAP = $(WC - 58) \times TG$ (19). VAI was calculated as

$$\text{VAI} = \left[\left(\frac{\text{WC}}{39.68}\right) + (1.88 \times BMI)\right] \times \left(\frac{\text{TG}}{1.03}\right) \times \left(\frac{1.31}{\text{HDL}}\right) \text{ for men and}$$

$$\text{VAI} = \left[\left(\frac{\text{WC}}{36.58}\right) + (1.89 \times BMI)\right] \times \left(\frac{\text{TG}}{0.81}\right) \times \left(\frac{1.52}{\text{HDL}}\right) \text{ for women (20).}$$

In both formulas, TG and HDL levels are expressed in mmol/l, WC in cm, and BMI in kg/m^2. The TyG index was calculated as

$$\text{Ln}\left(\text{TG} \times \frac{\text{FPG}}{2}\right) \text{ where both TG and FPG are expressed in mg/dl (15; 17).}$$

DXA measurements on body fat

Body composition was assessed using DXA. For the whole body DXA scans we used ProdigyTM total body-fan beam densitometer (GE Lunar Corp, Madison, WI, USA) (18). Total body weight (grams) was divided into bone mineral content, lean mass, and fat mass. In addition, we analyzed fat mass of the android body region and gynoid body region. Total fat mass percentage, android fat percentage, and gynoid fat percentage were calculated as percentages of total body weight. We also calculated the ratio of android to gynoid fat mass percentage.

Type 2 diabetes mellitus

Participants were followed from the date of baseline center visit onwards. Cases of type 2 diabetes were ascertained through active follow-up using general practitioners' records, hospital discharge letters and glucose measurements from RS visits which take place approximately every 4 years.

According to the current WHO guidelines, type 2 diabetes was defined as a fasting blood glucose ≥ 7.0 mmol/L, a non-fasting blood glucose ≥ 11.1 mmol/L (when fasting samples were unavailable), or the use of blood glucose lowering medication (21). Information regarding the use of blood glucose lowering medication was derived from both structured home interviews and linkage to pharmacy dispensing 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 an endocrinologist. Follow-up data was complete until January 1st 2012.

Covariates

Height and weight were measured with the participants standing without shoes and heavy outer garments. BMI was calculated by dividing weight in kilograms by height in meters squared. 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. Blood pressure was measured twice at the right brachial artery with a random-zero sphygmomanometer with the participant in sitting position, and the mean of 2 consecutive measurements was used. Insulin, glucose, HDL cholesterol, and TG were measured on the COBAS 8000 Modular Analyzer (Roche Diagnostics GmbH). HOMA-IR (the homeostatic model assessment to quantify insulin resistance) was calculated dividing the product of fasting glucose (in mmol/L) and fasting insulin (in mU/L) by 22.5. HOMA-B (the homeostatic model assessment of β-cell function) was calculated dividing the product of fasting insulin (in mU/L) and 20 by the difference of glucose (in mmol/L) with 3.5 (22). TG levels were not available in the same visit with DXA measures (RSI-4), thus they were taken from the closest previous visit (RSI-3).The corresponding interassay coefficients of variations are the following: insulin < 8%, glucose < 1.4% and lipids < 2.1%. Information on medication use and tobacco smoking behaviour was collected by trained research assistants via computerized questionnaires during home visits. Smoking was classified as current versus non-current smokers. History of cardiovascular disease (CVD) was defined as a history of coronary heart diseases (myocardial infarction, revascularization, coronary artery bypass graft surgery or percutaneous coronary intervention) or stroke and was verified from the medical records of the general practitioners.

Statistical analysis

Considering gender differences in fat distribution and that the formulas of metabolic indices differ by gender, all analysis were performed among women and men separately. Descriptive characteristics were presented as mean ± standard deviation (SD) for continuous variables and numbers (percentages) for dichotomous variables. To compare general characteristics between men and women, we used One Way ANOVA for continuous variables and Chi-Square test (Chi^2) for categorical variables. Markers with a right-skewed distribution were transformed to the natural logarithmic scale.

We used Cox proportional hazard models to investigate associations of different combined metabolic indices (VAI, LAP, TyG), the anthropometric (BMI, WC) or laboratory components (inverse HDL, TG) included in their formula, as well as DXA measurements on body fat (an-

droid, gynoid, total fat mass, the ratio of android to gynoid fat mass percentage) with incident type 2 diabetes. We used inverse HDL to compare the estimates easier. The proportional hazard assumption of the Cox model was checked by 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). The first model was adjusted for age and cohort. In the second model, we additionally adjusted for BMI. In the third model, we additionally adjusted for systolic blood pressure, medication for hypertension, smoking and prevalent CVD. In the fourth model, we added HDL, TG, serum lipid reducing agents. In the fifth model, we added fasting glucose. As glucose is a mean for diagnosis of type 2 diabetes, this model should be considered a conservative model. For each novel lipid index, the covariates that were already in the index formula were excluded from the multivariable adjusted model.

To check whether the association of different markers with incident diabetes differ by obesity status, we further stratified the analyses based on BMI cut-off of 30 and performed the analyses among non-obese (BMI < 30) and obese (BMI ≥ 30) individuals. To compare the estimates between women and men, we applied an interaction test in model four. The p-value is derived from the z- score calculated from the ratio between the difference of the two estimates and standard error of this difference (23). The p-value indicates whether the difference between the estimates is significant.

Multiple imputation procedure was performed (N = 5 imputations) to impute missing data in covariates. All analyses were conducted in SPSS software version 21 (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp). A P-value <0.05 was considered statistically significant.

RESULTS

Metabolic indices and incident type 2 diabetes

Baseline characteristics of 5576 women and 3988 men included in the study are shown in Table 1. Women were older, had lower levels of systolic blood pressure and glucose but higher levels of total cholesterol. A larger proportion of women were treated for hypertension. CVD was more prevalent among men and a larger proportion of men were receiving lipid reducing agents or were current smokers. BMI, HDL, TG and VAI were higher in women whereas WC, LAP and TyG were higher in men (Table 1).

The correlation coefficients for metabolic indices in relation to glycemic indices are shown in ESM Table 1. For both women and men, the correlation coefficients for VAI, LAP, and TyG ranged between 0.43-0.57 for HOMA-IR and between 0.04-0.28 for HOMA-B. The correlation coefficients for different visceral indices in relation to HOMA-IR were overall larger among women compared to men, albeit not statistically significant.

During a median follow-up of 6.5 years (maximum of 14.7 years) 899 incident type 2 diabetes cases were identified (511 women and 388 men). All indices were significantly associated with the risk of type 2 diabetes in age adjusted models. In the multivariable-adjusted model (model 4), TyG showed to be the strongest marker for type 2 diabetes in women (per 1 SD HR; 95% CI: 1.73;

Table 1. Baseline characteristics of study participants (N=9564).

Characteristic	Women (N = 5576)	Men (N = 3988)	p-value
Age (years)	65.1 ± 10.3	64.3 ± 9.5	< **0.001**
Systolic Blood pressure (mm/Hg)	136.2 ± 21.6	138.6 ± 20.2	< **0.001**
Treatment for hypertension	1225.0 (22.0%)	786.0 (19.7)	**0.011**
Prevalent cardiovascular disease (%)	282.0 (5.1)	564.0 (14.1)	< **0.001**
Serum lipid reducing agents use (%)	739.0 (13.3)	639.0 (16.0)	**0.001**
Current smokers	809.0 (14.5)	874.0 (21.9)	< **0.001**
Total cholesterol (mmol/l)	5.9 ± 0.9	5.5 ± 0.9	< **0.001**
*Insulin (pmol/l)	69.0 (30.0 – 182.0)	71.0 (30.0 – 188.0)	0.2
*Glucose (mmol/l)	5.3 (4.6 – 6.4)	5.5 (4.7 – 6.5)	< **0.001**
Metabolic indices			
BMI (kg/m^2)	27.1 ± 4.5	26.7 ± 3.4	< **0.001**
Waist circumference (cm)	89.1 ± 11.8	97.7 ± 10.0	< **0.001**
*High density lipoprotein cholesterol (mmol/l)	1.5 (0.9 – 2.3)	1.2 (0.8 – 1.9)	< **0.001**
*Triglycerides (moml/l)	1.3 (0.7 – 2.8)	1.3 (0.7- 3.1)	< **0.001**
*VAI	1.6 (0.6 – 4.8)	1.5 (0.6 – 4.8)	**0.008**
*LAP	38.1 (11.4 – 106.8)	42.6 (15.7 – 122.4)	< **0.001**
TyG	2.8 ± 0.5	2.9 ± 0.5	< **0.001**
DXA measurements	Women (N = 1734)	Men (N = 1228)	
*Android fat %	3.3 (1.8 – 4.5)	3.1 (1.6 – 4.3)	< **0.001**
*Gynoid fat %	6.3 (4.5 – 8.1)	3.9 (2.6 – 5.3)	< **0.001**
*Android/Gynoid	0.5 (0.3 – 0.7)	0.7 (0.5 – 1.1)	< **0.001**
*Total fat mass %	39.2 (27.2 – 48.6)	27.5 (16.9 – 37.0)	< **0.001**

Values are presented as means ± standard deviation,* median (inter-quartile range), or numbers (percentages).
Abbreviations: BMI, body mass index; VAI, visceral adiposity index; LAP, lipid accumulation product; TyG, the product of fasting glucose and triglycerides.

1.52, 1.98) and in men (1.43; 1.26, 1.62). Other markers that remained significantly associated with incident type 2 diabetes in both genders in the multivariable-adjusted model were BMI (1.37; 1.26, 1.49 in women and 1.45; 1.28, 1.65 in men), inverse HDL (per 1 SD naturally log-transformed HR; 95% CI: 1.29; 1.14, 1.46 in women and 1.32; 1.14, 1.52 in men), VAI (1.49; 1.36, 1.65 in women and 1.37; 1.22, 1.53 in men), LAP (1.35; 1.16, 1.56 in women and 1.19; 1.01, 1.42 in men). WC (1.24; 1.07, 1.45) and TG (1.24; 1.10, 1.39) remained strongly associated with the risk for type 2 diabetes only in women (Table 2). Associations of metabolic indices with diabetes were overall stronger among women compared to men. However, the difference of the estimates between women and men was statistically significant only for TyG (Table 2).

After additionally adjusting for fasting glucose (model 5), only BMI (1.27; 1.17, 1.38 for women and 1.25; 1.09, 1.43 for men), inverse HDL (1.29; 1.14, 1.47 for women and 1.41; 1.22, 1.63 for men), and VAI (1.29; 1.17, 1.43 for women and 1.23; 1.09, 1.38 for men) remained significantly associated with the risk for type 2 diabetes in both genders (Table 2).

In the analyses stratified for obesity status, in the multivariable adjusted model (model 4), BMI, inverse HDL, VAI, and TyG remained significantly associated with incident diabetes regardless of the obesity status. While LAP was significantly associated with incident diabetes among non-obese women and men, WC and TG remained strongly associated with the risk for type 2

Table 2. Associations between different metabolic indices and incident type 2 diabetes mellitus (N = 9564).

	Incident type 2 diabetes	
	HR(95%CI)	
Index	Women (511 cases)	Men (388 cases)
BMI		
Model 1	**1.51 (1.39, 1.63)**	**1.64 (1.45, 1.86)**
Model 2	NA	NA
Model 3	**1.49 (1.38, 1.62)**	**1.61 (1.42, 1.82)**
Model 4	**1.37 (1.26, 1.49)**	**1.45 (1.28, 1.65)**
Model 5	**1.27 (1.17, 1.38)**	**1.25 (1.09, 1.43)**
WC		
Model 1	**1.62 (1.49, 1.77)**	**1.44 (1.31, 1.58)**
Model 2	**1.39 (1.19, 1.61)**	1.15 (0.94, 1.39)
Model 3	**1.37 (1.18, 1.59)**	1.13 (0.92, 1.38)
Model 4	**1.24 (1.07, 1.45)**	1.04 (0.83, 1.31)
Model 5	1.04 (0.89, 1.22)	1.04 (0.82, 1.30)
[*]1/HDL		
Model 1	**1.58 (1.44, 1.74)**	**1.53 (1.36, 1.73)**
Model 2	**1.46 (1.33, 1.61)**	**1.42 (1.25, 1.61)**
Model 3	**1.46 (1.32, 1.61)**	**1.40 (1.24, 1.59)**
Model 4	**1.29 (1.14, 1.46)**	**1.32 (1.14, 1.52)**
Model 5	**1.29 (1.14, 1.47)**	**1.41 (1.22, 1.63)**
[*]Triglycerides		
Model 1	**1.58 (1.44, 1.74)**	**1.44 (1.30, 1.58)**
Model 2	**1.45 (1.31, 1.60)**	**1.30 (1.18, 1.45)**
Model 3	**1.41 (1.28, 1.56)**	**1.28 (1.15, 1.42)**
Model 4	**1.24 (1.10, 1.39)**	1.12 (0.99, 1.27)
Model 5	1.07 (0.95, 1.21)	0.94 (0.83, 1.06)
[*]VAI		
Model 1	**1.65 (1.51, 1.81)**	**1.52 (1.36, 1.69)**
Model 2	NA	NA
Model 3	**1.49 (1.35, 1.65)**	**1.37 (1.22, 1.53)**
Model 4	**1.49 (1.36, 1.65)**	**1.37 (1.22, 1.53)**
Model 5	**1.29 (1.17, 1.43)**	**1.23 (1.09, 1.38)**
[*]LAP		
Model 1	**1.83 (1.65, 2.03)**	**1.66 (1.47, 1.87)**
Model 2	**1.60 (1.41, 1.82)**	**1.47 (1.27, 1.70)**
Model 3	**1.55 (1.36, 1.76)**	**1.43 (1.24, 1.66)**
Model 4	**1.35 (1.16, 1.56)**	**1.19 (1.01, 1.42)**
Model 5	1.08 (0.93, 1.26)	0.96 (0.81, 1.15)
TyG		
Model 1	**2.06 (1.86, 2.29)**	**1.74 (1.56, 1.94)**
Model 2	**1.88 (1.69, 2.09)**	**1.58 (1.41, 1.77)**
Model 3	**1.82 (1.64, 2.04)**	**1.55 (1.38, 1.75)**
Model 4[$]	**1.73 (1.52, 1.98)**	**1.43 (1.26, 1.62)**
Model 5	NA	NA

Model 1: Adjusted for age and cohort Model 2: Additionally adjusted for BMI Model 3: Additionally adjusted for SBP, treatment for hypertension, smoking, prevalent CVD Model 4: Additionally adjusted for HDL, triglycerides, serum lipid reducing agents Model 5: Additionally adjusted for fasting glucose; Hazard ratios are presented per 1 standard deviation increase in the marker.
[*]Marker is naturally log-transformed.
[$] P-value for the difference in hazard ratio between women and men ≤ 0.05

diabetes only in non-obese women. Overall, the tendency for the association of visceral indices with diabetes was stronger among non-obese individuals (ESM Table 2).

DXA measurements on body fat and incident type 2 diabetes

Android fat, gynoid fat and total fat mass percentages were higher in women whereas the ratio of android to gynoid fat percentage was higher in men (Table 1). Complete baseline characteristics of 1734 women and 1228 men included in the analyses for DXA measures and type 2 diabetes are presented in ESM Table 3.

Among 1734 women and 1228 men included in the analyses for DXA measurements, 149 women and 107 men developed type 2 diabetes during a median follow-up of 8 years (maximum of 10 years). Among the DXA measurements on body fat, gynoid fat percentage (per 1 SD naturally log-transformed HR; 95% CI: 0.64; 0.45, 0.89) and the ratio of android to gynoid fat percentage (1.48; 1.14, 1.94) remained significantly associated with incident type 2 diabetes in the multivari-

Table 3. Associations between DXA measurements on body fat and incident type 2 diabetes (N=2962).

	Incident type 2 diabetes	
	HR(95%CI)	
DXA measurements	Women (149 cases)	Men (107 cases)
*__Android fat mass %__		
Model 1	**1.77 (1.42, 2.22)**	**1.43 (1.13, 1.81)**
Model 2	**1.42 (1.06, 1.89)**	**1.44 (1.06, 1.95)**
Model 3	**1.36 (1.02, 1.82)**	**1.43 (1.05, 1.93)**
Model 4	1.21 (0.89, 1.63)	1.33 (0.97, 1.83)
Model 5	1.09 (0.83, 1.45)	1.34 (0.99, 1.82)
*__Gynoid fat mass %__		
Model 1	1.01 (0.76, 1.35)	1.21 (0.91, 1.59)
Model 2	**0.56 (0.40, 0.78)**	1.03 (0.74, 1.44)
Model 3	**0.57 (0.41, 0.79)**	1.03 (0.74, 1.45)
Model 4[$]	**0.64 (0.45, 0.89)**	1.12 (0.78, 1.59)
Model 5	0.76 (0.54, 1.07)	1.08 (0.75, 1.54)
*__Android/Gynoid__ Model 1	**1.95 (1.55, 2.46)**	**1.53 (1.13, 2.07)**
Model 2	**1.73 (1.35, 2.21)**	**1.46 (1.07, 1.99)**
Model 3	**1.68 (1.31, 2.16)**	**1.45 (1.06, 1.98)**
Model 4	**1.48 (1.14, 1.94)**	1.24 (0.89, 1.73)
Model 5	1.26 (0.97, 1.64)	1.32 (0.93, 1.88)
*__Total fat mass %__		
Model 1	**1.56 (1.17, 2.08)**	**1.43 (1.11, 1.84)**
Model 2	0.76 (0.53, 1.11)	**1.43 (1.00, 2.04)**
Model 3	0.76 (0.52, 1.09)	1.42 (0.99, 2.03)
Model 4[$]	0.77 (0.52, 1.14)	1.45 (1.00, 2.11)
Model 5	0.86 (0.59, 1.27)	**1.45 (1.00, 2.11)**

Model 1: Adjusted for age and cohort Model 2: Additionally adjusted for BMI Model 3: Additionally adjusted for SBP, treatment for hypertension, smoking, prevalent CVD Model 4: Additionally adjusted for HDL, triglycerides, serum lipid reducing agents Model 5: Additionally adjusted for fasting glucose; Hazard ratios are presented per 1 standard deviation increase in the marker.

*Marker is naturally log-transformed.

[$] P-value for the difference in hazard ratio between women and men ≤ 0.05

able-adjusted model (model 4) in women. Total fat mass percentage was the only significant DXA measure associated with incident diabetes among men (1.45; 1.00, 2.11) (Table 3).
In the analyses stratified for obesity status, gynoid fat percentage (0.57; 0.38, 0.85) and the ratio of android to gynoid fat percentage (1.74; 1.27, 2.39) remained significantly associated with incident type 2 diabetes in the multivariable-adjusted model (model 4) only in non-obese women (ESM Table 4). After additionally adjusting for fasting glucose (model 5), only the ratio of android to gynoid fat mass percentage (1.47; 1.07, 2.02) remained associated with incident type 2 diabetes in non-obese women (ESM table 4).

DISCUSSION

In our large population-based Rotterdam Study, novel metabolic indices; VAI, LAP and TyG, were stronger risk markers for incident type 2 diabetes than the traditional anthropometric and lipid measures, particularly among women. The predictive value of these novel metabolic indices for type 2 diabetes was also comparable to DXA measured body fat compositions in women. Among men, neither combined metabolic indices nor DXA measures on body fat were superior to traditional anthropometric and lipid measures, in particular BMI, in association with type 2 diabetes.
VAT is a hormonally active component of total body fat. Among the two main fat depots; VAT and subcutaneous adipose tissue, VAT seems to be behind the term diabesity playing a key role in the association between adiposity and glucose metabolism (4; 8-10). Excess visceral adiposity has been linked to higher risk of type 2 diabetes, regardless of obesity (2; 3; 7; 12). The three combined metabolic indices: VAI, LAP and TyG have been introduced as indicators of "visceral adipose function" (20) and insulin resistance (15-17) and have been linked to cardio-metabolic risk (20), prediabetes (24) and diabetes (24) in cross-sectional studies. Our study is the first to simultaneously investigate the longitudinal associations of all these new indices, as well as their components, with incident type 2 diabetes among women and men. The three novel combined metabolic indices were all independently associated with increased risk of type 2 diabetes in our study. VAI and LAP combine both physical and metabolic parameters to evaluate body fatness, whereas TyG includes only metabolic parameters. By including both BMI and WC as well as TG and HDL cholesterol, VAI is a reflection of different obesity parameters as well as pro-atherogenic and anti-atherogenic properties of lipids. LAP includes WC and TG and is an index for excessive lipid accumulation. Being a product of combined TG and fasting glucose levels, TyG was the strongest risk marker for type 2 diabetes in our study. Since precise measurement of visceral fat content requires the use of expensive imaging techniques such as CT or MRI (2; 12), simple and economical quantification of these visceral adiposity indices could lead to improvements in identification of individuals at high risk for type 2 diabetes.
The counterbalance between insulin secretion and insulin resistance is critical for type 2 diabetes pathogenesis. VAI, LAP and TyG have been introduced as early indicators of insulin resistance (15-17). In our study, the three indices were all moderately correlated with insulin resistance (HOMA-IR) and showed a smaller correlation with insulin secretion (HOMA-B). As VAI and

LAP combine both lipid variables and adiposity status, they served as better surrogates for insulin resistance compared to either lipid or adiposity measures alone. The largest correlation of TyG with IR in our study is in line with other studies, supporting that both lipotoxicity and glucotoxicity have central role in modulation of insulin resistance (25). Since obesity has a strong impact on dyslipidemia, insulin resistance and development of type 2 diabetes, we further stratified the analyses based on obesity status. Correlation of different combined adiposity indices with HOMA measures did not materially differ between non-obese and obese individuals. The overall tendency towards stronger associations of these metabolic indices with incident diabetes among non-obese individuals might be due to their lower discriminatory power among higher risk obese individuals.

While the exact mechanisms responsible for the relationship between excess abdominal/visceral fat and cardiometabolic risk are still unclear, several hypotheses are proposed (26). Subcutaneous fat faces obesogenic stress with a limited capacity for regional adipocyte hypertrophy or hyperplasia. Once this capacity is overpassed, adipose tissue storage is forced into other regions, such as organs or compartments of the body, which are named ectopic. Visceral fat is considered the classic ectopic fat depot, sick fat and is associated to dysfunctional adiposity or adiposopathy (27; 28). Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men (29). Increased intramyocellular fat is associated with skeletal muscle insulin resistance (30). Other studies highlight the role of genes that predispose to preferential fat distribution in cardiometabolic diseases (26; 31). The direct effect of lipolysis products in omental and mesenteric fat may play a critical role in insulin resistance of type 2 diabetes and metabolic syndrome (32).

It is worth noting that WC, TG, VAI, LAP, and TyG showed a stronger association with incident type 2 diabetes in women than in men in our study. Similarly, the correlations between VAI, LAP, and TyG with HOMA-IR in our study were overall stronger among women. The greater association of VAT with diabetes and adverse cardiovascular risk profiles among women has been suggested by some studies (33; 34). Gender differences in adverse metabolic complications associated with visceral fat have been related to a significantly lower visceral fat area in nondiabetic women compared with nondiabetic men and a similar visceral fat area for both diabetic men and women (33). Among individuals with more visceral fat, a greater portion of hepatic free fatty acid delivery originates from visceral adipose tissue lipolysis (35). Contribution of the visceral lipolysis to hepatic free fatty acid delivery in relation to visceral fat has been found to be greater in women than in men (35). Moreover, correlation between visceral adipose tissue area and serum triglycerides has been found to be stronger in women than in men (36).

No previous study has investigated the associations of DXA measures on body fat with incident type 2 diabetes. Our study suggests gynoid fat percentage and android-gynoid percent fat ratio among women and total fat mass among men as independent risk markers for type 2 diabetes. Previous studies have shown important relationships between the android/gynoid fat and metabolic risk in healthy adults. Android or truncal obesity has been associated with the risk for metabolic disorders and cardiovascular disease (37), yet there is evidence that gynoid fat distribution may be protective (38). Android fat depot is the adipose tissue mainly around the trunk including, but not exclusively, visceral fat and has been found to be more closely associated

with metabolic syndrome than abdominal visceral fat in elderly people (5). High android-gynoid percent fat ratio seems to have a larger correlation with cardiometabolic dysregulation than android percent fat, gynoid percent fat, or body mass index, which is in line with our findings (13). Android obesity in women has been correlated with a higher incidence of glucose intolerance relative to women with a predominantly gynoid distribution (39).

Excess android fat mass has recently been associated with high triglycerides and low HDL cholesterol levels in men and high LDL and low HDL cholesterol levels in women. It was also reported that excess gynoid fat mass was positively correlated with total cholesterol in men, whereas gynoid fat mass in women showed a favorable association with triglycerides and HDL cholesterol (40). It therefore seems that regional fat distribution in the android and gynoid regions have varying effects on lipid profiles among women and men. This is in line with our findings regarding an inverse association of gynoid fat and the android to gynoid ratio with the risk for type 2 diabetes in women and a positive association of the total fat mass with type 2 diabetes risk in men. In line with our findings, another study provided evidence that increased gynoid fat mass may be protective against the progression of NAFLD in female Japanese patients with type 2 diabetes (41).

In our study, the magnitude of the association between DXA measures of body fat and diabetes was comparable to those of combined metabolic indices and traditional anthropometry and lipid measures. Therefore, considering the costs and radiation exposure associated with DXA measurement, its use as a screening tool for diabetes may not be justified.

To our knowledge, this is the first prospective population-based cohort study to simultaneously investigate the associations between novel metabolic indices as well as DXA measures with incident type 2 diabetes among women and men over a long follow-up. We used data from a well-characterized prospective cohort study, which allowed for direct comparison of several metabolic indices as well as correction for a wide range of covariates. The limitations of our study also warrant attention. Our population comprises 55 years and older individuals of European ancestry. Thus, generalization of the results to younger age groups and other ethnicities should be done with caution. Due to unavailability of CT or MRI in our population, comparison of our results against the gold standard measures for visceral fat is not possible.

In conclusion, novel combined metabolic indices; VAI, LAP and TyG, were stronger risk markers for incident type 2 diabetes, than the traditional anthropometric and lipid measures, particularly among women. The predictive value of these novel metabolic indices for type 2 diabetes was also comparable to DXA measured body fat compositions in women. Neither combined metabolic indices nor DXA measures on body fat were superior to traditional anthropometric and lipid measures in association with type 2 diabetes among men. Particularly, BMI remains the best marker for type 2 diabetes risk in men and among the best markers in women.

AUTHOR CONTRIBUTION

AB ran the analysis and wrote the manuscript. MK, EJGS and AD designed the study and critically revised the manuscript. All authors have read and approved the manuscript. OHF designed the study and provided resources. AB is the guarantor of this work.

Acknowledgments

The dedication, commitment, and contribution of inhabitants, general practitioners, and pharmacists of the Ommoord district to the Rotterdam Study are gratefully acknowledged. We thank Layal Chaker, Jolande Verkroost-van Heemst, and Ke-Xin Wen of Erasmus MC for their invaluable contribution to the collection of the diabetes data. AB is the guarantor of this work.

Funding

The Rotterdam Study is supported by the Erasmus MC and Erasmus University Rotterdam; the Netherlands Organization for Scientific Research (NWO); the Netherlands Organisation for Health Research and Development (ZonMw); the Research Institute for Diseases in the Elderly (RIDE); the Netherlands Genomics Initiative (NGI); the Ministry of Education, Culture and Science, the Ministry of Health, Welfare and Sports; the European Commission (DG XII); and the Municipality of Rotterdam.
None of the funders had any role in design and conduct of the study, collection, management, analysis, and interpretation of the data, and preparation, review, or approval of the manuscript.
MK is supported by the NWO VENI grant (VENI, 91616079).

Disclosures

OHF works in ErasmusAGE, a center for aging research across the life course funded by Nestlé Nutrition (Nestec Ltd.); Metagenics Inc.; and AXA. The other authors report no potential conflicts of interest.

REFERENCES

1. Despres J-P, Lemieux I: Abdominal obesity and metabolic syndrome. 2006;444:881-887
2. Wander PL, Boyko EJ, Leonetti DL, McNeely MJ, Kahn SE, Fujimoto WY: Change in visceral adiposity independently predicts a greater risk of developing type 2 diabetes over 10 years in Japanese Americans. Diabetes Care 2013;36:289-293
3. Neeland IJ, Turer AT, Ayers CR, Powell-Wiley TM, Vega GL, Farzaneh-Far R, Grundy SM, Khera A, McGuire DK, de Lemos JA: Dysfunctional adiposity and the risk of prediabetes and type 2 diabetes in obese adults. JAMA 2012;308:1150-1159
4. Boyko EJ, Fujimoto WY, Leonetti DL, Newell-Morris L: Visceral adiposity and risk of type 2 diabetes: a prospective study among Japanese Americans. Diabetes Care 2000;23:465-471
5. Kang SM, Yoon JW, Ahn HY, Kim SY, Lee KH, Shin H, Choi SH, Park KS, Jang HC, Lim S: Android fat depot is more closely associated with metabolic syndrome than abdominal visceral fat in elderly people. PLoS One 2011;6:e27694
6. Fu X, Song A, Zhou Y, Ma X, Jiao J, Yang M, Zhu S: Association of regional body fat with metabolic risks in Chinese women. Public Health Nutr 2014;17:2316-2324
7. DeNino WF, Tchernof A, Dionne IJ, Toth MJ, Ades PA, Sites CK, Poehlman ET: Contribution of abdominal adiposity to age-related differences in insulin sensitivity and plasma lipids in healthy nonobese women. Diabetes Care 2001;24:925-932
8. Fox CS, Massaro JM, Hoffmann U, Pou KM, Maurovich-Horvat P, Liu C-Y, Vasan RS, Murabito JM, Meigs JB, Cupples LA, D'Agostino RB, O'Donnell CJ: Abdominal Visceral and Subcutaneous Adipose Tissue Compartments. Association With Metabolic Risk Factors in the Framingham Heart Study 2007;116:39-48
9. McLaughlin T, Lamendola C, Liu A, Abbasi F: Preferential fat deposition in subcutaneous versus visceral depots is associated with insulin sensitivity. J Clin Endocrinol Metab 2011;96:E1756-1760
10. Preis SR, Massaro JM, Robins SJ, Hoffmann U, Vasan RS, Irlbeck T, Meigs JB, Sutherland P, D'Agostino RB, Sr., O'Donnell CJ, Fox CS: Abdominal subcutaneous and visceral adipose tissue and insulin resistance in the Framingham heart study. Obesity (Silver Spring) 2010;18:2191-2198
11. Camhi SM, Bray GA, Bouchard C, Greenway FL, Johnson WD, Newton RL, Ravussin E, Ryan DH, Smith SR, Katzmarzyk PT: The relationship of waist circumference and BMI to visceral, subcutaneous, and total body fat: sex and race differences. Obesity (Silver Spring) 2011;19:402-408
12. Shah RV, Murthy VL, Abbasi SA, Blankstein R, Kwong RY, Goldfine AB, Jerosch-Herold M, Lima JA, Ding J, Allison MA: Visceral adiposity and the risk of metabolic syndrome across body mass index: the MESA Study. JACC Cardiovasc Imaging 2014;7:1221-1235
13. Okosun IS, Seale JP, Lyn R: Commingling effect of gynoid and android fat patterns on cardiometabolic dysregulation in normal weight American adults. Nutr Diabetes 2015;5:e155
14. Doran DA, Mc Geever S, Collins KD, Quinn C, McElhone R, Scott M: The validity of commonly used adipose tissue body composition equations relative to dual energy X-ray absorptiometry (DXA) in gaelic games players. Int J Sports Med 2014;35:95-100
15. Simental-Mendia LE, Rodriguez-Moran M, Guerrero-Romero F: The product of fasting glucose and triglycerides as surrogate for identifying insulin resistance in apparently healthy subjects. Metab Syndr Relat Disord 2008;6:299-304
16. Du T, Yuan G, Zhang M, Zhou X, Sun X, Yu X: Clinical usefulness of lipid ratios, visceral adiposity indicators, and the triglycerides and glucose index as risk markers of insulin resistance. Cardiovasc Diabetol 2014;13:146
17. Guerrero-Romero F, Simental-Mendia LE, Gonzalez-Ortiz M, Martinez-Abundis E, Ramos-Zavala MG, Hernandez-Gonzalez SO, Jacques-Camarena O, Rodriguez-Moran M: The product of triglycerides and glucose, a simple measure of insulin sensitivity. Comparison with the euglycemic-hyperinsulinemic clamp. J Clin Endocrinol Metab 2010;95:3347-3351
18. Hofman A, Brusselle GG, Darwish Murad S, van Duijn CM, Franco OH, Goedegebure A, Ikram MA, Klaver CC, Nijsten TE, Peeters RP, Stricker BH, Tiemeier HW, Uitterlinden AG, Vernooij MW: The Rotterdam Study: 2016 objectives and design update. Eur J Epidemiol 2015;30:661-708
19. Kahn HS: The "lipid accumulation product" performs better than the body mass index for recognizing cardiovascular risk: a population-based comparison. BMC Cardiovasc Disord 2005;5:26
20. Amato MC, Giordano C, Galia M, Criscimanna A, Vitabile S, Midiri M, Galluzzo A, AlkaMeSy Study G: Visceral Adiposity Index: a reliable indicator of visceral fat function associated with cardiometabolic risk. Diabetes Care 2010;33:920-922

21. World Health Organization G: Definition and Diagnosis of DiabetesMellitus and Intermediate Hyperglycemia: Report of a WHO/IDF Consultation. 2006: p. 1–50
22. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412-419
23. Altman DG, Bland JM: Interaction revisited: the difference between two estimates. BMJ 2003;326:219
24. Liu PJ, Ma F, Lou HP, Chen Y: Visceral Adiposity Index Is Associated with Pre-Diabetes and Type 2 Diabetes Mellitus in Chinese Adults Aged 20-50. Ann Nutr Metab 2016;68:235-243
25. Er LK, Wu S, Chou HH, Hsu LA, Teng MS, Sun YC, Ko YL: Triglyceride Glucose-Body Mass Index Is a Simple and Clinically Useful Surrogate Marker for Insulin Resistance in Nondiabetic Individuals. PLoS One 2016;11:e0149731
26. Klein S, Allison DB, Heymsfield SB, Kelley DE, Leibel RL, Nonas C, Kahn R, Association for Weight M, Obesity P, Naaso TOS, American Society for N, American Diabetes A: Waist circumference and cardiometabolic risk: a consensus statement from Shaping America's Health: Association for Weight Management and Obesity Prevention; NAASO, The Obesity Society; the American Society for Nutrition; and the American Diabetes Association. Am J Clin Nutr 2007;85:1197-1202
27. Britton KA, Fox CS: Ectopic fat depots and cardiovascular disease. Circulation 2011;124:e837-841
28. Bays HE: Adiposopathy, diabetes mellitus, and primary prevention of atherosclerotic coronary artery disease: treating "sick fat" through improving fat function with antidiabetes therapies. Am J Cardiol 2012;110:4B-12B
29. Seppala-Lindroos A, Vehkavaara S, Hakkinen AM, Goto T, Westerbacka J, Sovijarvi A, Halavaara J, Yki-Jarvinen H: Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. J Clin Endocrinol Metab 2002;87:3023-3028
30. Jacob S, Machann J, Rett K, Brechtel K, Volk A, Renn W, Maerker E, Matthaei S, Schick F, Claussen CD, Haring HU: Association of increased intramyocellular lipid content with insulin resistance in lean nondiabetic offspring of type 2 diabetic subjects. Diabetes 1999;48:1113-1119
31. Scott RA, Fall T, Pasko D, Barker A, Sharp SJ, Arriola L, Balkau B, Barricarte A, Barroso I, Boeing H, Clavel-Chapelon F, Crowe FL, Dekker JM, Fagherazzi G, Ferrannini E, Forouhi NG, Franks PW, Gavrila D, Giedraitis V, Grioni S, Groop LC, Kaaks R, Key TJ, Kuhn T, Lotta LA, Nilsson PM, Overvad K, Palli D, Panico S, Quiros JR, Rolandsson O, Roswall N, Sacerdote C, Sala N, Sanchez MJ, Schulze MB, Siddiq A, Slimani N, Sluijs I, Spijkerman AM, Tjonneland A, Tumino R, van der AD, Yaghootkar H, Group RS, Consortium EP-I, McCarthy MI, Semple RK, Riboli E, Walker M, Ingelsson E, Frayling TM, Savage DB, Langenberg C, Wareham NJ: Common genetic variants highlight the role of insulin resistance and body fat distribution in type 2 diabetes, independent of obesity. Diabetes 2014;63:4378-4387
32. Yang YK, Chen M, Clements RH, Abrams GA, Aprahamian CJ, Harmon CM: Human mesenteric adipose tissue plays unique role versus subcutaneous and omental fat in obesity related diabetes. Cell Physiol Biochem 2008;22:531-538
33. Kanaya AM, Harris T, Goodpaster BH, Tylavsky F, Cummings SR, Health A, Body Composition S: Adipocytokines attenuate the association between visceral adiposity and diabetes in older adults. Diabetes Care 2004;27:1375-1380
34. Tanaka S, Togashi K, Rankinen T, Perusse L, Leon AS, Rao DC, Skinner JS, Wilmore JH, Despres JP, Bouchard C: Sex differences in the relationships of abdominal fat to cardiovascular disease risk among normal-weight white subjects. Int J Obes Relat Metab Disord 2004;28:320-323
35. Nielsen S, Guo Z, Johnson CM, Hensrud DD, Jensen MD: Splanchnic lipolysis in human obesity. J Clin Invest 2004;113:1582-1588
36. Smith SR, Lovejoy JC, Greenway F, Ryan D, deJonge L, de la Bretonne J, Volafova J, Bray GA: Contributions of total body fat, abdominal subcutaneous adipose tissue compartments, and visceral adipose tissue to the metabolic complications of obesity. Metabolism 2001;50:425-435
37. Direk K, Cecelja M, Astle W, Chowienczyk P, Spector TD, Falchi M, Andrew T: The relationship between DXA-based and anthropometric measures of visceral fat and morbidity in women. BMC Cardiovasc Disord 2013;13:25
38. Snijder MB, Visser M, Dekker JM, Goodpaster BH, Harris TB, Kritchevsky SB, De Rekeneire N, Kanaya AM, Newman AB, Tylavsky FA, Seidell JC, Health ABCS: Low subcutaneous thigh fat is a risk factor for unfavourable glucose and lipid levels, independently of high abdominal fat. The Health ABC Study. Diabetologia 2005;48:301-308

39. Hill MJ, Metcalfe D, McTernan PG: Obesity and diabetes: lipids, 'nowhere to run to'. Clin Sci (Lond) 2009;116:113-123
40. Min KB, Min JY: Android and gynoid fat percentages and serum lipid levels in United States adults. Clin Endocrinol (Oxf) 2015;82:377-387
41. Bouchi R, Fukuda T, Takeuchi T, Nakano Y, Murakami M, Minami I, Izumiyama H, Hashimoto K, Yoshimoto T, Ogawa Y: Gender difference in the impact of gynoid and android fat masses on the progression of hepatic steatosis in Japanese patients with type 2 diabetes. BMC Obes 2017;4:27

ELECTRONIC SUPPLEMENT MATERIAL (ESM):

ESM Table 1. Correlation of metabolic indices and DXA measures with glycemic indices.

	Pearson`s Correlation Coefficient				
Sex		FG	FI	HOMA-IR	HOMA-B
Women	BMI	0.270*	0.491*	0.494*	0.287*
	WC	0.295*	0.501*	0.510*	0.271*
	1/HDL	0.210*	0.352*	0.358*	0.198*
	Triglycerides	0.291*	0.398*	0.424*	0.193*
	VAI	0.295*	0.437*	0.455*	0.212*
	LAP	0.341*	0.535*	0.551*	0.273*
	TyG	0.596*	0.483*	0.574*	0.057*
	Android fat mass %	0.201*	NA	NA	NA
	Gynoid fat mass %	-0.001	NA	NA	NA
	Android/Gynoid	0.239*	NA	NA	NA
	Total fat mass %	0.122*	NA	NA	NA
Men	BMI	0.223*	0.485*	0.484*	0.314*
	WC	0.237*	0.478*	0.483*	0.291*
	1/HDL	0.145*	0.319*	0.318*	0.218*
	Triglycerides	0.246*	0.381*	0.399*	0.204*
	VAI	0.240*	0.417*	0.431*	0.242*
	LAP	0.290*	0.506*	0.523*	0.287*
	TyG	0.575*	0.443*	0.547*	0.045*
	Android fat mass %	0.157*	NA	NA	NA
	Gynoid fat mass %	0.116*	NA	NA	NA
	Android/Gynoid	0.098*	NA	NA	NA
	Total fat mass %	0.150*	NA	NA	NA

*Correlation is significant at the 0.01 level (2-tailed).
NA-Not available (insulin was not measured in RSI-4 and RSII-2).

ESM Table 2. Associations between different lipid indices and incident type 2 diabetes mellitus in obese/non-obese subjects (N = 9564).

		Incident type 2 diabetes			
		Women		Men	
		BMI < 30 (N=309)	BMI ≥ 30 (N=167)	BMI<30 (N=278)	BMI ≥ 30 (N=79)
Index		HR(95%CI)	HR(95%CI)	HR(95%CI)	HR(95%CI)
BMI	Model 1	**1.79 (1.47, 2.16)**	**1.46 (1.22, 1.74)**	**1.78 (1.42, 2.24)**	**1.61 (1.13, 2.27)**
	Model 2	NA	NA	NA	NA
	Model 3	**1.72 (1.42, 2.09)**	**1.47 (1.23, 1.76)**	**1.74 (1.55, 1.96)**	**1.54 (1.09, 2.18)**
	Model 4	**1.49 (1.22, 1.82)**	**1.42 (1.18, 1.71)**	**1.50 (1.18, 1.91)**	**1.45 (1.01, 2.06)**
	Model 5	**1.45 (1.19, 1.77)**	**1.24 (1.02, 1.50)**	1.23 (0.96, 1.58)	1.09 (0.75, 1.60)
WC	Model 1	**1.76 (1.51, 2.05)**	**1.38 (1.15, 1.64)**	**1.65 (1.37, 1.98)**	1.66 (0.91, 1.49)
	Model 2	**1.56 (1.28, 1.89)**	1.18 (0.93, 1.48)	**1.38 (1.05, 1.83)**	0.95 (0.65, 1.39)
	Model 3	**1.50 (1.23, 1.83)**	1.18 (0.94, 1.49)	**1.35 (1.02, 1.79)**	0.91 (0.62, 1.34)
	Model 4	**1.35 (1.10, 1.66)**	1.06 (0.83, 1.36)	1.26 (0.94, 1.68)	0.84 (0.55, 1.27)
	Model 5	1.14 (0.93, 1.41)	0.87 (0.68, 1.13)	1.26 (0.93, 1.71)	0.79 (0.49, 1.29)
***1/HDL**	Model 1	**1.49 (1.34, 1.68)**	**1.57 (1.32, 1.87)**	**1.47 (1.28, 1.69)**	**1.52 (1.18, 1.96)**
	Model 2	**1.42 (1.26, 1.59)**	**1.51 (1.27, 1.81)**	**1.39 (1.21, 1.61)**	**1.49 (1.15, 1.92)**
	Model 3	**1.41 (1.25, 1.60)**	**1.51 (1.27, 1.81)**	**1.38 (1.19, 1.59)**	**1.46 (1.12, 1.90)**
	Model 4	**1.22 (1.05, 1.42)**	**1.38 (1.12, 1.71)**	**1.28 (1.09, 1.51)**	**1.40 (1.04, 1.89)**
	Model 5	**1.23 (1.05, 1.43)**	**1.40 (1.13, 1.74)**	**1.39 (1.17, 1.64)**	**1.49 (1.09, 2.06)**
***Triglycerides**	Model 1	**1.57 (1.39, 1.77)**	**1.39 (1.17, 1.65)**	**1.41 (1.25, 1.59)**	**1.26 (1.03, 1.54)**
	Model 2	**1.23 (1.05, 1.43)**	**1.40 (1.13, 1.74)**	**1.39 (1.17, 1.64)**	**1.49 (1.09, 2.06)**
	Model 3	**1.44 (1.28, 1.63)**	**1.36 (1.14, 1.62)**	**1.30 (1.15, 1.47)**	**1.23 (1.00, 1.51)**
	Model 4	**1.29 (1.12, 1.50)**	1.17 (0.95, 1.44)	1.14 (0.99, 1.32)	1.08 (0.86, 1.36)
	Model 5	1.10 (0.95, 1.28)	1.03 (0.84, 1.27)	0.98 (0.84, 1.13)	0.90 (0.71, 1.16)
***VAI**	Model 1	**1.62 (1.44, 1.82)**	**1.50 (1.27, 1.78)**	**1.48 (1.31, 1.69)**	**1.44 (1.14, 1.80)**
	Model 2	NA	NA	NA	NA
	Model 3	**1.57 (1.39, 1.77)**	**1.49 (1.26, 1.77)**	**1.45 (1.27, 1.64)**	**1.38 (1.09, 1.75)**
	Model 4	**1.57 (1.39, 1.77)**	**1.50 (1.27, 1.77)**	**1.45 (1.27, 1.64)**	**1.38 (1.09, 1.75)**
	Model 5	**1.35 (1.19, 1.52)**	**1.34 (1.13, 1.59)**	**1.29 (1.14, 1.48)**	1.20 (0.94, 1.54)
***LAP**	Model 1	**1.76 (1.54, 2.02)**	**1.66 (1.34, 2.07)**	**1.65 (1.42, 1.90)**	**1.49 (1.12, 1.98)**
	Model 2	**1.64 (1.41, 1.91)**	**1.51 (1.20, 1.91)**	**1.53 (1.29, 1.81)**	**1.39 (1.03, 1.88)**
	Model 3	**1.57 (1.35, 1.84)**	**1.49 (1.19, 1.89)**	**1.48 (1.24, 1.76)**	1.35 (0.99, 1.85)
	Model 4	**1.39 (1.17, 1.67)**	0.92 (0.75, 1.12)	**1.25 (1.03, 1.52)**	1.09 (0.77, 1.55)
	Model 5	1.11 (0.92, 1.33)	0.99 (0.75, 1.32)	1.04 (0.85, 1.27)	0.88 (0.61, 1.26)
TyG	Model 1	**2.02 (1.77, 2.29)**	**1.84 (1.53, 2.22)**	**1.72 (1.51, 1.96)**	**1.55 (1.24, 1.93)**
	Model 2	**1.91 (1.67, 2.18)**	**1.81 (1.49, 2.19)**	**1.62 (1.42, 1.86)**	**1.53 (1.23, 1.92)**
	Model 3	**1.84 (1.61, 2.10)**	**1.80 (1.49, 2.18)**	**1.59 (1.38, 1.83)**	**1.51 (1.20, 1.89)**
	Model 4	**1.79 (1.52, 2.10)**	**1.67 (1.32, 2.12)**	**1.47 (1.26, 1.72)**	**1.36 (1.05, 1.76)**
	Model 5	NA	NA	NA	NA

Model 1: Adjusted for age and cohort Model 2: Additionally adjusted for BMI Model 3: Additionally adjusted for SBP, treatment for hypertension, smoking, prevalent CVD Model 4: Additionally adjusted for HDL, triglycerides, serum lipid reducing agents Model 5: Additionally adjusted for fasting glucose; Hazard ratios are presented per 1 standard deviation increase in the marker. *Marker is naturally log-transformed.

ESM Table 3. Baseline characteristics of study participants with DXA measurements on body fat (N = 2962).

Characteristic	Women (1734) (557(6259)	Men (1228)	p-value
Age (years)	63.4 ± 5.9	63.2 ± 5.8	0.3
Systolic Blood pressure (mm/Hg)	148.8 ± 21.6	147.0 ± 20.3	**0.022**
Treatment for hypertension, n (%)	12.0 (0.7)	13.0 (1.1)	0.3
Prevalent cardiovascular disease, n (%)	142.0 (8.2)	251.0 (20.4)	< **0.001**
Serum lipid reducing agents use, n (%)	349.0 (20.1)	282.0 (23)	0.06
Current smokers, n (%)	229.0 (13.2)	164.0 (13.4)	< **0.001**
Total cholesterol (mmol/l)	5.9 ± 0.9	5.4 ± 0.9	< **0.001**
*Glucose (mmol/l)	5.4 (4.7 – 6.5)	5.4 (4.7 – 6.5)	**0.001**
BMI (kg/m^2)	27.4 ± 4.3	26.9 ± 3.2	**0.003**
Waist circumference (cm)	88.1 ± 10.7	98.2 ± 9.8	< **0.001**
*High density lipoprotein cholesterol (mmol/l)	1.5 (1.0 – 2.3)	1.3 (0.8 – 1.9)	< **0.001**
*Triglycerides (moml/l)	1.3 (0.7 – 2.7)	1.3 (0.7 – 2.9)	0.2
DXA measurements	Women (1734)	Men (1228)	
*Android fat %	3.3 (1.8 – 4.5)	3.1 (1.6 – 4.3)	< **0.001**
*Gynoid fat %	6.3 (4.5 – 8.1)	3.9 (2.6 – 5.3)	< **0.001**
*Android/Gynoid	0.5 (0.3 – 0.7)	0.7 (0.5 – 1.1)	< **0.001**
*Total fat mass %	39.2 (27.2 – 48.6)	27.5 (16.9 – 37.0)	< **0.001**

Values are presented as means ± standard deviation or * median (inter-quartile range). Abbreviations: BMI, body mass index. Triglycerides were not available in RSI-4 and RSII-2 and were taken from RSI-3 (the closest previous visit).

ESM Table 4. Associations between DXA measurements on body fat and incident type 2 diabetes in obese/non-obese subjects (N=2962).

	Incident type 2 diabetes			
	Women		Men	
	BMI < 30 (N=98)	BMI ≥ 30 (N=50)	BMI < 30 (N=85)	BMI ≥ 30 (N=20)
DXA measurements	HR(95%CI)	HR(95%CI)	HR(95%CI)	HR(95%CI)
*__Android fat mass %__				
Model 1	**1.80 (1.35, 2.41)**	1.38(0.78, 2.43)	**1.58 (1.19, 2.09)**	0.87 (0.36, 2.14)
Model 2	**1.54 (1.09, 2.19)**	1.04 (0.57, 1.89)	**1.39 (1.00, 1.94)**	1.09 (0.41, 2.93)
Model 3	**1.49 (1.05, 2.12)**	0.97 (0.53, 1.77)	**1.40 (1.01, 1.94)**	1.05 (0.62, 1.76)
Model 4	1.37 (0.95, 1.97)	0.77 (0.41, 1.45)	1.33 (1.12, 1.59)	0.93 (0.32, 2.69)
Model 5	1.34 (0.94, 1.89)	0.59 (0.31, 1.16)	1.32 (0.92, 1.87)	1.15 (0.36, 3.65)
*__Gynoid fat mass %__				
Model 1	0.82 (0.59, 1.13)	0.89 (0.45, 1.77)	1.23 (0.88, 1.70)	0.99 (0.41, 1.42)
Model 2	**0.50 (0.34, 0.74)**	0.61 (0.30, 1.25)	0.98 (0.68, 1.41)	1.25 (0.47, 3.29)
Model 3	**0.51 (0.35, 0.75)**	0.62 (0.31, 1.26)	0.98 (0.68, 1.41)	1.23 (0.46, 3.33)
Model 4	**0.57 (0.38, 0.85)**	0.75 (0.35, 1.61)	1.09 (0.74, 1.60)	1.04 (0.38, 2.87)
Model 5	0.78 (0.52, 1.18)	0.70 (0.33, 1.49)	1.05 (0.71, 1.55)	1.07 (0.38, 3.02)
*__Android/Gynoid__				
Model 1	**2.10 (1.59, 2.77)**	1.33 (0.82, 2.15)	**1.61 (1.17, 2.22)**	0.87 (0.34, 2.26)
Model 2	**1.93 (1.44, 2.59)**	1.27 (0.78, 2.07)	**1.46 (1.05, 2.05**)	0.87 (0.34, 2.26)
Model 3	**1.89 (1.41, 2.55)**	1.24 (0.75, 2.02)	**1.46 (1.05, 2.06)**	0.85 (0.51, 1.41)
Model 4	**1.74 (1.27, 2.39)**	0.96 (0.56, 1.65)	1.26 (0.88, 1.80)	0.90 (0.33, 2.51)
Model 5	**1.47 (1.07, 2.02)**	0.85 (0.48, 1.51)	1.32 (0.90, 1.92)	1.05 (0.35, 3.16)
*__Total fat mass %__				
Model 1	1.39 (0.96, 1.99)	0.87 (0.33, 2.31)	**1.56 (1.14, 2.14)**	1.17 (0.42, 3.23)
Model 2	0.70 (0.43, 1.13)	0.34 (0.11, 1.00)	1.30 (0.89, 1.91)	1.71 (0.54, 5.42)
Model 3	0.71 (0.45, 1.13)	**0.35 (0.12, 0.97)**	1.31 (0.89, 1.92)	1.66 (0.55, 5.05)
Model 4	0.75 (0.46, 1.23)	0.37 (0.13, 1.09)	1.39 (0.92, 2.09)	1.34 (0.40, 4.46)
Model 5	1.03 (0.62, 1.69)	0.38 (0.21, 0.67)	1.37 (0.91, 2.06)	1.64 (0.46, 5.86)

Model 1: Adjusted for age and cohort Model 2: Additionally adjusted for BMI Model 3: Additionally adjusted for SBP, treatment for hypertension, smoking, prevalent CVD Model 4: Additionally adjusted for HDL, triglycerides, serum lipid reducing agents Model 5: Additionally adjusted for fasting glucose; Hazard ratios are presented per 1 standard deviation increase in the marker.
*Marker is naturally log-transformed.

Chapter 3.3

Epicardial fat volume and the risk for cardiovascular disease and incident type 2 diabetes: the Rotterdam Study

Adela Brahimaj
Daniel Bos
Meike W. Vernooij
M. Arfan Ikram
Abbas Dehghan
Oscar H. Franco
Maryam Kavousi

ABSTRACT

Background

Epicardial fat volume (EFV) may be a marker of unfavorable cardio metabolic risk profile.
Objectives: We aimed to investigate the associations of EFV with incident type 2 diabetes (T2D), coronary heart disseat (CHD) and stroke in the large prospective population-based Rotterdam Study.

Methods

Among 2318 subjects, we used Cox proportional hazard models to investigate associations between EFV and incident T2D, CHD, and stroke. Associations were adjusted for traditional cardiometabolic risk factors.

Results

We identified 160, 91, and 109 incident T2D, CHD, and stroke cases respectively. In age and sex adjusted model, EFV was positively associated with T2D (per 1 SD naturally log-transformed, HR = 1.58; 95% CI: 1.33, 1.89) and CHD (1.26; 1.01, 1.58). In the multivariable adjusted model, the association remained significant only between the third tertile of EFV and T2D (2.70; 1.12, 6.49). EFV was associated with a high risk for stroke in multivariable adjusted model (per 1 SD naturally log-transformed, HR = 1.58; 1.02, 2.45).

Conclusions

Increased amount of EFV may be associated with excessive risk for T2D, CHD and stroke in total population independent of traditional cardiometabolic risk factors.

INTRODUCTION

Epicardial fat (EF), the visceral fat portion within the pericardial sac around the heart, located between the visceral pericardium and the outer wall of the myocardium (1), has been recently associated with cardio-metabolic risk (2-6). The contribution of EF to unfavorable cardio-metabolic risk profile may be due to its endocrine and paracrine activities as well as its close proximity to the heart and coronary vasculature (7).
We have previously shown that larger volumes of EF are associated with larger amounts of coronary and extracranial carotid artery atherosclerosis in men (3). Furthermore, we reported an association of EF with atrial fibrillation (AF) in individuals free of cardiovascular disease (CVD) that was independent of coronary atherosclerosis (4). EF has been associated with fatal and nonfatal coronary events in the general population (2) and with cerebral ischemic stroke in a cross-sectional setting (8). Moreover, EF was found to be a significant marker of increased insulin resistance (9; 10). Recent findings indicated that increased EF thickness is independently associated with prevalence of diabetes in Korean men (11). However, to date large-population–based longitudinal data on the prognostic value of EF for incident stroke and incident type 2 diabetes events are lacking.
Therefore, we studied the associations of epicardial fat volume with incident events of type 2 diabetes, coronary heart disease, as well as stroke, among women and men from the large prospective population-based Rotterdam Study.

RESEARCH DESIGN AND METHODS

Study population

The study was performed in the framework of the Rotterdam Study (RS), a prospective population-based cohort study in Ommoord, a district of Rotterdam, the Netherlands. The design of RS has been described in more details elsewhere (12). The original study (RSI) started in 1989 when all residents within the well-defined study area aged 55 years or older were invited to participate of whom 78% (7983 out of 10275) agreed. There were no other eligibility criteria to enter the RS except minimum age and residential area based on postal code. The first examination of the original cohort (RSI-1) took place from 1990 to 1993. The cohort has been extended twice (RSII and RSIII) to include the participants who were 45 years or older or moved to the study research area. For all 3 cohorts of RS, follow-up examinations were conducted every 3-5 years. For the current study, we used data from the fourth visit of the original cohort (RSI-4) and second visit of the extended cohort (RS-II-2) for whom the EF data was available. From 2524 subjects with available EF data, 154 were excluded due to image artefacts or segmentation errors. We further excluded 52 subjects because of missing diabetes data. Hence, the current study population consists of 2318 participants with complete data for EF volume and for outcomes of interest including diabetes, coronary heart disease (CHD), and stroke.
The RS 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 Netherlands. All participants provided written informed consent to participate in the study and to obtain information from their treating physicians.

Assessment of epicardial fat

From 2003 until 2006, all participants who completed a regular visit at the research centre were invited to undergo non-enhanced 16-slice (n ¼ 785) or 64-slice (n ¼ 1739) multidetector computed tomography (MDCT) scan of the coronary arteries, aortic arch, extracranial carotid arteries, and intracranial carotid arteries (Somatom Sensation, Siemens, Forchheim, Germany). Using a cardiac scan and a scan that reached from the aortic arch to the intracranial vasculature (1 cm above the sella turcica). Scans with image artefacts or segmentation errors were excluded. Detailed information regarding imaging parameters of both scans is provided elsewhere (13).

We used a fully automatic tool for quantification of epicardial fat in milliliters (14) . This quantification method consisted of two steps: (i) whole heart segmentation and (ii) epicardial fat volume quantification. For whole heart segmentation, we used a multi-atlas-based segmentation approach. In this approach, eight manually segmented contrast-enhanced cardiac scans (atlases) were registered (spatially aligned) with every participant's CT scan. The atlas scans and subject's scan were initially aligned using an affine transformation, which was followed by a non-rigid registration. After registration, the segmentations of the atlases were mapped onto the subject's scan to obtain the whole heart segmentation. All registrations were performed using Elastix (15), a publicly available software package. Next, we used the obtained whole heart segmentation as a region of interest and a threshold window of 230 to 2200 Hounsfield Units for the quantification of the amount of fat (16). A connected-component analysis was applied to all adipose tissue voxels using an 18-neighbourhood rule, to remove regions smaller than 10 voxels (2.8 mm3) in size, which we considered to be noise. This fully automatic method was validated by an expert reviewer panel and proved to be as good as manual segmentation (14).

Dedicated commercially available software (Syngo CalciumScoring, Siemens, Germany) was used to automatically quantify atherosclerotic calcification in the coronary arteries, the aortic arch, and the extracranial internal carotid arteries (13). Calcification in the intracranial internal carotid arteries was quantified using a semi-automated method (3). Calcification volumes were expressed in cubic millimetres.

Incident Type 2 diabetes mellitus

Participants were followed from the date of baseline center visit onwards. Cases of type 2 diabetes were ascertained through active follow-up using general practitioners' records, hospital discharge letters and glucose measurements from RS visits which take place approximately every 4 years. According to the current WHO guidelines, type 2 diabetes was defined as a fasting blood glucose ≥ 7.0 mmol/L, a non-fasting blood glucose ≥ 11.1 mmol/L (when fasting samples were unavailable), or the use of blood glucose lowering medication (17). Information regarding the use of blood glucose lowering medication was derived from both structured home interviews and linkage to pharmacy dispensing records. At baseline, more than 95% of the RS 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 an endocrinologist. Follow-up data was complete until January 1st 2012.

Incident CHD and stroke

Coronary heart disease was defined as a compound outcome including fatal or nonfatal myocardial infarction or cardiovascular mortality (18). Ischemic strokes were diagnosed when a patient had typical symptoms and a CT or MRI within four weeks that ruled out other diagnoses or when indirect evidence (deficit limited to 1 limb or completely resolved within 72 h, atrial fibrillation in the absence of anticoagulants) pointed at an ischemic nature of the stroke. Hemorrhagic stroke was diagnosed when a relevant hemorrhage was shown on CT or MRI. If a stroke did not match any of these criteria, it was classified as unspecified (19).

Covariates

Height and weight were measured with the participants standing without shoes and heavy outer garments. Body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared. 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. Blood pressure was measured twice at the right brachial artery with a random-zero sphygmomanometer with the participant in sitting position, and the mean of 2 consecutive measurements was used. Insulin, glucose, high-density lipoprotein (HDL) cholesterol were measured on the COBAS 8000 Modular Analyzer (Roche Diagnostics GmbH). The corresponding interassay coefficients of variations are the following: insulin < 8%, glucose < 1.4% and lipids < 2.1%. Information on medication use and tobacco smoking behaviour was collected by trained research assistants via computerized questionnaires during home visits. Smoking was classified as current versus non-current smokers. History of cardiovascular disease (CVD) was defined as a history of coronary heart diseases (myocardial infarction, revascularization, coronary artery bypass graft surgery or percutaneous coronary intervention) or stroke and was verified from the medical records of the general practitioner. A diagnosis of Atrial fibrillation (AF) was only accepted when it was supported by a ECG diagnosis.The ECGs done at baseline and during follow-up were stored digitally, and analyzed by the modular ECG analysis system (MEANS), which has high specificity (99.5%) and high sensibility (96.6%) for detection of arrhythmias (20).

Statistical analysis

Considering gender differences in EF, all analysis were performed in total population as well as among women and men separately. Descriptives were presented as mean ± standard deviation (SD) for continuous variables and numbers (percentages) for dichotomous variables. Markers with a right-skewed distribution were transformed to the natural logarithmic scale.

We used Cox proportional hazard models to investigate associations of EF volume with incident events of stroke, CHD, CVD as well as type 2 diabetes. According to the outcome of interest, were excluded prevalent diabetes cases, prevalent revascularization and myocardial infarction cases or prevalent stroke cases. The proportional hazard assumption of the Cox model was checked by 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). The first model was adjusted for age and

sex. In the second model, we additionally adjusted for BMI, systolic blood pressure, medication for hypertension, smoking, lipid lowering medication, total and HDL cholesterol, According to the outcome of interest, we additionally adjusted for parental history of DM, prevalent CVD, prevalent diabetes or prevalent atrial fibrillation. In the third model, we added fasting glucose for the models with type 2 diabetes as an outcome and coronary artery calcification (CAC) for cardiovascular outcomes. As glucose is a mean for diagnosis of type 2 diabetes, this model should be considered a conservative model.
Since AF is a major risk factor for ischemic stroke, we performed sensitivity analyses removing prevalent AF cases and AF cases preceding a stroke event as well as competing risk analysis for stroke and AF.
All analyses were conducted in SPSS software version 21 (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp). A p-value lower than 0.05 was considered statistically significant.

RESULTS

Baseline characteristics of the study population are shown for the total of 2318 subjects in Table 1. The mean (SD) age at baseline was 68.9 (6.6) years and 47.7 % of the population were men. The mean (SD) BMI was 27.7 (3.9) kg/m2 and 23.7 % of the study population used lipid lowering medication.
Baseline characteristics among 1212 women and 1106 men are presented in ESM Table 1. Women had higher BMI, total cholesterol and HDL cholesterol than men, whereas men had higher waist circumference, fasting glucose, prevalence of cardiovascular disease and prevalence of diabetes than women.
The correlation coefficients for epicardial fat volume in relation to BMI, waist circumference, total cholesterol, HDL cholesterol, triglycerides, CRP, fasting glucose, extracranial and intracranial carotid artery calcification are shown in ESM Table 2.

Epicardial fat volume and the risk for type 2 diabetes

Among 1989 subjects free of diabetes at baseline, 160 incident diabetes cases were identified during a median follow-up of 7 years (12 cases per 1000 person-years). In age and sex adjusted model, EF volume was positively associated with the risk for type 2 diabetes (per 1 SD naturally log-transformed, HR = 1.58; 95% CI: 1.33, 1.89). In multivariate adjusted model, the association remained significant only between the third tertile of epicardial fat volume and the risk for type 2 diabetes (HR = 2.70; 95% CI: 1.12, 6.49) (Table 2).
In the gender specific analysis, the epicardial fat volume was associated with the risk for type 2 diabetes only in age adjusted model in women (per 1 SD naturally log-transformed, HR = 2.14; 95% CI: 1.63, 2.81), but no association was found between the epicardial fat volume and the risk for type 2 diabetes among men (ESM Table 3).

Table 1. Baseline characteristics of the study participants.

Characteristics	Total (n= 2318)
Age, y	68.9 ± 6.6
Men, n (%)	1106 (47.7)
Parental history of diabetes, n (%)	227 (9.8)
Waist circumference, cm	94.3 ± 11.6
Body mass index, kg/m^2	27.7 ± 3.9
Systolic blood pressure, mmHg	164.9 ± 22.9
Diastolic blood pressure, mmHg	98.6 ± 14.7
Hypertension medication, n (%)	241 (10.4)
Total cholesterol, mmol/L	5.6 ± 0.9
*HDL cholesterol, mmol/L	1.4 (1.2-1.7)
Current smokers, n (%)	298 (12.9)
Prevalent cardiovascular disease, n (%)	249 (10.7)
Prevalent atrial fibrillation, n (%)	59 (2.5)
Prevalent diabetes, n (%)	329 (14.2)
* Coronary artery calcification	69.3 (2.6-354.4)
Lipid lowering medication, n (%)	549 (23.7)
*Fasting glucose, mmol/L	5.4 (5.1-5.9)
Epicardial fat volume, mm^3	101.9 (80.5-130.7)
Extracranial carotid artery calcification, mm^3	22.0 (0.0-114.9)
Intracranial carotid artery calcification, mm^3	40.8 (6.2-136.2)

Plus-minus values are means ± SD or
* median (inter-quartile range)

Epicardial fat volume and the risk for CHD

Among 2143 subjects free of prevalent CHD at baseline, 91 incident CHD cases were identified during a median follow-up of 6 years (7 cases per 1000 person-years). EF volume was associated with a high risk for CHD in the age and sex adjusted model (per 1 SD naturally log-transformed, HR = 1.26; 95% CI: 1.01, 1.58). The association could not survive further adjustment (Table 2). No association was observed between epicardial fat volume and the risk for CHD in the gender specific analysis (ESM Tale 4).

Epicardial fat volume and the risk for stroke

Among 2223 subjects free of stroke at baseline, 109 incident stroke cases were identified during a median follow-up of 9 years (5 cases per 1000 person-years). In multivariate adjusted model, epicardial fat volume was associated with a high risk for stroke (per 1 SD naturally log-transformed, HR = 1.58; 95% CI: 1.02, 2.45) in total population (Table 2), but not among women and men separately (ESM Tale 5). Removing prevalent AF cases and AF cases preceding a stroke event as well as competing risk analysis for stroke and AF did not change the results.

Table 2. Epicardial fat volume and the risk for incident type 2 diabetes, coronary heart disease and stroke.

	Epicardial fat volume					
	Tertile 1	Tertile 2	Tertile 3	*Continuous*	*P-value*	*P-trend*
Type 2 diabetes						
Cases	34	56	70			
Model 1, HR, 95% CI	1	**1.82 (1.19, 2.81)**	**2.55 (1.68, 3.87)**	1.58 (1.33, 1.89)	**4.2×10^{-7}**	**9.0×10^{-6}**
[d] Model 2, HR, 95% CI	1	1.65 (0.69, 3.89)	**2.70 (1.12, 6.49)**	1.46 (0.98, 2.17)	0.6×10^{-1}	2.0×10^{-2}
[d] Model 3, HR, 95% CI	1	1.37 (0.56, 3.32)	2.21 (0.89, 5.46)	1.26 (0.83, 1.90)	0.2	6.3×10^{-2}
CHD						
Cases	23	32	36			
Model 1, HR, 95% CI	1	1.49 (0.87, 2.55)	1.57 (0.93, 2.66)	1.26 (1.01, 1.58)	**0.045**	7.9×10^{-2}
[c] Model 2, HR, 95% CI	1	1.43 (0.51, 4.01)	1.36 (0.46, 3.99)	1.11 (0.68, 1.83)	0.6	3.5×10^{-1}
[c] Model 3, HR, 95% CI	1	1.32 (0.47, 3.68)	1.19 (0.39, 3.59)	1.03 (0.62, 1.72)	0.8	4.6×10^{-1}
Stroke						
Cases	32	37	40			
Model 1, HR, 95% CI	1	1.12 (0.70, 1.80)	1.16 (0.73, 1.85)	1.07 (0.87, 1.32)	0.5	5.3×10^{-1}
[s] Model 2, HR, 95% CI	1	1.98 (0.76, 5.14)	2.24 (0.83, 6.02)	1.58 (1.02, 2.45)	**0.04**	1.3×10^{-1}
[s] Model 3, HR, 95% CI	1	1.95 (0.75, 5.08)	2.22 (0.82, 5.98)	1.59 (1.08, 2.35)	**0.019**	1.4×10^{-1}

Model 1: age and sex adjusted
Model 2: + body mass index, systolic blood pressure, medication for hypertension, smoking, [c]prevalent diabetes or [d]parental history of diabetes + prevalent cardiovascular disease, lipid lowering medication, total and high-density lipoprotein cholesterol, [s]prevalent atrial fibrillation
Model 3: + [c,s]coronary artery calcification, [s]extra-cranial carotid artery calcification or [d]fasting glucose
HR: hazard ratio; CI: confidence interval

DISCUSSION

In this prospective population-based cohort study, we investigated the associations of epicardial fat volume with incident type 2 diabetes, CHD and stroke in total population and among women and men. Epicardial fat volume was positively associated with the risk for type 2 diabetes in total population and among women and with the risk for CHD in total population in age and sex adjusted model. After further adjustment for relevant cardiometabolic risk factors, epicardial fat volume remained positively associated only with the risk for stroke in total population.

To date, large population–based longitudinal data on the value of epicardial fat for assessment of type 2 diabetes risk are lacking. In the current study, we suggest a graded association of EF volume with incident type 2 diabetes in total population and among women, which becomes statistically non-significant after further adjustment for cardiometabolic risk factors. Increased epicardial fat thickness, but not abdominal fat or total body fat, was found to be independently associated with prevalence of diabetes in Korean men (11). Among women with gestational diabetes mellitus, the echocardiographically measured epicardial fat tissue has also shown to be significantly higher (21). Human epicardial adipose tissue, as the portion of visceral fat located between the heart and the pericardium, surrounding the heart and the coronary arteries (22), originates from brown fat and is a source of inflammatory mediators (7). Although it accounts for only 1% of total body fat, EF has been shown to have close correlation with insulin resistance (23; 24). The association between EF volume and the risk for diabetes in our study diminished

in particular when HDL cholesterol or the ratio of total to HDL cholesterol was additionally adjusted for in our multivariate adjusted model. When investigating the correlation of EF volume with lipids, we observed a negative correlation coefficient with both HDL cholesterol, which was expected, and total cholesterol, which was not expected. This might suggest that EF volume may indirectly play a role in the development of type 2 diabetes through lowering HDL cholesterol.

We have previously reported that larger volumes of epicardial fat are associated with larger amounts of coronary and extracranial carotid artery atherosclerosis in men, independent of traditional cardiovascular risk factors (3). In the current longitudinal study, we suggest an association between EFV with CHD risk in total population. Our study confirms a previous report regarding the independent association of EF with coronary events in the general population and complements information from cardiac computed tomography above the CAC score (2). Similar to our analysis with diabetes, the association between EF volume and CHD risk in our study diminished in particular after taking into account HDL cholesterol in the analysis. Considering the importance of HDL cholesterol in the development of both CHD and diabetes, this observation further supports the possibility that the role of EF in development of unfavorable metabolic profiles might be mediated through its effect on lowering HDL cholesterol.

In the current study, we report a novel association between epicardial fat volume with incident stroke. We have also recently reported an association between epicardial fat with atrial fibrillation in individuals free of CVD that was independent of traditional cardiovascular risk factors, coronary atherosclerosis, left atrial size, and various measures of adiposity (4). Considering that atrial fibrillation is a major risk for ischemic stroke, we performed sensitivity analyses removing prevalent AF cases and AF cases preceding a stroke event as well as competing risk analysis for stroke and atrial fibrillation. These results were similar to our main analysis, suggesting that the association of EF volume with stroke risk is independent of AF (4). Also, as carotid artery atherosclerosis is an independent predictor of stroke risk (25), we additionally adjusted the association of EF with stroke for extracranial and intracranial carotid artery calcification. Our results suggest that larger volumes of EF increase the risk for incident stroke events independent of carotid artery atherosclerosis.

This study has certain strengths and limitations. To our knowledge, this is the first prospective population-based cohort study to investigate the association between EF volume and incident type 2 diabetes, CHD and stroke during a long-term follow-up. We used data from a well-characterized prospective cohort study, which allowed us to correct for a wide range of covariates. The image-based quantification of the EF volume is another strength of our study. Although the majority of previous studies performed ultrasound measurements of EF, CT-based measurements are superior for accurate detection and quantification of EF volume (2; 26). Moreover, we were the first to develop and apply a fully automatic method to quantify EF volume on non-enhanced CT-scans (27). However, our population is 55 years and older and mainly comprised of individuals of European ancestry. Thus, generalization of our results to younger age groups and other ethnicities should be done with caution. The definition of EF which is used in the literature is a matter of debate (2; 28-30). Especially, pericardial fat and epicardial fat are interchangeably used, which should be taken into account in comparison of results between different studies. In our study, we applied the definition as proposed by Iacobellis et al. (1).

In conclusion, increased amount of epicardial fat volume may be associated with excessive risk for type 2 diabetes, CHD and stroke in total population independent of traditional cardiometabolic risk factors.

AUTHOR CONTRIBUTION

AB ran the analysis and wrote the manuscript. DB contributed to the analysis. OHF designed the study and provided resources. MK and AD designed the study and critically revised the manuscript. All authors have read and approved the manuscript. AB is the guarantor of this work.

Acknowledgments

The dedication, commitment, and contribution of inhabitants, general practitioners, and pharmacists of the Ommoord district to the Rotterdam Study are gratefully acknowledged. We thank Layal Chaker, Jolande Verkroost-van Heemst, and Ke-Xin Wen of Erasmus MC for their invaluable contribution to the collection of the diabetes data.

Funding

The Rotterdam Study is supported by the Erasmus MC and Erasmus University Rotterdam; the Netherlands Organization for Scientific Research (NWO); the Netherlands Organisation for Health Research and Development (ZonMw); the Research Institute for Diseases in the Elderly (RIDE); the Netherlands Genomics Initiative (NGI); the Ministry of Education, Culture and Science, the Ministry of Health, Welfare and Sports; the European Commission (DG XII); and the Municipality of Rotterdam.
None of the funders had any role in design and conduct of the study, collection, management, analysis, and interpretation of the data, and preparation, review, or approval of the manuscript. MK is supported by the NWO VENI grant (VENI, 91616079).

Disclosures

OHF works in ErasmusAGE, a center for aging research across the life course funded by Nestlé Nutrition (Nestec Ltd.); Metagenics Inc.; and AXA. The other authors report no potential conflicts of interest.

REFERENCES

1. Iacobellis G: Epicardial and pericardial fat: close, but very different. Obesity (Silver Spring) 2009;17:625; author reply 626-627
2. Mahabadi AA, Berg MH, Lehmann N, Kalsch H, Bauer M, Kara K, Dragano N, Moebus S, Jockel KH, Erbel R, Mohlenkamp S: Association of epicardial fat with cardiovascular risk factors and incident myocardial infarction in the general population: the Heinz Nixdorf Recall Study. J Am Coll Cardiol 2013;61:1388-1395
3. Bos D, Shahzad R, van Walsum T, van Vliet LJ, Franco OH, Hofman A, Niessen WJ, Vernooij MW, van der Lugt A: Epicardial fat volume is related to atherosclerotic calcification in multiple vessel beds. Eur Heart J Cardiovasc Imaging 2015;16:1264-1269
4. Bos D, Vernooij MW, Shahzad R, Kavousi M, Hofman A, van Walsum T, Deckers JW, Ikram MA, Heeringa J, Franco OH, van der Lugt A, Leening MJ: Epicardial Fat Volume and the Risk of Atrial Fibrillation in the General Population Free of Cardiovascular Disease. JACC Cardiovasc Imaging 2017;
5. Mookadam F, Goel R, Alharthi MS, Jiamsripong P, Cha S: Epicardial fat and its association with cardiovascular risk: a cross-sectional observational study. Heart Views 2010;11:103-108
6. Yorgun H, Canpolat U, Hazirolan T, Ates AH, Sunman H, Dural M, Sahiner L, Kaya EB, Aytemir K, Tokgozoglu L, Kabakci G, Oto A: Increased epicardial fat tissue is a marker of metabolic syndrome in adult patients. Int J Cardiol 2013;165:308-313
7. Mazurek T, Zhang L, Zalewski A, Mannion JD, Diehl JT, Arafat H, Sarov-Blat L, O'Brien S, Keiper EA, Johnson AG, Martin J, Goldstein BJ, Shi Y: Human epicardial adipose tissue is a source of inflammatory mediators. Circulation 2003;108:2460-2466
8. Akil E, Akil MA, Varol S, Ozdemir HH, Yucel Y, Arslan D, Akyuz A, Alan S: Echocardiographic epicardial fat thickness and neutrophil to lymphocyte ratio are novel inflammatory predictors of cerebral ischemic stroke. J Stroke Cerebrovasc Dis 2014;23:2328-2334
9. Manco M, Morandi A, Marigliano M, Rigotti F, Manfredi R, Maffeis C: Epicardial fat, abdominal adiposity and insulin resistance in obese pre-pubertal and early pubertal children. Atherosclerosis 2013;226:490-495
10. Iacobellis G, Leonetti F: Epicardial adipose tissue and insulin resistance in obese subjects. J Clin Endocrinol Metab 2005;90:6300-6302
11. Chun H, Suh E, Byun AR, Park HR, Shim KW: Epicardial fat thickness is associated to type 2 diabetes mellitus in Korean men: a cross-sectional study. Cardiovasc Diabetol 2015;14:46
12. Hofman A, Brusselle GG, Darwish Murad S, van Duijn CM, Franco OH, Goedegebure A, Ikram MA, Klaver CC, Nijsten TE, Peeters RP, Stricker BH, Tiemeier HW, Uitterlinden AG, Vernooij MW: The Rotterdam Study: 2016 objectives and design update. Eur J Epidemiol 2015;30:661-708
13. Odink AE, van der Lugt A, Hofman A, Hunink MG, Breteler MM, Krestin GP, Witteman JC: Association between calcification in the coronary arteries, aortic arch and carotid arteries: the Rotterdam study. Atherosclerosis 2007;193:408-413
14. Shahzad R, Bos D, Metz C, Rossi A, Kirisli H, van der Lugt A, Klein S, Witteman J, de Feyter P, Niessen W, van Vliet L, van Walsum T: Automatic quantification of epicardial fat volume on non-enhanced cardiac CT scans using a multi-atlas segmentation approach. Med Phys 2013;40:091910
15. Klein S, Staring M, Murphy K, Viergever MA, Pluim JP: elastix: a toolbox for intensity-based medical image registration. IEEE Trans Med Imaging 2010;29:196-205
16. Yoshizumi T, Nakamura T, Yamane M, Islam AH, Menju M, Yamasaki K, Arai T, Kotani K, Funahashi T, Yamashita S, Matsuzawa Y: Abdominal fat: standardized technique for measurement at CT. Radiology 1999;211:283-286
17. Geneva WH: Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia: Report of a WHO/IDF Consultation. 2006:p. 1–50
18. Leening MJ, Kavousi M, Heeringa J, van Rooij FJ, Verkroost-van Heemst J, Deckers JW, Mattace-Raso FU, Ziere G, Hofman A, Stricker BH, Witteman JC: Methods of data collection and definitions of cardiac outcomes in the Rotterdam Study. Eur J Epidemiol 2012;27:173-185
19. Alberts VP, Bos MJ, Koudstaal P, Hofman A, Witteman JC, Stricker B, Breteler M: Heart failure and the risk of stroke: the Rotterdam Study. Eur J Epidemiol 2010;25:807-812
20. Vitezova A, Cartolano NS, Heeringa J, Zillikens MC, Hofman A, Franco OH, Kiefte-de Jong JC: Vitamin D and the risk of atrial fibrillation--the Rotterdam Study. PLoS One 2015;10:e0125161
21. Nar G, Inci S, Aksan G, Unal OK, Nar R, Soylu K: The relationship between epicardial fat thickness and gestational diabetes mellitus. Diabetol Metab Syndr 2014;6:120

22. Bertaso AG, Bertol D, Duncan BB, Foppa M: Epicardial fat: definition, measurements and systematic review of main outcomes. Arq Bras Cardiol 2013;101:e18-28
23. Wajchenberg BL, Giannella-Neto D, da Silva ME, Santos RF: Depot-specific hormonal characteristics of subcutaneous and visceral adipose tissue and their relation to the metabolic syndrome. Horm Metab Res 2002;34:616-621
24. Perseghin G, Lattuada G, De Cobelli F, Esposito A, Belloni E, Ntali G, Ragogna F, Canu T, Scifo P, Del Maschio A, Luzi L: Increased mediastinal fat and impaired left ventricular energy metabolism in young men with newly found fatty liver. Hepatology 2008;47:51-58
25. Nandalur KR, Baskurt E, Hagspiel KD, Finch M, Phillips CD, Bollampally SR, Kramer CM: Carotid artery calcification on CT may independently predict stroke risk. AJR Am J Roentgenol 2006;186:547-552
26. Nichols JH, Samy B, Nasir K, Fox CS, Schulze PC, Bamberg F, Hoffmann U: Volumetric measurement of pericardial adipose tissue from contrast-enhanced coronary computed tomography angiography: a reproducibility study. J Cardiovasc Comput Tomogr 2008;2:288-295
27. Mathur KS, Kashyap SK, Kumar V: Correlation of the extent and severity of atherosclerosis in the coronary and cerebral arteries. Circulation 1963;27:929-934
28. Dey D, Wong ND, Tamarappoo B, Nakazato R, Gransar H, Cheng VY, Ramesh A, Kakadiaris I, Germano G, Slomka PJ, Berman DS: Computer-aided non-contrast CT-based quantification of pericardial and thoracic fat and their associations with coronary calcium and Metabolic Syndrome. Atherosclerosis 2010;209:136-141
29. Rosito GA, Massaro JM, Hoffmann U, Ruberg FL, Mahabadi AA, Vasan RS, O'Donnell CJ, Fox CS: Pericardial fat, visceral abdominal fat, cardiovascular disease risk factors, and vascular calcification in a community-based sample: the Framingham Heart Study. Circulation 2008;117:605-613
30. Ding J, Kritchevsky SB, Harris TB, Burke GL, Detrano RC, Szklo M, Jeffrey Carr J, Multi-Ethnic Study of A: The association of pericardial fat with calcified coronary plaque. Obesity (Silver Spring) 2008;16:1914-1919

ESM Table 1. Baseline characteristics among women and men in the study.

Characteristics	Women (n_w=1212)	Men (n_m=1106)	p-value
Age, y	68.9 ± 6.7	69.1 ± 6.5	0.5
Parental history of diabetes, n (%)	120 (9.9)	107 (9.7)	0.4
Waist circumference, cm	89.7 ± 10.9	99.4 ± 10.0	**< 0.001**
Body mass index, kg/m^2	27.9 ± 4.4	27.5 ± 3.4	**0.014**
Systolic blood pressure, mmHg	165.6 ± 23.2	164.3 ± 22.7	0.1
Diastolic blood pressure, mmHg	97.5 ± 14.6	99.9 ± 14.8	**< 0.001**
Hypertension medication, n (%)	126 (10.4)	115 (10.4)	0.1
Total cholesterol, mmol/L	5.9 ± 0.9	5.4 ± 0.9	**< 0.001**
*HDL cholesterol, mmol/L	1.5 (1.3 – 1.8)	1.3 (1.1 – 1.5)	**< 0.001**
Current smokers, n (%)	162 (13.4)	136 (12.3)	**< 0.001**
Prevalent cardiovascular disease, n (%)	79 (6.5)	170 (15.4)	**< 0.001**
Prevalent atrial fibrillation, n (%)	19 (1.6)	40 (3.6)	**0.002**
Prevalent diabetes, n (%)	155 (12.8)	174 (15.7)	**0.04**
*CAC	22.5 (0.2 – 160.5)	161.8 (22.5 – 595.1)	**< 0.001**
Lipid lowering medication, n (%)	290 (23.9)	259 (23.4)	0.8
*Fasting glucose, mmol/L	5.3 (5.0 – 5.8)	5.5 (5.1 – 6.0)	**0.001**
Epicardial fat volume, mm^3	90.1 (71.8- 110.2)	121.1 (95.9-152.3)	**< 0.001**
Extracranial carotid artery calcification, mm^3	13.5 (0.0-77.7)	40.0 (1.4-154.4)	**< 0.001**
Intracranial carotid artery calcification, mm^3	35.3 (5.3-117.8)	47.7 (8.8-164.6)	**< 0.001**

Plus-minus values are means ± SD or

* median (inter-quartile range).

CAC: coronary artery calcification

ESM Table 2. Correlation coefficients of epicardial fat volume with other covariates.

Pearson`s Correlation Coefficient										
	BMI	WC	Total cholesterol	HDL	TG	CRP	FG	CAC	ECAC	ICAC
EFV	0.463**	0.702**	-0.150**	-0.347**	0.248**	0.193**	0.222**	0.298**	0.217**	0.130**

**Correlation is significant at the 0.01 level (2-tailed).

Abbreviations: BMI, body mass index; WC, waist circumference; HDL, high density lipoproteins cholesterol; TG, triglycerides; CRP, chain reaction protein; FG, fasting glucose; CAC, coronary artery calcification; ECAC, extracranial carotid artery calcification; ICAC, intracranial carotid artery calcification.

TG and CRP are taken as a proxy from the closest visits of the Rotterdam Study with the visit in which epicardial fat volume was available and this study was performed.

ESM Table 3. Epicardial fat and the risk for type 2 diabetes in women and men.

	EFV				
	Tertile 1	Tertile 2	Tertile 3	*Continuous*	*P-value*
Women					
Cases	10	29	40		
Model 1, HR, 95% CI	1	**3.21 (1.56, 6.59)**	**4.92 (2.45, 9.89)**	2.14 (1.63, 2.81)	**3.7×10^{-8}**
Model 2, HR, 95% CI	1	1.91 (0.40, 9.07)	3.26 (0.66, 16.09)	1.65 (0.79, 3.43)	0.1
Model 3, HR, 95% CI	1	1.78 (0.37, 8.67)	3.11 (0.55, 17.53)	1.34 (0.62, 2.89)	0.4
Men					
Cases	24	27	30		
Model 1, HR, 95% CI	1	1.24 (0.71, 2.17)	1.55 (0.89, 2.68)	1.25 (0.99, 1.59)	0.06
Model 2, HR, 95% CI	1	1.55 (0.49, 4.90)	2.66 (0.88, 8.00)	1.52 (0.91, 2.53)	0.1
Model 3, HR, 95% CI	1	1.26 (0.39, 4.06)	2.27 (0.75, 6.83)	1.35 (0.79, 2.28)	0.2

Model 1: age and sex adjusted
Model 2: +body mass index, systolic blood pressure, medication for hypertension, parental history of diabetes, smoking, prevalent cardiovascular disease, lipid lowering medication, total and high-density lipoprotein cholesterol
Model 3: + fasting glucose
HR: hazard ratio; CI: confidence interval

ESM Table 4. Epicardial fat volume and the risk for hard coronary heart disease in women and men.

	EFV				
	Tertile 1	Tertile 2	Tertile 3	*Continuous*	*P-value*
Women					
Cases	7	12	13		
Model 1, HR, 95% CI	1	1.54 (0.61, 3.93)	1.57 (0.62, 3.95)	1.33 (0.87, 2.02)	0.1
Model 2, HR, 95% CI	1	1.19 (0.22, 6.54)	0.70 (0.09, 5.76)	0.85 (0.30, 2.39)	0.7
Model 3, HR, 95% CI	1	1.14 (0.20, 6.39)	0.58 (0.07, 4.96)	0.77 (0.27, 2.19)	0.6
Men					
Cases	16	20	23		
Model 1, HR, 95% CI	1	1.46 (0.76, 2.83)	1.58 (0.83, 3.00)	1.23 (0.95, 1.61)	0.1
Model 2, HR, 95% CI	1	1.58 (0.43, 5.86)	1.91 (0.54, 6.79)	1.23 (0.69, 2.18)	0.4
Model 3, HR, 95% CI	1	1.39 (0.38, 5.17)	1.88 (0.51, 6.89)	1.18 (0.65, 2.12)	0.5

Model 1: age and sex adjusted
Model 2: + body mass index, systolic blood pressure, medication for hypertension, smoking, prevalent diabetes, lipid lowering medication, total and high-density lipoprotein cholesterolModel 3: + coronary artery calcification
HR: hazard ratio; CI: confidence interval

ESM Table 5. Epicardial fat volume and the risk for stroke in women and men.

	EFV				
	Tertile 1	Tertile 2	Tertile 3	*Continuous*	*P-value*
Women					
Cases	18	18	16		
Model 1, HR, 95% CI	1	0.93 (0.48, 1.78)	0.77 (0.39, 1.52)	0.92 (0.67, 1.27)	0.9
Model 2, HR, 95% CI	1	1.92 (0.47, 7.78)	1.58 (0.32, 7.89)	1.48 (0.61, 3.59)	0.3
Model 3, HR, 95% CI	1	1.95 (0.47, 8.11)	1.68 (0.33, 8.57)	1.54 (0.62, 3.80)	0.3
Men					
Cases	14	19	24		
Model 1, HR, 95% CI	1	1.38 (0.69, 2.75)	1.71 (0.88, 3.32)	1.21 (0.91, 1.59)	0.1
Model 2, HR, 95% CI	1	1.66 (0.44, 6.29)	2.55 (0.67, 9.67)	1.58 (0.89, 2.78)	0.1
Model 3, HR, 95% CI	1	1.62 (0.43, 6.16)	2.50 (0.65, 9.56)	1.57 (0.88, 2.80)	0.1

Model 1: age and sex adjusted
Model 2: + body mass index, systolic blood pressure, medication for hypertension, smoking, prevalent diabetes, lipid lowering medication, total and high-density lipoprotein cholesterol, prevalent atrial fibrillation
Model 3: + coronary artery calcification, extra-cranial carotid artery calcification
HR: hazard ratio; CI: confidence interval

ESM Table 6. Competing risk analysis for epicardial fat volume and incident stroke and atrial fibrillation.

	Incident stroke		Competing risk with AF	
EFV	HR(95%CI)	p-value	HR(97.5%CI)	p-value
Model 1	1.07 (0.87, 1.32)	0.5	1.043 (0.81, 1.35)	0.5
Model 2	1.58 (1.02, 2.45)	**0.04**	1.82 (1.18, 2.80)	**0.0068**
Model 3	1.57 (1.01, 2.44)	**0.044**	1.82 (1.18, 2.80)	**0.0069**

Model 1: age and sex adjusted
Model 2: + body mass index, systolic blood pressure, medication for hypertension, smoking, prevalent diabetes, lipid lowering medication, total and high-density lipoprotein cholesterol
Model 3: + coronary artery calcification
HR: hazard ratio; CI: confidence interval

Chapter 4

Sex hormones and type 2 diabetes

Chapter 4.1

Serum dehydroepiandrosterone levels are associated with lower risk of type 2 diabetes: the Rotterdam Study.

Adela Brahimaj
Taulant Muka
Maryam Kavousi
Joop S. E. Laven
Abbas Dehghan
Oscar H. Franco

Diabetologia 2016

ABSTRACT

Aims/hypothesis

Previous literature documents controversial results about the impact of DHEA in glucose metabolism. We aimed to assess the associations between serum levels of DHEA, its main derivatives- DHEAS and androstenedione as well as the DHEAS-to-DHEA ratio with the risk of type 2 diabetes.

Methods

We used data of serum levels of DHEA, DHEAS and androstenedione in 5189 middle-aged and elderly men and women from the prospective population based Rotterdam Study. Type 2 diabetes was defined as a fasting blood glucose ≥7.0 mmol/l or a non-fasting blood glucose ≥ 11.1 mmol/l.

Results

During a median follow-up time of 10.9 years, 643 incident type 2 diabetes cases were identified. After adjusting for age, sex, cohort, fasting status, fasting glucose and insulin, BMI, both serum DHEA levels (per 1 unit naturally log-transformed, HR= 0.76, 95% CI: 0.67, 0.87) and serum DHEAS levels (per 1 unit naturally log-transformed, HR= 0.82, 95% CI: 0.73, 0.92) were inversely associated with the risk of type 2 diabetes in the total population. Further adjustment for alcohol, smoking, physical activity, prevalent CVD, serum total cholesterol, anti-lipids use, SBP, treatment for hypertension, CRP, estradiol, testosterone, did not substantially affect the association between DHEA and incident type 2 diabetes (per 1 unit naturally log-transformed, HR= 0.80, 95% CI: 0.65, 0.99), but abolished the association between DHEAS and type 2 diabetes. Androstenedione was not associated with the risk of type 2 diabetes, nor the DHEAS to DHEA ratio.

Conclusions/Interpretation

DHEA serum levels might be an independent marker for type 2 diabetes.

Key words

androstenedione, DHEA, DHEAS, food supplement, independent marker, type 2 diabetes.

Abbreviations

BMI, body mass index; CVD, cardiovascular diseases; CRP, chain reaction protein; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone; SBP, systolic blood pressure.

INTRODUCTION

Dehydroepiandrosterone (DHEA), mostly present as its sulfated ester (DHEAS), is the most abundant circulating adrenal steroid hormone in healthy adults. In both men and women, the peak serum levels of DHEA and DHEAS occur around 25 years of age and decline steadily from the third decade onwards (1). Similarly, both glucose tolerance and insulin sensitivity, decrease with aging (2).

DHEA is available as a health food supplement in the United States, but previous literature about the effects of supplemental DHEA on glucose metabolism in healthy humans is controversial (3-6).

Evidence from animal studies, indicate that DHEA treatment could result in increased insulin-induced glucose uptake in type 2 diabetes rat models and moderate the severity of diabetes (7; 8). Along the same line, a randomized controlled trial (RCT) of elderly women and men, with age-related DHEA decrease, showed that DHEA replacement, reduced abdominal fat and improved insulin sensitivity (9). Further RCTs reported positive effects of DHEA supplementation on reducing body fat and improving glucose metabolism, but not all (10-14). In observational studies, DHEAS plasma levels have also been associated with lower body mass index and visceral fat in women (15), and glucose and insulin concentration in non-diabetic men (16). Despite extensive research on DHEA and insulin action, information about the role of DHEA on type 2 diabetes risk remains scarce. Studies investigating prospectively the association between DHEA or DHEAS and type 2 diabetes are limited and have been conducted mainly in women (17; 18). Sex-differences have been suggested and it remains unclear whether DHEA effects on type 2 diabetes risk are different in women and men (11-14; 19; 20).

Therefore, we aimed to prospectively examine the associations of DHEA and its main derivatives- DHEAS and androstenedione and the ratio of DHEAS to DHEA with the incident type 2 diabetes in healthy middle-aged and elderly men and women.

MATERIALS AND METHODS

Study population

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 (21). 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. Follow-up visits were held every 3-5years. For this study, we used DHEA and it derivatives measurements done in the third visit of the first cohort (RSI-3) and the baseline examinations of the second cohort (RSII-1). 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 of analysis

The present study used data from the third visit of the first cohort (RSI-3) and the baseline examinations of the second cohort (RSII-1). Overall, there were 6923 subjects eligible for blood measurements and available type 2 diabetes follow-up (3923 postmenopausal woman and 3000 males). Among them 937 subjects (483 postmenopausal woman and 454 males) with prevalent diabetes were excluded. Furthermore 797 (422 postmenopausal women and 375 males) did not have information on DHEA and its derivatives, and were therefore excluded from the analysis, leaving 5189 subjects for our analysis (supplementary figure 1). Among them, in 270 participants sex steroids were assessed in non-fasting samples. A sensitivity analysis was performed excluding these subjects from the analysis.

Ascertainment of type 2 diabetes

The participants were followed from the date of baseline center visit onwards. At baseline and during follow-up, cases of type 2 diabetes 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 (22). Type 2 diabetes was defined according to current 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 (23). Information regarding the use of blood glucose lowering medication was derived from both structured home interviews and linkage to pharmacy records (22). At baseline, more than 95% of the Rotterdam Study population was covered by the pharmacies in the study area. All potential events of type 2 diabetes 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.

Hormone measurements:

Sex hormone-binding globulin (SHBG) was measured on the Immulite 2000Xpi platform (Siemens, Los Angeles, USA), while TSH was measured on the Vitros Eci (Ortho Diagnostics). Estradiol was measured on the COBAS 8000 Modular Analyzer (Roche Diagnostics GmbH). The corresponding interassay coefficients of variations were < 7%.

Androstenedione, testosterone and dehydroepiandrosterone sulfate (DHEAS), were measured on a Waters XEVO-TQ-S system (Waters, Milford, MA, USA) using the CHS™ MSMS Steroids Kit (Perkin Elmer, Turku, Finland). The inter-assay coefficients of variation of androstenedione, testosterone and DHEA-S were < 6.5%, < 5%, and < 5.9%, respectively. While assessment of blood measurements, including sex steroids, was performed in fasting samples, some of the participants came to the research center in a non-fasting state.

Covariates

Information on current health status, medical history, medication use and smoking behavior was obtained at baseline of the study for all the participants. Participants were asked whether they were currently smoking cigarettes, cigars, or pipes. Alcohol intake was assessed in grams of ethanol per day. History of cardiovascular disease was defined as a history of coronary heart diseases (myocardial infarction, revascularization, coronary artery bypass graft surgery or percutaneous coronary intervention) and was verified from the medical records of the general practitioner. Information regarding the use of hormone replacement therapy was derived from structured home interviews.

Parental history of diabetes was collected by trained research assistants during home visits. Blood pressure was measured in the sitting position at the right upper arm with a random-zero-sphygmomanometer. Physical height (m) and body weight (kg) were measured at baseline with the participants standing without shoes and heavy outer garments. Body mass index (BMI) was calculated as weight divided by height squared (kg/m2). All biochemical parameters were assessed in fasting serum. Insulin, glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triacylglycerol (TG) and C-reactive protein (CRP) were measured on the COBAS 8000 Modular Analyzer (Roche Diagnostics GmbH). The corresponding interassay coefficients of variations are the following: insulin < 8%, glucose < 1.4%, lipids < 2.1% and CRP < 16.9%. LDL-C level was estimated indirectly from measurements of total cholesterol, HDL-C and triacylglycerols by means of the Friedewald equation (24). Physical activity was assessed with an adapted version of the Zutphen Physical Activity Questionnaire (25). Every activity mentioned in the questionnaire was attributed a MET-value according to the 2011 (26).

Statistical analysis

Person years of follow-up were calculated from study entrance (March 1997- December 1999 for RSI-3, February 2000-December 2001 for RSII-1) to the date of diagnosis of type 2 diabetes, death or the censor date (date of last contact of the living), whichever occurred first. Follow-up was until January 1st 2012. Cox proportional hazard modelling was used to evaluate whether DHEA, DHEAS, androstenedione and DHEAS to DHEA ratio were associated with type 2 diabetes. 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 account for age and sex differences in serum levels of DHEA, DHEAS and androstenedione, we used sex hormone levels adjusted for age and sex by using the residual method (27). All sex hormones variables were assessed in separate models, continuously and in tertiles. To study the relations across increasing tertiles, trend tests were computed by entering the categorical variables as continuous variables in multivariable Cox's proportional hazard models. To achieve approximately normal distribution, skewed variables (DHEA, DHEAS, androstenedione, testosterone, estradiol, SHBG, CRP, TSH, glucose, insulin) were naturally log-transformed. In the base model (Model 1), we adjusted for age, sex, cohort (I and II), fasting status (fasting sample vs. non-fasting sample). Model 2 included all factors in model 1 and body mass index (BMI)

(continuous), glucose (continuous) and insulin (continuous). Model 3 included all covariates in model 2 and further potential intermediate factors including: metabolic risk factors (total cholesterol, systolic blood pressure (continuous), treatment 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), physical activity), prevalent coronary heart disease (yes vs. no) and CRP (continuous). We also controlled for upstream precursor hormones (supplementary figure 2) which may act as confounders. Thus, DHEA was adjusted in all models that included either DHEAS or androstenedione. Effect modifications of sex hormones by BMI and sex were tested in the final multivariable model in addition to performing stratified analysis. We also performed several sensitivity analyses: (i) further adjusting for hormones including the downstream metabolites that might be casual intermediates such as estradiol and testosterone; (ii) further adjusting for SHBG; (iii) substituting BMI for waist circumference; (iv) substituting total cholesterol with HDL-C, triacylglycerols and LDL-C; (v) further adjusting for parental history of diabetes (vi); further adjusting for TSH and (vii) excluding the type 2 diabetes cases within the first three years of follow up (n=99/643 cases); (viii) excluding participants with non-fasting samples (n=270); (ix) further adjustment for hormone replacement therapy; (x) further adjustment for free androgen index. Multiple imputation procedure was used (N= 5 imputations) to impute the missing data.

Moreover, we compared the ability of DHEA and its derivatives in 10 year type 2 diabetes risk prediction by studying the discrimination. Discrimination is the ability of a prediction model to assign a higher risk to the individuals who will develop an event in 10 years compared with those who will not develop an event. We quantified discrimination for both models by calculating the c-statistic difference between based model and models that additionally included DHEA or its derivatives (28). For type 2 diabetes there is no yet an unique established risk prediction model, therefore, as a base model, we used Wilson`s risk score including age, sex, parental history of diabetes and body mass index (29). To perform this analysis we used a combination of "foreign" and "survC1" R packages. All other analyses were done using SPSS statistical software (SPSS, version 21.0; SPSS Inc, Chicago, Illinois) and R V.3.0.1 (R Foundation for Statistical Computing, Vienna, Austria). A p-value of less than 0.05 was considered as statistically significant.

RESULTS

Table 1 summarizes the baseline characteristics of 5189 subjects free of diabetes at baseline, including 3018 postmenopausal women and 2171 males.

Survival analysis

During a median follow-up time of 10.9 years, 643 incident type 2 diabetes cases were identified. After adjusting for age, sex, cohort, fasting status, fasting glucose and insulin, BMI, both serum DHEA levels (per 1 unit naturally log-transformed, HR= 0.76, 95% CI: 0.67, 0.87) and serum DHEAS levels (per 1 unit naturally log-transformed, HR= 0.82, 95% CI: 0.73, 0.92) were inversely associated with the risk of type 2 diabetes (**table 2**) in the total population. Further adjustment

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

Characteristic	N = 5189
Age (years)	69 ± 8.3
Males, n (%)	2171 (41.8)
Fasting status, n (%)	4911 (94.6)
Current smokers, n (%)	632 (12.2)
*Alcohol intake (g/day)	2.1 (11.6)
BMI (kg/m^2)	26.7 ± 3.8
Waist circumference (cm)	92.7 ± 11.5
Prevalent cardiovascular disease, n (%)	586 (11.3)
Parental history of diabetes, n (%)	463 (8.9)
*Estradiol (pmol/l)	62.8 (137.5)
*Total testosterone (nmol/l)	1.4 (24.1)
*Sex-hormone binding globulin (nmol/l)	55.5 (93.12)
*Thyroid-stimulating hormone (mU/l)	1.8 (4.9)
*Insulin (pmol/l)	66 (145)
*Glucose (mmol/l)	5.5 (1.8)
*C-reactive protein (mg/ml)	1.7 (10.3)
Total cholesterol (mmol/l)	5.8 ± 0.9
Low density lipoprotein cholesterol (mmol/l)	3.7 ± 0.9
High density lipoprotein cholesterol (mmol/l)	1.4 ± 0.4
Serum lipid reducing agents use, n (%)	605 (11.7)
Hormone replacement therapy, n (%)	140 (2.7)
Free androgen index	2.5 (31.8)
*Triacylglycerols (moml/l)	1.4 (0.8)
Systolic Blood pressure (mm/Hg)	142 ± 21
Treatment for hypertension, n (%)	1087 (20.9)
*DHEA, nmol/l	8.3 (20.58)
*DHEAS, nmol/l	1819.2 (4672.71)
*Androstenedione, nmol/l	2.5 (4.05)

BMI, body mass index; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate. Plus minus values are mean ± SD; * Median (interquartile range)

for alcohol intake, smoking status, physical activity, prevalent CVD, serum total cholesterol, serum lipid reducing agents use, systolic blood pressure, treatment for hypertension, CRP did not materially affect the association between DHEA and incident type 2 diabetes (per 1 unit naturally log-transformed, HR= 0.76, 95% CI: 0.67, 0.88), but abolished the association between DHEAS and type 2 diabetes (table 2). Androstenedione was not associated with the risk of type 2 diabetes, nor the DHEAS to DHEA ratio (**table 2**).

Sensitivity analysis

In the sensitivity analysis, (i) further adjusting for hormones including the downstream metabolites that might be casual intermediates such as estradiol and testosterone; (ii) further adjustment for SHBG; (iii) substituting BMI for waist circumference; (iv) substituting total cholesterol for HDL-C, triacylglycerols and LDL-C; (v) further adjustment for parental history of diabetes (vi)

Table 2. Associations of androstenedione, DHEA, DHEAS with the risk of type 2 diabetes in postmenopausal women and men, the Rotterdam Study (N = 5189).

	Androstenedione			*Continuous*	*P-value*	*Ptrend*
	Tertile 1	Tertile 2	Tertile 3			
Cases	216	230	197			
Model 1, HR, 95% CI	1	1.09 (0.90, 1.31)	0.92 (0.76, 1.12)	0.92 (0.77, 1.09)	0.3	0.4
Model 2, HR, 95% CI	1	1.07 (0.89, 1.29)	0.82 (0.67, 0.99)*	0.82 (0.69, 0.97)	1.8×10^{-2}	4.5×10^{-2}
Model 3, HR, 95% CI	1	1.19 (0.97, 1.46)	0.98 (0.77, 1.26)	0.98 (0.78, 1.24)[d]	0.9	0.9
	DHEA					
Cases	236	206	201			
Model 1, HR, 95% CI	1	0.79 (0.66, 0.96)	0.76 (0.63, 0.92)*	0.79 (0.69, 0.89)	3.0×10^{-4}	5.0×10^{-3}
Model 2, HR, 95% CI	1	0.84 (0.69, 1.02)	0.73 (0.60, 0.89)*	0.76 (0.67, 0.87)	5.6×10^{-5}	1.0×10^{-3}
Model 3, HR, 95% CI	1	0.84 (0.69, 1.02)	0.73 (0.60, 0.89)*	0.76 (0.67, 0.87)[d]	8.7×10^{-5}	2.0×10^{-3}
	DHEAS					
Cases	226	203	214			
Model 1, HR, 95% CI	1	0.86 (0.71, 1.04)	0.91 (0.76, 1.09)	0.88 (0.79, 0.99)	3.5×10^{-2}	0.3
Model 2, HR, 95% CI	1	0.79 (0.65, 0.96)*	0.83 (0.69, 1.01)	0.82 (0.73, 0.92)	1×10^{-3}	6.0×10^{-2}
Model 3, HR, 95% CI	1	0.91 (0.74, 1.12)	1.13 (0.88, 1.45)	0.94 (0.79, 1.12)[d]	0.5	0.3
	DHEAS/DHEA					
Cases	198	215	230			
Model 1, HR, 95% CI	1	1.08 (0.89, 1.31)	1.23 (1.02, 1.49)*	1.14 (0.96, 1.34)	0.1	3.0×10^{-2}
Model 2, HR, 95% CI	1	1.11 (0.92, 1.35)	1.15 (0.95, 1.39)	1.03 (0.88, 1.22)	0.6	0.1
Model 3, HR, 95% CI	1	1.11 (0.92, 1.36)	1.15 (0.95, 1.41)	1.03 (0.87, 1.22)[d]	0.7	0.1

DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate.

Model 1: Adjusted for age, sex, cohort, fasting status Model 2: Model 1 + insulin, glucose and body mass index

Model 3: Model 2 + alcohol intake, smoking status, physical activity, prevalent CVD, serum total cholesterol, serum lipid reducing agents use, systolic blood pressure, treatment for hypertension, C-reactive protein and sex hormones for each other (Androstenedione adjust for DHEA; DHEAS adjust for DHEA).

[d] No significant interaction between sex and hormone or ratio ($p > 0.05$)

* $p < 0.05$

further adjustment for TSH; (vii) exclusion of the type 2 diabetes cases within the first three years of follow up; (viii) excluding participants with non-fasting samples (n=270); (ix) further adjustment for hormone replacement therapy; (x) further adjustment for free androgen index did not affect any of the associations (**table 3**). Also, in the stratification analysis, no significant interactions were found for any of the hormones or ratio with gender and BMI (**table 3**).

The c-statistic of the Wilson's base model for 10-year type 2 diabetes risk was 0.637 (0.609, 0.666). When we added DHEA to the model, the c-statistic improved to 0.638 (0.612, 0.665) with a difference of 0.001 (-0.003, 0.005). DHEA and it derivatives did not improve Wilson`s base model (supplementary table 1).

DISCUSSION

In this large prospective population-based cohort study, we found that DHEA serum level was inversely associated with the risk of type 2 diabetes, independent of established diabetes risk

Table 3. Sensitivity analysis of sex hormones and the risk of type 2 diabetes in postmenopausal women and men, the Rotterdam Study (N = 5189).

	Androstenedione	DHEA	DHEAS	DHEAS/DHEA
Multivariable model	0.99 (0.79, 1.24)	0.76 (0.67, 0.88)*	0.94 (0.80, 1.11)	1.03 (0.87, 1.21)
Multivariable model + sex hormones for each other(estradiol and testosterone included in the model)	1.01 (0.80, 1.27)	0.80 (0.65, 0.99)*	0.94 (0.79, 1.10)	Not included
Multivariable model +SHBG	0.96 (0.78, 1.19)	0.77 (0.67, 0.88)*	0.89 (0.77, 1.03)	0.96 (0.83, 1.12)
Multivariable model + waist circumference	0.98 (0.78, 1.23)	0.76 (0.67, 0.87)*	0.95 (0.80, 1.12)	1.04 (0.88, 1.22)
Multivariable model + HDL-C + TG + LDL-C	0.98 (0.78, 1.24)	0.77 (0.68, 0.88)*	0.97 (0.82, 1.14)	1.06 (0.89, 1.25)
Multivariable model + serum thyroid stimulating hormone	0.97 (0.77, 1.22)	0.76 (0.67, 0.87)*	0.96 (0.81, 1.13)	1.05 (0.89, 1.23)
Multivariable model + parental history of diabetes	0.98 (0.79, 1.22)	0.77 (0.67, 0.88)*	0.95 (0.82, 1.09)	1.02 (0.88, 1.18)
Multivariable model excluding the first 3years of follow-up	1.06 (0.83 - 1.36)	0.76 (0.66, 0.88)*	0.97 (0.81, 1.16)	1.06 (0.89, 1.27)
Multivariable model excluding non-fasting subjects	0.96 (0.77, 1.21)	0.76 (0.67, 0.88)*	0.91 (0.78, 1.06)	0.98 (0.84, 1.15)
Multivariable model + hormone replacement therapy	0.98 (0.79, 1.23)	0.76 (0.67, 0.87)*	0.95 (0.82, 1.11)	1.03 (0.88, 1.19)
Multivariable model + free androgen index	0.95 (0.75, 1.20)	0.76 (0.67, 0.87)*	0.90 (0.76, 1.07)	1.01 (0.85, 1.19)
BMI (kg/m^2)[b]				
<25 643 (130)	0.75 (0.45, 1.24)[a]	0.81 (0.59, 1.11)[a]	1.21 (0.82, 1.78)[a]	1.29 (0.89, 1.87)[a]
25-29.9 643 (321)	1.20 (0.87, 1.66)[a]	0.80 (0.66, 0.97)[a*]	0.96 (0.77, 1.21)[a]	1.02 (0.81, 1.29)[a]
≥30 643 (184)	0.84 (0.54, 1.30)[a]	0.67 (0.53, 0.86)[a*]	0.75 (0.55, 1.03)[a]	0.88 (0.65, 1.21)[a]
Sex[b]				
Male 643 (270)	1.17 (0.80, 1.71)	0.82 (0.66, 1.03)	1.02 (0.77, 1.35)	1.10 (0.84, 1.44)
Female 643 (373)	0.88 (0.66, 1.17)	0.74 (0.63, 0.88)*	0.91 (0.74, 1.12)	0.99 (0.80, 1.22)

DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triacylglycerols.

[b]Multivariable model adjusted for variables in model 3 of Table 2.

Values are + 1 natural log increase

[a] P-interaction >0.05; [b] Results are adjusted for variables in model 3 of Table 2.

*$p < 0.05$

factors including BMI, fasting glucose, insulin and CRP. We did not find an evidence regarding gender differences in this association. The association remained significant also after including in the model downstream sex steroid metabolites such as testosterone and estradiol, cited as risk factors for type 2 diabetes (30). In our study, no independent association was found between DHEAS, androstenedione or DHEAS to DHEA ratio with the risk of type 2 diabetes.

A study of 1612 postmenopausal women examined prospectively the association between DHEA and the risk of type 2 diabetes and showed no effect (17). Our study included both men and women, a larger number of incident type 2 diabetes cases (643 vs116) with a longer follow-up (median: 10.9 vs 4.7 years) and a comprehensive assessment of hormones. Another study by Mather et al. showed no association between DHEA and diabetes incidence, however it was conducted in subjects at high risk for diabetes and with a short follow-up (median 3.0 years) (31). Similar to our findings, while an inverse association was suggested in a nested case-control study

of 718 postmenopausal women, DHEAS was not statistically significantly associated with lower risk of type 2 diabetes (18). Also, a recent study, including 1258 community-dwelling men and women reported no association between DHEAS and type 2 diabetes overall, but after stratification by gender, higher serum DHEAS levels were protective against type 2 diabetes in men, but not in women (19).

Previous animal studies have documented therapeutic effects of DHEA in diabetic mice (7). Due to the striking beneficial effects of DHEA, observed mostly in animals, against diabetes, but also other chronic conditions such as cancer, obesity, cardiovascular disease, DHEA is now available as a food supplement in retail stores (32-34). The afore mentioned conditions, are mostly observed in aging and accompanying the natural steady decline of DHEA from the third decade onward (1; 35).

However, animal data should be translated with caution to human physiology, taking into consideration the contrast between the lower amounts of DHEA in laboratory animals and the distinctly human role of DHEA, which has a pattern of biosynthesis specific for higher primates (36). Until recently, there is conflicting evidence in the literature concerning the effect of DHEA on glucose metabolism in healthy humans. Human data have shown that DHEA administration increases (9; 11), has no effect (13; 20) or decreases insulin sensitivity (37), leaving unclear the role of DHEA on glucose metabolism and development of type 2 diabetes. Moreover, Villareal et al. (9) found that DHEA supplementation for 6 months decreased visceral adiposity, while Nair et al. (20), in a 2 year trial, did not observe any benefits of DHEA.

Our findings on a protective role of DHEA against type 2 diabetes, provide epidemiological evidence in agreement to previous claims on positive effects of DHEA in type 2 diabetes (DHEA is previously called also "elixir of youth"). Pertaining to its mechanisms of action, DHEA is a PPARα agonist [30]. Tenenbaum et al found that bezafibrate, a PPARα receptor ligand, reduced the incidence and delayed the onset of type 2 diabetes in patients with impaired fasting glucose levels (38). DHEA and DHEAS have been shown to be insulin sensitizers (39), whereas there is less evidence that insulin alters DHEA or DHEAS levels (40). Perrini et al found that DHEA increases glucose uptake in both human and 3T3-L1 adipocytes by stimulating GLUT4 and GLUT1 translocation to the plasma membrane (41). Another study in type 2 diabetes patients concluded that DHEA administration counteracts oxidative imbalance and advanced glycation end product formation (42). Also, in rat models, it has been shown that DHEA improves glucose uptake via activations of protein kinase C and phosphatidylinositol 3-kinase (43). Another possible mechanism of prevention of type 2 diabetes by DHEA is the improvement of endothelial function (14), which is implicated in the development of insulin resistance (44).

Previous studies have suggested gender differences of DHEA and its derivatives in type 2 diabetes (11; 12; 19; 45). DHEA concentrations are approximately twice higher in women, whereas DHEAS levels are higher in men (46). In our study, we observed the protective effect of DHEA in men and women, slightly prominent in women than in men, however the interaction with sex was not significant. The hazard ratios had the same direction in both women and men, but the association was not significant in men. This might be explained by the smaller number of incident type 2 diabetes cases in males (n = 270) compared to women (n = 373) in our study.

Very little is known regarding the role of androstenedione plasma levels in the pathology of type 2 diabetes. O`Reilli et al. showed that serum androstenedione level, other than its role as precursor of testosterone, is a useful tool for predicting metabolic risk in PCOS women (47). Our study is the first study to investigate the association of serum androstenedione levels with incident type 2 diabetes, showing no association.

ACTH regulates production of both DHEA and DHEAS, which once secreted into the blood stream, are carried bound to albumin. Although DHEAS, the sulphated form of DHEA, can be converted to DHEA or vice-versa, they have some important peculiarities (48). Concentrations of DHEAS are between 250 and 500 times higher than the concentrations of DHEA, in women and men respectively. This difference in concentrations between DHEA and DHEAS depends mainly on the fact that DHEAS is only slowly cleared from the blood with a clearance rate of 13L/day, while DHEA is rapidly cleared at a rate of approximately 2000L/day. Therefore, DHEAS has a half-life of 10 to 20 hours while the half-life of DHEA is 1 to 3 hours. Diurnal variation of DHEA exhibits a similar pattern as cortisol secretion, reaching the peak in the early morning. DHEAS levels are considered to not have a diurnal variation (49).

Strengths of our study include its prospective design, the long follow-up and comprehensive adjustment for a broad range of possible confounders. Moreover, this is the first population-based study to investigate the associations between DHEA and its main derivatives serum levels and incident type 2 diabetes in both men and women. We also performed several sensitivity analyses such as excluding the first three years of follow up to avoid potential bias of undiagnosed disease at baseline. Furthermore, the diagnosis of incident diabetes was done by standardized blood glucose measurements at the repeated study center visits and electronic linkage with pharmacy dispensing records in the study area.

Yet our study has some limitations. Our population is 55 years and older and therefore generalization of the results to a younger age should be done with caution. We did not have hemoglobin A1c (HbA1c) or an oral glucose tolerance test measured in our study population, which would have strengthened our results. In contrast to DHEAS and its other derivatives, DHEA has a pronounced diurnal rhythm and exhibits morning elevation similar to cortisol. However, the secretory pattern of DHEA is more stable and less stable to day-to-day variability (50). Furthermore, this study is done in elderly and in older age hormone levels are more stable over time (intraclass correlation = 0.75, 0.88 and 0.66 for DHEA, DHEAS and androstenedione respectively) (50). Also, the Rotterdam Study mainly includes individuals from European Ancestry (98%). Thus, our findings may not be extended to non-Caucasian groups.

We conclude that higher serum levels of DHEA are independently associated with decreased risk of developing type 2 diabetes in healthy population of both men and postmenopausal women. These prospective data suggest that DHEA may play a role in the pathogenesis of type 2 diabetes, which may have important implications for preventive interventions.

Author contribution:

AB ran the analysis and wrote the manuscript. TM designed the study and contributed to the analyses. J.S.E. Laven, MK and AD designed the study and critically revised the manuscript. O.H.F designed the study, critically revised the manuscript and provided resources.

Funding/Support:

The Rotterdam Study is supported by the Erasmus MC and Erasmus University Rotterdam; the Netherlands Organisation for Scientific Research (NWO); the Netherlands Organisation for Health Research and Development (ZonMw); the Research Institute for Diseases in the Elderly (RIDE); the Netherlands Genomics Initiative (NGI); the Ministry of Education, Culture and Science, the Ministry of Health, Welfare and Sports; the European Commission (DG XII); and the Municipality of Rotterdam.

Acknowledgements

AB has been financially supported by Erasmus Mundus Western Balkans (ERAWEB), a project funded by the European Commission. TM and O.H.F work in ErasmusAGE, a center for aging research across the life course funded by Nestlé Nutrition (Nestec Ltd.), Metagenics Inc. and AXA. O.H.F reported receiving grants or research support from Metagenics Inc. These funding sources had no role in design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review or approval of the manuscript. AD is supported by NWO grant (veni, 916.12.154) and the EUR Fellowship. J.S.E. Laven has nothing to disclose. MK is supported by the NOW VENI grant (VENI, 91616079).

REFERENCES

1. Orentreich N, Brind JL, Rizer RL, Vogelman JH: Age changes and sex differences in serum dehydroepiandrosterone sulfate concentrations throughout adulthood. J Clin Endocrinol Metab 1984;59:551-555
2. DeFronzo RA: Glucose intolerance and aging. Diabetes Care 1981;4:493-501
3. Morales AJ, Nolan JJ, Nelson JC, Yen SS: Effects of replacement dose of dehydroepiandrosterone in men and women of advancing age. J Clin Endocrinol Metab 1994;78:1360-1367
4. Yen SS, Morales AJ, Khorram O: Replacement of DHEA in aging men and women. Potential remedial effects. Ann N Y Acad Sci 1995;774:128-142
5. Lasco A, Frisina N, Morabito N, Gaudio A, Morini E, Trifiletti A, Basile G, Nicita-Mauro V, Cucinotta D: Metabolic effects of dehydroepiandrosterone replacement therapy in postmenopausal women. Eur J Endocrinol 2001;145:457-461
6. Jakubowicz D, Beer N, Rengifo R: Effect of dehydroepiandrosterone on cyclic-guanosine monophosphate in men of advancing age. Ann N Y Acad Sci 1995;774:312-315
7. Coleman DL, Leiter EH, Schwizer RW: Therapeutic effects of dehydroepiandrosterone (DHEA) in diabetic mice. Diabetes 1982;31:830-833
8. Byrne JJ, Bradlow HL: DHEA-PC slows the progression of type 2 diabetes (non-insulin-dependent diabetes mellitus) in the ZDF/Gmi-fa/fa rat. Diabetes Technol Ther 2001;3:211-219
9. Villareal DT, Holloszy JO: Effect of DHEA on abdominal fat and insulin action in elderly women and men: a randomized controlled trial. JAMA 2004;292:2243-2248
10. Nestler JE, Clore JN, Strauss JF, 3rd, Blackard WG: The effects of hyperinsulinemia on serum testosterone, progesterone, dehydroepiandrosterone sulfate, and cortisol levels in normal women and in a woman with hyperandrogenism, insulin resistance, and acanthosis nigricans. J Clin Endocrinol Metab 1987;64:180-184
11. Dhatariya K, Bigelow ML, Nair KS: Effect of dehydroepiandrosterone replacement on insulin sensitivity and lipids in hypoadrenal women. Diabetes 2005;54:765-769
12. Nestler JE, Barlascini CO, Clore JN, Blackard WG: Dehydroepiandrosterone reduces serum low density lipoprotein levels and body fat but does not alter insulin sensitivity in normal men. J Clin Endocrinol Metab 1988;66:57-61
13. Basu R, Dalla Man C, Campioni M, Basu A, Nair KS, Jensen MD, Khosla S, Klee G, Toffolo G, Cobelli C, Rizza RA: Two years of treatment with dehydroepiandrosterone does not improve insulin secretion, insulin action, or postprandial glucose turnover in elderly men or women. Diabetes 2007;56:753-766
14. Kawano H, Yasue H, Kitagawa A, Hirai N, Yoshida T, Soejima H, Miyamoto S, Nakano M, Ogawa H: Dehydroepiandrosterone supplementation improves endothelial function and insulin sensitivity in men. J Clin Endocrinol Metab 2003;88:3190-3195
15. Moran C, Arriaga M, Arechavaleta-Velasco F, Moran S: Adrenal androgen excess and body mass index in polycystic ovary syndrome. J Clin Endocrinol Metab 2015;100:942-950
16. Haffner SM, Valdez RA, Mykkanen L, Stern MP, Katz MS: Decreased testosterone and dehydroepiandrosterone sulfate concentrations are associated with increased insulin and glucose concentrations in nondiabetic men. Metabolism 1994;43:599-603
17. Kalyani RR, Franco M, Dobs AS, Ouyang P, Vaidya D, Bertoni A, Gapstur SM, Golden SH: The association of endogenous sex hormones, adiposity, and insulin resistance with incident diabetes in postmenopausal women. J Clin Endocrinol Metab 2009;94:4127-4135
18. Ding EL, Song Y, Manson JE, Rifai N, Buring JE, Liu S: Plasma sex steroid hormones and risk of developing type 2 diabetes in women: a prospective study. Diabetologia 2007;50:2076-2084
19. Veronese N, Trevisan C, De Rui M, Bolzetta F, Maggi S, Zambon S, Corti MC, Baggio G, Perissinotto E, Crepaldi G, Manzato E, Sergi G: Serum Dehydroepiandrosterone Sulfate and Risk for Type 2 Diabetes in Older Men and Women: The Pro.V.A Study. Can J Diabetes 2016;
20. Nair KS, Rizza RA, O'Brien P, Dhatariya K, Short KR, Nehra A, Vittone JL, Klee GG, Basu A, Basu R, Cobelli C, Toffolo G, Dalla Man C, Tindall DJ, Melton LJ, 3rd, Smith GE, Khosla S, Jensen MD: DHEA in elderly women and DHEA or testosterone in elderly men. N Engl J Med 2006;355:1647-1659
21. Hofman A, Brusselle GG, Darwish Murad S, van Duijn CM, Franco OH, Goedegebure A, Ikram MA, Klaver CC, Nijsten TE, Peeters RP, Stricker BH, Tiemeier HW, Uitterlinden AG, Vernooij MW: The Rotterdam Study: 2016 objectives and design update. Eur J Epidemiol 2015;30:661-708
22. Leening MJ, Kavousi M, Heeringa J, van Rooij FJ, Verkroost-van Heemst J, Deckers JW, Mattace-Raso FU, Ziere G, Hofman A, Stricker BH, Witteman JC: Methods of data collection and definitions of cardiac outcomes in the Rotterdam Study. Eur J Epidemiol 2012;27:173-185

23. Organization WH: Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation. Geneva: World Health Organization: 1-50 2006;
24. 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;18:499-502
25. Caspersen CJ, Bloemberg BP, Saris WH, Merritt RK, Kromhout D: 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;133:1078-1092
26. Ainsworth BE, Haskell WL, Herrmann SD, Meckes N, Bassett DR, Jr., Tudor-Locke C, Greer JL, Vezina J, Whitt-Glover MC, Leon AS: 2011 Compendium of Physical Activities: a second update of codes and MET values. Med Sci Sports Exerc 2011;43:1575-1581
27. Willett WC, Howe GR, Kushi LH: Adjustment for total energy intake in epidemiologic studies. Am J Clin Nutr 1997;65:1220S-1228S; discussion 1229S-1231S
28. Haibe-Kains B, Desmedt C, Sotiriou C, Bontempi G: A comparative study of survival models for breast cancer prognostication based on microarray data: does a single gene beat them all? Bioinformatics 2008;24:2200-2208
29. Wilson PW, Meigs JB, Sullivan L, Fox CS, Nathan DM, D'Agostino RB, Sr.: Prediction of incident diabetes mellitus in middle-aged adults: the Framingham Offspring Study. Arch Intern Med 2007;167:1068-1074
30. Ding EL, Song Y, Malik VS, Liu S: Sex differences of endogenous sex hormones and risk of type 2 diabetes: a systematic review and meta-analysis. JAMA 2006;295:1288-1299
31. Mather KJ, Kim C, Christophi CA, Aroda VR, Knowler WC, Edelstein SE, Florez JC, Labrie F, Kahn SE, Goldberg RB, Barrett-Connor E, Diabetes Prevention P: Steroid Sex Hormones, Sex Hormone-Binding Globulin, and Diabetes Incidence in the Diabetes Prevention Program. J Clin Endocrinol Metab 2015;100:3778-3786
32. Baulieu EE: Dehydroepiandrosterone (DHEA): a fountain of youth? J Clin Endocrinol Metab 1996;81:3147-3151
33. Schwartz AG, Pashko LL: Cancer prevention with dehydroepiandrosterone and non-androgenic structural analogs. J Cell Biochem Suppl 1995;22:210-217
34. Khorram O, Vu L, Yen SS: Activation of immune function by dehydroepiandrosterone (DHEA) in age-advanced men. J Gerontol A Biol Sci Med Sci 1997;52:M1-7
35. Watson RR, Huls A, Araghinikuam M, Chung S: Dehydroepiandrosterone and diseases of aging. Drugs Aging 1996;9:274-291
36. Sapolsky RM, Vogelman JH, Orentreich N, Altmann J: Senescent decline in serum dehydroepiandrosterone sulfate concentrations in a population of wild baboons. J Gerontol 1993;48:B196-200
37. Mortola JF, Yen SS: The effects of oral dehydroepiandrosterone on endocrine-metabolic parameters in postmenopausal women. J Clin Endocrinol Metab 1990;71:696-704
38. Tenenbaum A, Motro M, Fisman EZ, Schwammenthal E, Adler Y, Goldenberg I, Leor J, Boyko V, Mandelzweig L, Behar S: Peroxisome proliferator-activated receptor ligand bezafibrate for prevention of type 2 diabetes mellitus in patients with coronary artery disease. Circulation 2004;109:2197-2202
39. Casson PR, Hornsby PJ, Buster JE: Adrenal androgens, insulin resistance, and cardiovascular disease. Semin Reprod Endocrinol 1996;14:29-34
40. Nestler JE: Regulation of human dehydroepiandrosterone metabolism by insulin. Ann N Y Acad Sci 1995;774:73-81
41. Perrini S, Natalicchio A, Laviola L, Belsanti G, Montrone C, Cignarelli A, Minielli V, Grano M, De Pergola G, Giorgino R, Giorgino F: Dehydroepiandrosterone stimulates glucose uptake in human and murine adipocytes by inducing GLUT1 and GLUT4 translocation to the plasma membrane. Diabetes 2004;53:41-52
42. Brignardello E, Runzo C, Aragno M, Catalano MG, Cassader M, Perin PC, Boccuzzi G: Dehydroepiandrosterone administration counteracts oxidative imbalance and advanced glycation end product formation in type 2 diabetic patients. Diabetes Care 2007;30:2922-2927
43. Ishizuka T, Kajita K, Miura A, Ishizawa M, Kanoh Y, Itaya S, Kimura M, Muto N, Mune T, Morita H, Yasuda K: DHEA improves glucose uptake via activations of protein kinase C and phosphatidylinositol 3-kinase. Am J Physiol 1999;276:E196-204
44. Pinkney JH, Stehouwer CD, Coppack SW, Yudkin JS: Endothelial dysfunction: cause of the insulin resistance syndrome. Diabetes 1997;46 Suppl 2:S9-13
45. Weiss EP, Villareal DT, Fontana L, Han DH, Holloszy JO: Dehydroepiandrosterone (DHEA) replacement decreases insulin resistance and lowers inflammatory cytokines in aging humans. Aging (Albany NY) 2011;3:533-542

46. Zumoff BV, Bradlow HL: Sex difference in the metabolism of dehydroisoandrosterone sulfate. J Clin Endocrinol Metab 1980;51:334-336
47. O'Reilly MW, Taylor AE, Crabtree NJ, Hughes BA, Capper F, Crowley RK, Stewart PM, Tomlinson JW, Arlt W: Hyperandrogenemia predicts metabolic phenotype in polycystic ovary syndrome: the utility of serum androstenedione. J Clin Endocrinol Metab 2014;99:1027-1036
48. Rosenfeld RS, Rosenberg BJ, Fukushima DK, Hellman L: 24-Hour secretory pattern of dehydroisoandrosterone and dehydroisoandrosterone sulfate. J Clin Endocrinol Metab 1975;40:850-855
49. Longcope C: Dehydroepiandrosterone metabolism. J Endocrinol 1996;150 Suppl:S125-127
50. Hucklebridge F, Hussain T, Evans P, Clow A: The diurnal patterns of the adrenal steroids cortisol and dehydroepiandrosterone (DHEA) in relation to awakening. Psychoneuroendocrinology 2005;30:51-57

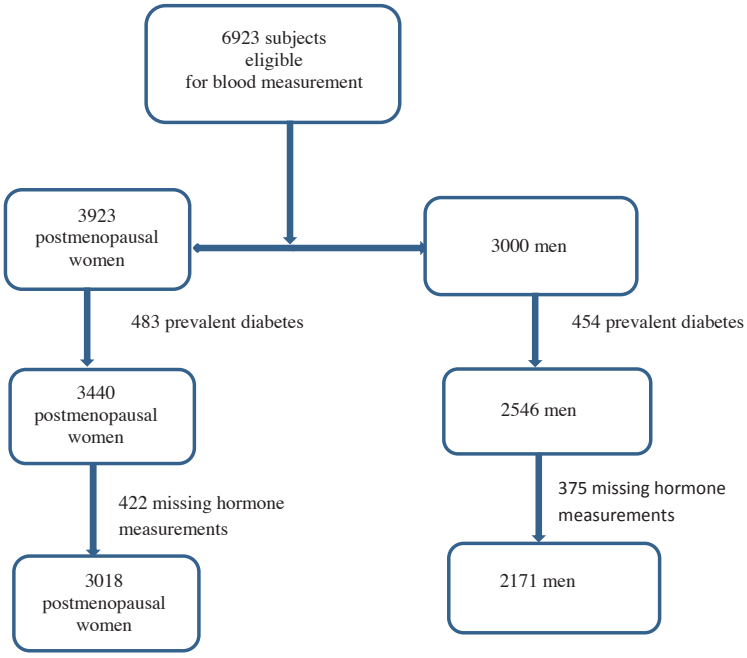
ELECTRONIC SUPPLEMENTARY MATERIAL (ESM)

Supplementary table 1. Comparison of the predictive value of DHEA with its derivatives.

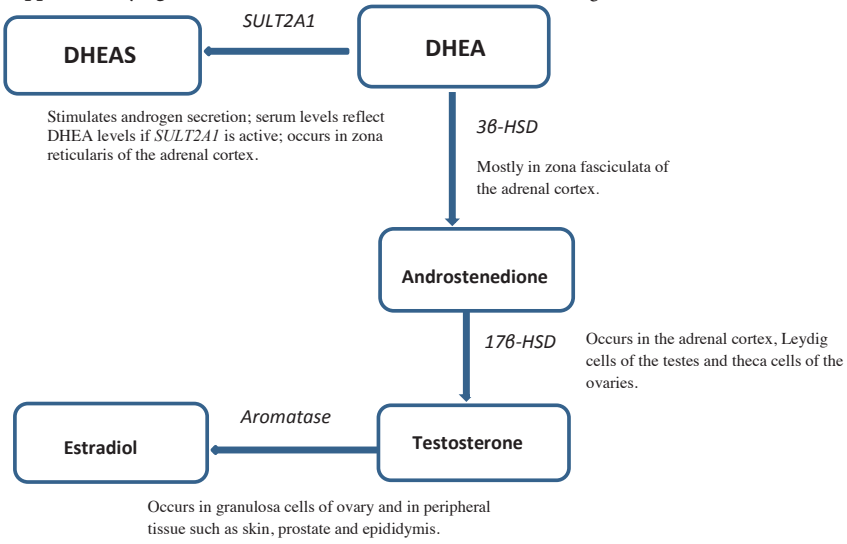
	c-statistics (95%CI)	c-statistics changes (95% CI) from base model
Base model	0.637 (0.609, 0.666)	NA
+DHEA	0.638 (0.612, 0.665)	0.001 (-0.003, 0.005)
+DHEAS	0.637 (0.610, 0.666)	0.000 (-0.004, 0.005)
+androstenedione	0.637 (0.608, 0.667)	0.000 (-0.002, 0.002)
+DHEAS/DHEA	0.637 (0.609, 0.666)	0.000 (-0.001, 0.001)

Base model: age, sex, parental history of diabetes, body mass index (Wilson`s base model).

Supplementary figure 1. Flow chart of subjects eligibility.



Supplementary figure 2. DHEA and its main derivatives in steroidogenesis.



Chapter 4.2

Endogenous sex steroid levels and SHBG relate with 5-year changes in body composition in postmenopausal women.

Najada Stringa
Adela Brahimaj*
Katerina Trajanoska*
Jessica C. Kiefte-de Jong
M. Arfan Ikram
Joop S. E. Laven
Fernando Rivadeneira
Taulant Muka
Oscar H. Franco

**Authors contributed equally to the work.*

ABSTRACT

Objective

The objective of this study was to investigate the association between endogenous sex steroid levels and changes in body composition in postmenopausal women.

Methods

A total of 1183 postmenopausal women participating in the Rotterdam Study were eligible for analysis. At baseline, serum levels of estradiol (E2), serum testosterone (T) and sex hormone-binding globulin (SHBG) were assessed. Body composition was assessed using dual-energy X-ray absorptiometry at baseline and 5 years later. Total weight, total fat mass (%) (FM), lean mass (%) (LM), FM/LM ratio, android fat (%) (AF) and android/gynoid fat mass ratio (A/G ratio) were calculated.

Results

No association was found between E_2 and body weight or E_2 and LM. High E_2 levels were associated with a decrease in FM, FM/LM ratio and an increase in AF. High testosterone levels were associated with a decrease in FM, FM/LM ratio and an increase in LM. High SHBG levels were associated with an increase in weight, FM, FM/LM ratio and a decrease in LM and AF. No association was found for any of the sex hormones or SHBG with A/G ratio.

Conclusion

These findings support the concept that changes in endogenous sex steroid levels are closely associated with changes in body composition in postmenopausal women.

Key words

body composition; body fat distribution; endogenous sex steroids; post-menopausal women; sex-hormone binding globulin

INTRODUCTION

Menopause is associated with adverse changes in body composition. Women during menopause tend to gain body fat and shift the fat storage from hips and thighs (pear-shaped body) to the abdominal region (apple-shaped) (1). At the same time, women experience an accelerated loss of muscle mass around the time of menopause (2). Both the accumulation of visceral fat in the abdomen and loss of muscle mass have been linked with an increased risk of cardiometabolic disease, disability and mortality (3-5).

The menopausal transition is characterized by a marked decline in endogenous estradiol (E_2) levels and a period of relative androgen excess (6), which may account for the changes in body composition during the post-menopausal period. Estrogens increase the number of alpha-adrenergic receptors in the lower body which decelerate fat release (7). Estrogens also exhibit anti-cortisol effects and sensitize the body to insulin (8). These mechanisms prevent fat accumulation in the abdominal region and may be responsible for the pear-shaped body most women have before menopause. Androgens, including testosterone are suggested to be involved in building and maintaining the muscle mass. The decrease of androgen levels after menopause, due to the loss of ovarian function, may reduce the muscles mass; a phenomenon observed in women after menopause (9). However, whether endogenous sex steroids affect body fat, its regional distribution and muscle mass remains unclear. Previous studies have been mainly cross-sectional (10) and findings from long-term studies examining the association between endogenous sex hormones, body fat and lean mass remain scarce. Furthermore, they have not taken into account the role that age and years since menopause may play in the association between endogenous sex steroids and body composition. Recent evidence shows that the effect of endogenous sex steroids, and in particular of E_2, may depend on the number of post-menopausal years (12).

Therefore, we aimed to investigate the association between endogenous E_2, androgens and SHBG levels and changes in body composition in postmenopausal women, and whether these associations depend on the number of post-menopausal years or BMI.

MATERIALS AND METHODS

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 (13). 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 (RS-III). Follow-up visits were held every 3-5years. The present study includes data from postmenopausal women from the first visit of the third cohort of RS (RS III-1, February 2006- December 2008) and from the second visit of the third cohort (RS III-2, March 2012- June 2014). 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.

Assessment of endogenous sex hormones

At baseline (RS III-1), fasting blood samples were collected and serum levels of sex steroids and sex-hormone binding globulin (SHBG) were determined. Estradiol (E_2) levels were estimated using the ultrasensitive RIA. The mean minimum detection limit of this test was 18.35pmol/ liter. Testosterone was estimated in single measurements by liquid chromatography-tandem mass spectrometry (LC-MS/MS). SHBG was measured with the Immulite platform (*Diagnostic Products Corporation, Breda, the Netherlands*). The Free androgen index (FAI), calculated as (T/ SHBG) * 100 was used as a surrogate measure of bioavailable T (14).

Assessment of body composition

Dual-energy X-ray absorptiometry (DXA) was used to assess the body composition of the participants at the first visit of the third cohort (RS III-1, February 2006- December 2008) and at the second visit of the third cohort (RS III-2, March 2012- June 2014). Whole body DXA scans were performed using a ProdigyTM and iDXA total body fan beam densitometers (GE Lunar Corp, Madison, WI, USA). Body weight (grams) was divided into fat mass, lean (non-fat) mass and bone mineral content. Android and gynoid fat mass was also assessed. The outcomes used were calculated as follow: total fat mass percentage = (total fat mass/ weight) *100; lean mass percentage = (lean mass/weight) *100; fat mass/lean mass ratio = total fat mass/ lean mass; android fat percentage = (android fat/total body fat) * 100; android/gynoid ratio = android fat/ gynoid fat. Assessment of covariates is described in details in the supplemental material **(Web Appendix)**.

Population for analyses

The present study uses data from the first and second visit of the third cohort (RS III-1, February 2006 – December 2008; RS III-2, March 2012- June 2014). There were 2252 women included in RS III-1, from which 567 were excluded because they were still in their pre-menopausal years, 242 women were excluded because they had no data on sex hormone levels and 260 women were excluded because of missing data on DXA scans during the first visit. Overall, we had 1183 women included in our analysis. Yet, 279 women had no data on DXA scans during the second visit and thus leaving 904 individuals with complete data on sex-steroids, SHBG and body composition (in both visits).

Statistical analyses

Continuous normally distributed data are presented as mean ±standard deviation, continuous non-normally distributed data are presented as median (interquartile range) and categorical variables are presented in percentages. To better approximate a normal distribution, natural log-transformation was used for the following variables: testosterone, SHBG, FAI, glucose, insulin and CRP. Estradiol was coded as 0 if it was lower than the detection limit (≤ 18.35pmol/L), otherwise as 1 (>18.35pmol/L). We fitted linear regression models using Generalized Estimating Equa-

tions with exchangeable correlation structure adjusting for the within-subject correlations due to repeated measurements of the outcomes in the same individuals (inter-class correlations for total fat mass percentage, lean mass percentage, android/gynoid ratio and weight are respectively 0.908; 0.877; 0.918; 0.956). To assess the annual changes in body composition related with levels of sex hormones and SHBG, we included in the model the interaction term of respective sex hormones and SHBG with time (coded as 1 for the baseline measurement and 5 for the second measurement which was done approximately 5 years after the baseline). Regression coefficients (βs) and 95% confidence intervals (95% CI) were obtained on the basis of robust standard errors. First, we calculated age, ethnicity and BMI in both visits (for fat mass percentage, lean mass percentage, fat mass/lean mass ratio, android fat percentage and android/gynoid ratio as outcome) (or height for weight as outcome) adjusted coefficients (Model 1). Subsequently, we adjusted for possible confounders (Model 2) including age at menopause, smoking status (current, never or former), alcohol intake (continuous), DHDI (continuous), systolic blood pressure (continuous), diastolic blood pressure (continuous), chronic diseases until the second DXA scan (yes, no), total cholesterol (continuous), hormone replacement therapy (yes, no), use of lipid lowering medication (yes, no), physical activity (continuous), testosterone and SHBG (for estradiol as exposure), SHBG for testosterone as exposure, testosterone and E_2 (for SHBG as exposure), E_2 (for FAI as exposure). In Model 3, we further adjusted for glucose, insulin and C-reactive protein (natural log-transformed, continuous) as we considered them as possible mediators. BMI, sex steroid levels and SHBG were considered time dependent covariates and the interaction term with time was included in the model. In the sensitivity analysis, to eliminate the differences of the body fat and lean mass associated with an individual's body size (surface), we assessed body fat mass index (BFMI) and fat-free mass index (FFMI) as outcomes. BFMI was calculated as total body fat mass (kg) divided by body surface (the square of the height in m i.e. m^2) whereas FFMI was calculated as difference between weight and total fat mass divided by square of height (15). We also performed stratified analyses by years since menopause comparing women in the first ten years of menopause and the ones with more than 10 years since menopause and by strata of baseline BMI (<25 and ≥25) since BMI might affect hormone levels in post menopause. Also, as sensitivity analysis, we restricted the analysis of estradiol only for women with estradiol levels above the detection limit. A P-value lower than 0.05 was considered as statistically significant. However, as sensitivity analysis, to account for multiple testing, we adjusted the p-value from 0.05 to 0.0166 by applying the Bonferroni correction for the number of exposures studied (N=3). To adjust for potential bias associated with missing data from the covariates we used multiple imputation procedure (N=5 imputations). All analyses were done using SPSS statistical software (SPSS, version 21.0; SPSS Inc., Chicago, Ilinois).

RESULTS

The main characteristics of the study population are shown in **Table 1**. E_2 levels were ≤ 18.35 pmol/l in 483 participants (40.8%). Median E_2, testosterone, SHBG and FAI levels were respectively 25.61pmol/l, 0.78 nmol/l, 56.15nmol/l and 1.41.

Table 1. Baseline characteristics of the study population: the Rotterdam Study.

Variable	
Age at entering Rotterdam Study (years)	59.25 (6.77)
Age at menopause (years)	47.96 (6.45)
Ethnicity	
Caucasian, N (%)	1132 (95.69%)
Non Caucasian, N (%)	51 (4.31%)
Chronic disease	
Yes, N (%)	270 (22.8%)
No, N (%)	913 (77.2%)
Hormone replacement therapy	
Yes, N (%)	34 (2.9%)
No, N (%)	1149 (97.1%)
Lipid lowering medication use	
Yes, N (%)	270 (22.8%)
No, N (%)	913 (77.2%)
Systolic blood pressure (mmHg)	131.57 (20.00)
Diastolic blood pressure (mmHg)	82.12 (11.13)
Alcohol* (g/day)	6.16 (0.69; 16.07)
Smoking status	
Current, N (%)	296 (25%)
Never or former, N (%)	887 (75%)
Dutch Healthy Diet Index	49.23 (9.34)
Serum total cholesterol (mmol/l)	5.84 (1.07)
Physical activity (MET hours/week)	70.47 (61.33)
Glucose* (mmol/l)	5.30 (4.90; 5.70)
Insulin* (pmol/l)	75.00 (54.00; 108.00)
C reactive protein* (mg/l)	1.30 (0.60; 2.80)
Estradiol* (pmol/l)	25.61 (18.35; 47.27)
Estradiol ≤ 18.35 pmol/l, N(%)	483 (40.8%)
Estradiol >18.35 pmol/l, N (%)	700 (59.2%)
Testosterone* (nmol/l)	0.78 (0.56; 1.08)
Sex hormone binding globulin(SHBG)* (nmol/l)	56.15 (40.22; 77.62)
Free androgen index (FAI)*	1.41 (0.94; 2.04)
Weight (kg) (RS III-1)	73.81 (13.15)
Fat mass percentage (RS III-1)	39.21 (6.94)
Lean mass percentage (RS III-1)	56.76 (7.04)
Android/Gynoid ratio (RS III-1)	0.52 (0.13)
Fat Mass Index (RS III-1)	11.05 (3.66)
Fat-free Mass Index (RS III-1)	16.45 (1.60)
Body mass index (kg/m^2)	27.50 (4.75)
Height (m)	163.86 (6.22)

*Median (Interquartile range)

After adjustment for age, BMI, ethnicity, chronic diseases, serum total cholesterol, blood pressure, age at menopause, hormone replacement therapy use and lifestyle factors, estradiol above the detection limit was associated with an annual decrease of 0.12% (95% CI -0.22, -0.03) in total fat mass, 0.003 (95% CI -0.006, -0.001) in fat mass/lean mass ratio, and an annual increase of 0.033% (95% CI 0.004, 0.062) in android fat (**Table 2**). No association was found between estradiol and weight or estradiol and lean mass (**Table 2**).

One log-transformed unit increase in T was associated with an annual decrease of 0.16% (95% CI -0.27, -0.02) in total fat mass, 0.006 (95% CI -0.008, -0.003) in fat mass/lean mass ratio and an annual increase of 0.21% (95% CI 0.10, 0.32) in lean mass (**Table 2**). There was no association between T and weight or T and android fat (**Table 2**). One log-transformed unit increase in FAI was associated with an annual decrease of 0.20kg (95% CI -0.37, -0.03) in weight, 0.18% (95% CI -0.27, -0.10) in total fat mass, 0.006 (95% CI -0.008, -0.003) in fat mass/lean mass ratio; an increase of 0.21% (95% CI 0.12,0.30) in lean mass and 0.04% (95% CI 0.01, 0.06) in android fat (**Table 2**).

One log-transformed unit increase in SHBG was associated with an annual increase of 0.25kg (95% CI 0.03, 0.47) in weight, 0.20% (95% CI 0.09, 0.30) in total fat mass, 0.006 (95% CI 0.003, 0.008) in fat mass/lean mass ratio and an annual decrease of 0.20% (95% CI -0.31, -0.09) in lean mass and 0.06% (95% CI -0.09, -0.03) in android fat (**Table 2**). These associations did not materially change when adjusting further for glucose, insulin and CRP. There was no association between endogenous sex hormone levels or SHBG and 5 years changes in android /gynoid fat ratio (**Table 2**).

Sensitivity Analysis

After applying Bonferroni correction, the associations between estradiol and fat mass and fat mass/lean mass ratio remained significant. Similarly, the associations between T and fat mass, lean mass and fat mass/lean mass ratio also remained significant. In addition, the associations of SHBG and FAI with fat mass, lean mas, fat mass/lean mass ratio and android fat were significant after Bonferroni correction. The associations between SHBG and weight, FAI and weight, estradiol and android fat did not reach the significance level at a p-value of 0.0166.

To evaluate the consistency of our findings, we reanalyzed the data using BFMI and FFMI as the outcomes. The results were similar to the ones observed when using total body fat mass (%) and lean mass (%) as outcomes (**Web Tables, Supplementary Table S1**). One log-transformed unit increase in T and FAI and one log-transformed unit decrease in SHBG were associated with annual decrease of 0.09 (95% CI: -0.15, -0.03), 0.11 (95% CI: -0.16, -0.05), 0.13 (95% CI: 0.06, 0.19) in fat mass index. Estradiol levels above the detection limit and one-log transformed unit increase in FAI were related to annual increase of 0.04 (95% CI: 0.01, 0.07) and 0.03 (95% CI: 0.002, 0.06) in fat-free mass index. The analysis on sex hormones and BMI provided similar results to body weight; one log-transformed unit increase in SHBG and one log-transformed unit decrease in FAI were related to annual increase of 0.09 (95% CI: 0.01, 0.17) and 0.07 (95% CI: -0.14, -0.01) in BMI, while no associations between estradiol or T and BMI was observed (**Web Tables, Supplementary Table S1**).

Table 2. Endogenous sex hormone levels and changes in body composition in postmenopausal women: the Rotterdam Study.

	Weight β (95% CI)			Fat mass % β (95% CI)		
Sex hormones	MODEL 1	MODEL 2	MODEL 3	MODEL 1	MODEL 2	MODEL 3
Estradiol	-0.008 (-0.179,0.163)	0.043 (-0.125,0.212)	0.048 (-0.120,0.217)	**-0.164 (-0.259,-0.068)***	**-0.123 (-0.220,-0.025)***	**-0.121 (-0.291,-0.023)***
Testosterone	-0.103 (-0.297,0.092)	-0.161 (-0.363,0.042)	-0.148 (-0.350,0.053)	**-0.122 (-0.227,-0.016)**	**-0.163 (-0.271,-0.055)***	**-0.163 (-0.272,-0.055)***
SHBG	**0.227 (0.016,0.438)**	**0.250 (0.029,0.471)**	**0.235 (0.015,0.455)**	**0.166 (0.063,0.269)***	**0.195 (0.089,0.301)***	**0.197 (0.091,0.302)***
FAI	**-0.198 (-0.369,-0.026)**	**-0.196 (-0.368,-0.025)**	**-0.186 (-0.337,-0.035)**	**-0.178 (-0.263,-0.093)***	**-0.180 (-0.265,-0.095)***	**-0.182 (-0.266,-0.097)***

	Lean mass % β (95% CI)			Fat mass/lean mass ratio β (95% CI)		
	MODEL 1	MODEL 2	MODEL 3	MODEL 1	MODEL 2	MODEL 3
Estradiol	**0.105 (0.006,0.203)**	0.052 (-0.048,0.152)	0.051 (-0.050,0.151)	**-0.005 (-0.007,-0.002)***	**-0.003 (-0.006,-0.001)***	**-0.003 (-0.006,-0.001)***
Testosterone	**0.169 (0.061,0.276)***	**0.211 (0.102,0.320)***	**0.211 (0.102,0.321)***	**-0.004 (-0.007,-0.002)***	**-0.006 (-0.008,-0.003)***	**-0.006 (-0.008,-0.003)***
SHBG	**-0.163 (-0.272,-0.055)***	**-0.203 (-0.315,-0.092)***	**-0.205 (-0.315,-0.094)***	**0.005 (0.002,0.007)***	**0.006 (0.003,0.008)***	**0.006 (0.003,0.008)***
FAI	**0.207 (0.119,0.294)***	**0.208 (0.121,0.296)***	**0.210 (0.122,0.298)***	**-0.006 (-0.008,-0.003)***	**-0.006 (-0.008,-0.003)***	**-0.006 (-0.008,-0.003)***

	Android fat % β (95% CI)			Android/gynoid ratio β (95% CI)		
	MODEL 1	MODEL 2	MODEL 3	MODEL 1	MODEL 2	MODEL 3
Estradiol	**0.040 (0.012,0.068)***	**0.033 (0.004,0.062)**	**0.033 (0.004,0.063)**	0.001 (-0.001,0.003)	0.001 (-0.001,0.003)	0.001 (-0.001,0.003)
Testosterone	0.004 (-0.029,0.037)	0.015 (-0.018,0.047)	0.014 (-0.019,0.047)	-0.001 (-0.004,0.001)	-0.001 (-0.003,0.001)	-0.001 (-0.003,0.001)
SHBG	**-0.055 (-0.085,-0.025)***	**-0.059 (-0.090,-0.028)***	**-0.059 (-0.089,-0.028)***	**-0.002 (-0.004,0.000)**	-0.002 (-0.004,0.000)	-0.002 (-0.004,0.000)
FAI	**0.037 (0.012,0.062)***	**0.037 (0.013,0.062)***	**0.036 (0.011,0.061)***	0.001 (-0.001,0.002)	0.001 (-0.001,0.002)	0.000 (-0.001,0.002)

FAI, Free androgen ratio, SHBG, Sex-hormone binding globulin.

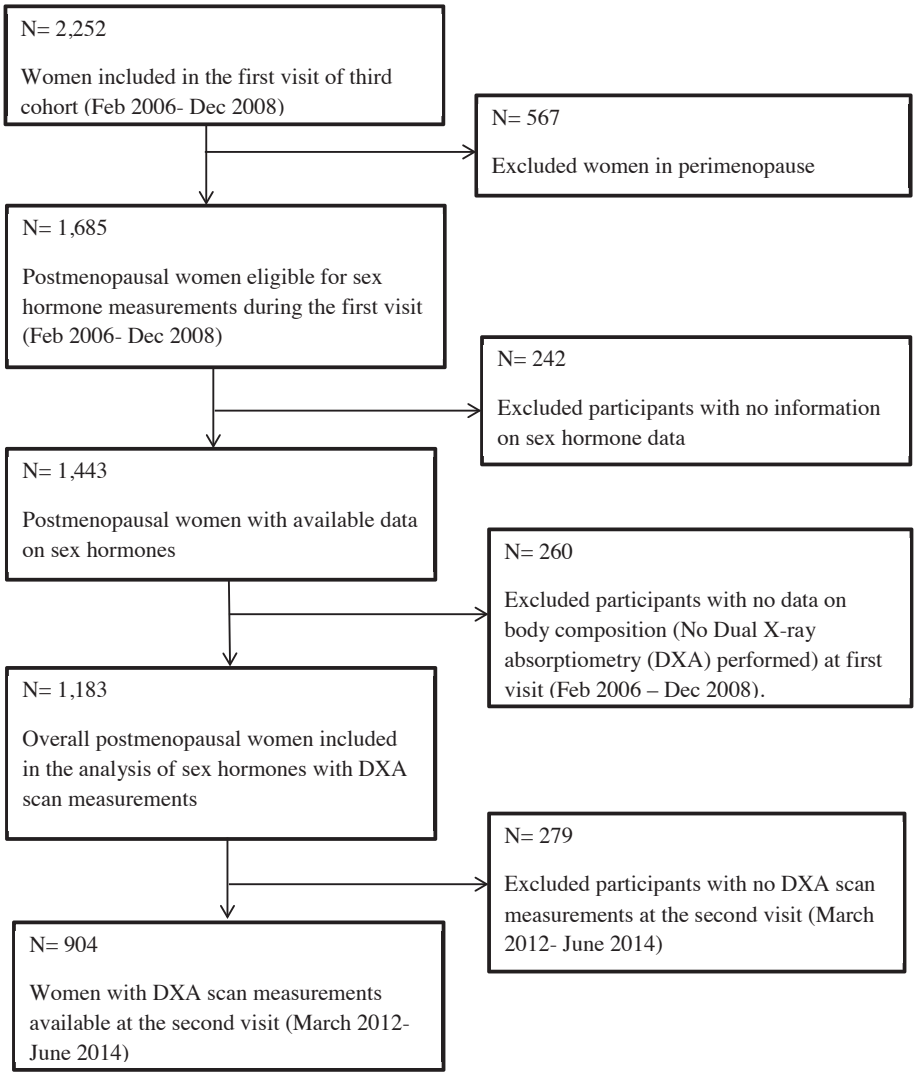
Model 1: Age, ethnicity, time, body mass index (trans_BMI) (for fat mass percentage, lean mass percentage, android/gynoid ratio as outcome), height (for weight as outcome)

Model 2: Model 1+ chronic disease, hormone replacement therapy, lipid lowering medication use, systolic blood pressure, diastolic blood pressure, age at menopause, alcohol, smoking status, Dutch Healthy Diet Index, serum total cholesterol, physical activity; total testosterone + total testosterone*time + SHBG + SHBG*time (for estradiol), SHBG + SHBG*time (for testosterone), total testosterone + total testosterone*time + estradiol + estradiol*time (for SHBG), estradiol + estradiol*time (for FAI)

Model 3: Model 2 + glucose, insulin, C-reactive protein

* Remains significant after Bonferroni correction

Figure 1. Flow chart of participants included in the analysis of endogenous sex hormones and body composition in postmenopausal women.



Stratified analyses for years since menopause (less than 10 years since menopause, more than 10 years since menopause) or by BMI (< 25, ≥ 25) found no difference between groups (**Web Tables, Supplemental table 2 and 3**). Analyses restricted to the subgroup of participants with estradiol levels above the detection limit (estradiol levels used as continuous) provided similar results as in overall population (data not shown).

DISCUSSION

The results of this prospective study suggest that endogenous sex steroid levels and SHBG are associated with 5 year changes in body composition in postmenopausal women. High E_2 levels were associated with a decrease in total fat mass, fat mass/lean mass ratio and an increase in android fat; high T levels were associated with a decrease in total fat mass, fat mass/lean mass ratio and an increase in lean mass; High SHBG levels were associated with an increase in weight, total fat mass, fat mass/lean mass ratio and a decrease in lean mass and android fat; high FAI levels were associated with a decrease in weight, total fat mass, fat mass/lean mass ratio and an increase in lean mass and android fat. No association was found for any of the sex steroids or SHBG with android/gynoid ratio.

Our null findings on E_2 levels, body weight and BMI are in line with randomized clinical trials in postmenopausal women showing no effect of hormone replacement therapy on these outcomes. The current study also provides evidence that E_2 levels are associated with decrease in total body fat and increase in lean mass. Studies in female mice have shown that supplementation with 17β-estradiol is not associated with changes in body weight but decreases adipose tissue, and that in ovariectomized mice, E_2 supplementation leads to higher phosphorylated levels of protein kinase A and hormone sensitive lipase, markers associated with lipolysis (16; 17). Limited data with conflicting results are available from observational studies in humans that examine the association between E_2 and changes in total body fat mass and lean mass in postmenopausal women (18; 19). Also, to date, few randomized controlled trials (RCTs) designed to examine the effect of hormone replacement therapy (HRT) on body fat mass and lean mass have included a small number of postmenopausal women, providing contradictory results (17). In line with our findings, a RCT including 33 postmenopausal women reported a decrease in FM:FFM ratio with estrogen therapy (20).

Estrogens have been suggested to affect distribution of fat in the body rather than total weight. In the current study we found that higher E_2 levels are associated with increased abdominal fat, however it remains unclear whether this increase is due to increases in visceral or subcutaneous fat or an increase in both compartments. Compared to subcutaneous fat, visceral adiposity remains more strongly associated with an adverse metabolic risk profile (21). Data from experimental studies show that E_2 promotes and maintains the typical female fat distribution through increasing the number of antilypolitic α2A-adrenergic receptors in subcutaneous fat tissue and has little or no effect on these receptors in the visceral fat tissue (7). This is supported from studies in premenopausal women showing no association between E_2 and 13 week changes in visceral fat (22). On the other hand, menopause, a state of extremely low estradiol levels, is associated with accumulation of visceral but not subcutaneous fat in women (23). Data from animal studies have suggested that, in postmenopausal women, estrogens accelerate the activation of 11 A hydroxysteroid dehydrogenase type 1 (11β-HSD1), which catalyzes the conversion of cortisol into cortisone resulting in an increase in visceral fat (24). However, studies in postmenopausal women have found no such association between E_2 and adipose 11β-HSD1 mRNA expression (25). On the contrary, two studies in postmenopausal women have reported positive correlations between 11β-HSD1 and aromatase mRNA; aromatase is the main enzyme responsible for the

conversion of androgens into estrogens, and it is highly expressed in fat tissue of postmenopausal women (25; 26). Also, E_2 acts through binding mainly with its nuclear receptors ERα and ERβ, both involved in obesity (27). However, the expression of these receptors may change depending on the menopausal status (28), and therefore, may impact on the role of E_2 in changing body fat. There are limited data however in postmenopausal women examining the expression of estrogen receptors in adipose tissue and the role of E_2 in the distribution of abdominal fat. Future research should focus on how E_2 and expression of its receptors in adipose tissue relate to changes in visceral and subcutaneous fat in postmenopausal women.

A recent study from Janssen et al. (29) investigating menopause-related changes in sex steroids with changes in adiposity in 243 women undergoing their menopausal transition, revealed that an increase of bioavailable testosterone was positively associated with changes in computed tomography-assessed visceral adipose tissue and subcutaneous abdominal adipose tissue but not with changes in total body fat as assessed by DXA. Similarly, we found that the FAI is associated with increase abdominal fat. However, contrary to the findings from Janssen et al., our data showed that testosterone and the FAI were both associated with a decreased total fat mass percentage and an increase in FFM. A RCT of postmenopausal women showed that both E_2 and testosterone supplementation increased FFM (20). The differences in the results between our study and Janssen et al. may be due to the fact that our population consisted only of postmenopausal women with median years since menopause around 10 years. Moreover, testosterone levels were lower in the current study compared to the ones measured in the Janssen's study because they were assessed during the menopausal transition. Finally, these authors only adjusted for baseline age, race/ethnicity, physical activity and smoking, while did not take into account BMI or adjustment of sex hormones for each other.

In our study, higher SHBG levels were associated with increased weight and total fat mass and decreased android fat. In contrast, another longitudinal study in 18 non-HRT postmenopausal women which used computed tomography measurements (30) reported that higher SHBG levels were positively associated with gain in intra-abdominal adipose tissue. However, the results of this study are limited due to the small number of participants and they only adjusted for baseline intra-abdominal adipose tissue and 2-year total body fat.

Initially the effect of SHBG was attributed to the amount of sex steroids it binds and thus less sex steroids are available to bind with the receptor and express their functions. Due to the higher affinity of testosterone for SHBG compared to estradiol, it was thought that it decreases the amount of bioavailable testosterone (decreases androgenicity) during the relative excess in menopause and post menopause. In our study, the association of SHBG remained significant even after adjusting for sex steroid levels which reveals an independent effect of SHBG on body composition. The mechanisms underlying this association are not clear yet and need further investigation.

Our study is unique among other investigations because of its prospective design, large population and adjustment for numerous confounders. However, possible limitations might be present. First, E_2 levels were under the detection limit in 40.8% of the population and the analyses were performed by categorizing the values into 0 if estradiol levels were ≤ 18.35 pmol/l and 1 if the values were < 18.35 pmol/l. Categorization of a continuous variable introduces loss of information and power. Nevertheless, sensitivity analyses using E_2 as a continuous variable in 59.2% of the

population showed similar results (data not shown.) Secondly, these measurements include E_2 and no data on bioavailable estradiol were available. Thirdly, only data on E_2 levels were available whereas estrone (E_1) is the dominant estrogen in postmenopausal women (31) but it has lower affinity for the estrogen receptors compared to E_2. A study from Goss et al. (30) in 53 women concluded that a high proportion of weak estrogens may promote fat partitioning to the intra-abdominal cavity over time. Fourth, because epidemiological studies might be prone to unmeasured confounding and reverse causality, our findings do not provide a causal direction of the association between sex hormones and body composition. Last but not least, we did not find any association between sex steroids and AF/GF ratio which may reflect no role of sex steroids in the distribution of body fat. However, our null findings might be attributed to very small changes in A/G ratio and larger longitudinal studies with more variation in A/G ratio might provide more insights into the role of sex steroids in the distribution of body fat. Therefore, we might lack statistical power to detect such changes.

To conclude, these findings support the notion that endogenous sex steroid levels may lead to changes in body composition in postmenopausal women. Further studies are needed to reinforce our findings and to identify the potential mechanisms behind these associations.

AKNOWLEDGEMENTS:

The contributions of the authors were as follows: Dr. Najada Stringa Dr. Taulant Muka and Dr. Oscar H Franco participated in the manuscript conception, statistical analyses, data interpretation, manuscript writing and revising and have primary responsibility for the final content of the manuscript; Dr. Jessica Kiefte-de Jong and Dr. Adela Brahimaj participated in data interpretation and revising the manuscript. Dr. Fernando Rivadeneira provided the data of the DXA scans; Dr. Katerina Trajanoska did the data cleaning of DXA scans. Dr. Joop Laven provided the data of the sex steroids. All authors critically reviewed, read and approved the final manuscript.

FUNDING SOURCES:

The Rotterdam Study is funded by Erasmus MC and Erasmus University, Rotterdam, the Netherlands; the Netherlands Organization for Scientific Research (NWO); the Netherlands Organization for the Health Research and Development (ZonMw); the Research Institute for Diseases in the Elderly (RIDE); the Ministry of Education, Culture and Science; the Ministry for Health, Welfare and Sports; the European Commission (DG XII); and the Municipality of Rotterdam.

REFERENCES

1. Milewicz A, Tworowska U, Demissie M: Menopausal obesity--myth or fact? Climacteric 2001;4:273-283
2. Douchi T, Yamamoto S, Nakamura S, Ijuin T, Oki T, Maruta K, Nagata Y: The effect of menopause on regional and total body lean mass. Maturitas 1998;29:247-252
3. Fu X, Song A, Zhou Y, Ma X, Jiao J, Yang M, Zhu S: Association of regional body fat with metabolic risks in Chinese women. Public health nutrition 2014;17:2316-2324
4. Kang SM, Yoon JW, Ahn HY, Kim SY, Lee KH, Shin H, Choi SH, Park KS, Jang HC, Lim S: Android fat depot is more closely associated with metabolic syndrome than abdominal visceral fat in elderly people. PLoS One 2011;6:e27694
5. Peder W, Fredrik T, Lars W, Göran H, Paul WF, Anna N, Peter N: Abdominal and Gynoid Fat Mass Are Associated with Cardiovascular Risk Factors in Men and Women. The Journal of Clinical Endocrinology & Metabolism 2008;93:4360-4366
6. Davison SL, Bell R, Donath S, Montalto JG, Davis SR: Androgen levels in adult females: changes with age, menopause, and oophorectomy. J Clin Endocrinol Metab 2005;90:3847-3853
7. Pedersen SB, Kristensen K, Hermann PA, Katzenellenbogen JA, Richelsen B: Estrogen controls lipolysis by up-regulating alpha2A-adrenergic receptors directly in human adipose tissue through the estrogen receptor alpha. Implications for the female fat distribution. J Clin Endocrinol Metab 2004;89:1869-1878
8. Biundo B, Gogola M: Estradiol: THE EMERGING EVIDENCE FOR A PROTECTIVE ROLE AGAINST INSULIN RESISTANCE AND OBESITY. Int J Pharm Compd 2015;19:289-293
9. Notelovitz M: Androgen effects on bone and muscle. Fertility and Sterility 2002;77, Supplement 4:34-41
10. Toth MJ, Tchernof A, Sites CK, Poehlman ET: Menopause-related changes in body fat distribution. Ann N Y Acad Sci 2000;904:502-506
11. Bowling MR, Xing D, Kapadia A, Chen YF, Szalai AJ, Oparil S, Hage FG: Estrogen effects on vascular inflammation are age dependent: role of estrogen receptors. Arterioscler Thromb Vasc Biol 2014;34:1477-1485
12. Rossouw JE, Prentice RL, Manson JE, Wu L, Barad D, Barnabei VM, Ko M, LaCroix AZ, Margolis KL, Stefanick ML: Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. Jama 2007;297:1465-1477
13. Hofman A, Brusselle GG, Darwish Murad S, van Duijn CM, Franco OH, Goedegebure A, Ikram MA, Klaver CC, Nijsten TE, Peeters RP, Stricker BH, Tiemeier HW, Uitterlinden AG, Vernooij MW: The Rotterdam Study: 2016 objectives and design update. Eur J Epidemiol 2015;30:661-708
14. Rosner W, Auchus RJ, Azziz R, Sluss PM, Raff H: Position statement: Utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement. J Clin Endocrinol Metab 2007;92:405-413
15. VanItallie TB, Yang MU, Heymsfield SB, Funk RC, Boileau RA: Height-normalized indices of the body's fat-free mass and fat mass: potentially useful indicators of nutritional status. Am J Clin Nutr 1990;52:953-959
16. Stubbins RE, Najjar K, Holcomb VB, Hong J, Nunez NP: Oestrogen alters adipocyte biology and protects female mice from adipocyte inflammation and insulin resistance. Diabetes Obes Metab 2012;14:58-66
17. Norman RJ, Flight IH, Rees MC: Oestrogen and progestogen hormone replacement therapy for peri-menopausal and post-menopausal women: weight and body fat distribution. Cochrane Database Syst Rev 2000:CD001018
18. Davis SR, Castelo-Branco C, Chedraui P, Lumsden MA, Nappi RE, Shah D, Villaseca P: Understanding weight gain at menopause. Climacteric 2012;15:419-429
19. Douchi T, Yamamoto S, Yoshimitsu N, Andoh T, Matsuo T, Nagata Y: Relative contribution of aging and menopause to changes in lean and fat mass in segmental regions. Maturitas 2002;42:301-306
20. Davis SR, Walker KZ, Strauss BJ: Effects of estradiol with and without testosterone on body composition and relationships with lipids in postmenopausal women. Menopause 2000;7:395-401
21. Fox CS, Massaro JM, Hoffmann U, Pou KM, Maurovich-Horvat P, Liu CY, Vasan RS, Murabito JM, Meigs JB, Cupples LA, D'Agostino RB, Sr., O'Donnell CJ: Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study. Circulation 2007;116:39-48
22. Leenen R, van der Kooy K, Seidell JC, Deurenberg P, Koppeschaar HP: Visceral fat accumulation in relation to sex hormones in obese men and women undergoing weight loss therapy. J Clin Endocrinol Metab 1994;78:1515-1520
23. Lovejoy JC, Champagne CM, de Jonge L, Xie H, Smith SR: Increased visceral fat and decreased energy expenditure during the menopausal transition. Int J Obes (Lond) 2008;32:949-958

24. Tagawa N, Yuda R, Kubota S, Wakabayashi M, Yamaguchi Y, Kiyonaga D, Mori N, Minamitani E, Masuzaki H, Kobayashi Y: 17Beta-estradiol inhibits 11beta-hydroxysteroid dehydrogenase type 1 activity in rodent adipocytes. J Endocrinol 2009;202:131-139
25. Andersson T, Simonyte K, Andrew R, Strand M, Buren J, Walker BR, Mattsson C, Olsson T: Tissue-specific increases in 11beta-hydroxysteroid dehydrogenase type 1 in normal weight postmenopausal women. PLoS One 2009;4:e8475
26. Yamatani H, Takahashi K, Yoshida T, Takata K, Kurachi H: Association of estrogen with glucocorticoid levels in visceral fat in postmenopausal women. Menopause 2013;20:437-442
27. Brown LM, Clegg DJ: Central Effects of Estradiol in the Regulation of Adiposity. The Journal of steroid biochemistry and molecular biology 2010;122:65-73
28. Sakaguchi H, Fujimoto J, Aoki I, Tamaya T: Expression of estrogen receptor alpha and beta in myometrium of premenopausal and postmenopausal women. Steroids 2003;68:11-19
29. Janssen I, Powell LH, Jasielec MS, Kazlauskaite R: Covariation of change in bioavailable testosterone and adiposity in midlife women. Obesity (Silver Spring) 2015;23:488-494
30. Goss AM, Darnell BE, Brown MA, Oster RA, Gower BA: Longitudinal associations of the endocrine environment on fat partitioning in postmenopausal women. Obesity (Silver Spring) 2012;20:939-944
31. Grodin J, Siiteri P, MacDonald P: Source of Estrogen Production in Postmenopausal Women 1. The Journal of Clinical Endocrinology & Metabolism 1973;36:207-214

WEB APPENDIX

Supplementary methods

Assessment of covariates

Information on current health status, medical history, medication use and smoking status were assessed at the home interview in RS III-1. Physical height (at RS III-1) was measured with the participants standing without shoes and heavy outer garments. BMI was calculated as weight divided by height squared (kg/m^2). Participants were asked if they were currently smoking cigarettes, cigars or pipe and were subsequently categorized in current smokers vs. never or former smokers. Alcohol intake was converted in grams of ethanol per day. The Dutch Healthy Diet Index (DHDI) was used to take into account overall dietary quality. The DHDI represents compliance to the Dutch guidelines for a healthy diet as assessed from the Food Frequency Questionnaire (FFQ) (1) at baseline (RS III-1). Blood pressure was measured in sitting position at the right upper arm with a random-zero-sphygmomanometer. Cardiovascular diseases were defined as a history of myocardial infarction, coronary artery bypass, or percutaneous transluminal coronary angioplasty (2). Type 2 diabetes mellitus was diagnosed if a random serum glucose level was ≥ 11mmol/L or if a person used glucose lowering drugs (3). Development of chronic diseases was defined as the presence of cardiovascular diseases, type 2 diabetes mellitus or cancer at baseline (RS III-1) and until the date of the second DXA measurement. Total cholesterol, C-reactive protein (CRP), glucose and insulin levels were determined by automated enzymatic procedures in non-fasting blood samples. Physical activity was assessed using the LASA Physical Activity Questionnaire (LAPAQ) and is expressed in MET hours/week (4).

Supplementary Table 1. Endogenous sex hormone levels in relation with body fat mass index, fat-free mass index and BMI: the Rotterdam Study.

	Body fat mass index						**Fat-free mass index**					
	Model 1		Model 2		Model 3		Model 1		Model 2		Model 3	
Sex hormones	**β**	**95% CI**	**β**	**95% CI**	**β**	**95% CI**	**β**	**95% CI**	**β**	**95% CI**	**β**	**95% CI**
Estradiol	**-0.056**	**-0.108, -0.003**	-0.031	-0.083, 0.021	-0.029	-0.082, 0.023	**0.381**	**0.193,0.569**	**0.042**	**0.012,0.073**	**0.043**	**0.013,0.073**
Testosterone	-0.062	-0.125, 0.001	**-0.089**	**-0.153, -0.025**	**-0.085**	**-0.150, -0.021**	0.021	-0.011,0.052	0.025	-0.007,0.057	0.027	-0.005,0.059
SHBG	**0.110**	**0.049, 0.172**	**0.125**	**0.061, 0.189**	**0.121**	**0.057, 0.184**	-0.024	-0.059,0.011	-0.030	-0.066,0.007	-0.032	-0.069,0.004
FAI	**-0.105**	**-0.156, -0.054**	**-0.105**	**-0.156, -0.053**	**-0.101**	**-0.151, -0.052**	**0.028**	**0.001,0.056**	**0.029**	**0.002,0.056**	**0.031**	**0.003,0.058**
			BMI									
	Model 1		Model 2		Model 3							
	β	**95% CI**	**β**	**95% CI**	**β**	**95% CI**						
Estradiol	-0.006	-0.070, 0.057	0.013	-0.050, 0.075	0.015	-0.048, 0.077						
Testosterone	-0.042	-0.114, 0.031	-0.063	-0.138, 0.012	-0.058	-0.133, 0.017						
SHBG	**0.082**	**0.003, 0.160**	**0.091**	**0.009,0.173**	**0.085**	**0.004, 0.166**						
FAI	**-0.074**	**-0.137, -0.011**	**-0.073**	**-0.136,-0.010**	**-0.069**	**-0.125,-0.014**						

Model 1: Age, ethnicity, time, height

Model 2: Model 1+ chronic disease, hormone replacement therapy, lipid lowering medication use, systolic blood pressure, diastolic blood pressure, age at menopause, alcohol, smoking status, Dutch Healthy Diet Index, serum total cholesterol, physical activity; total testosterone + total testosterone*time + SHBG + SHBG*time (for estradiol), SHBG + SHBG*time (for testosterone), total testosterone + total testosterone*time + estradiol + estradiol*time (for SHBG), estradiol + estradiol*time (for FAI)

Model 3: Model 2 + glucose, insulin, C-reactive protein

Supplementary Table 2. Endogenous sex hormone levels and changes in body composition in postmenopausal women, stratified by years since menopause: the Rotterdam Study.

		MODEL 3 β (95% CI)					
Sex hormones	**Years since menopause**	Weight	Fat mass %	Lean mass %	Fat mass/ lean mass ratio	Android fat %	Android/ Gynoid ratio
Estradiol	**<10 years**	0.047 (-0.163,0.257)	-0.101 (-0.227,0.025)	0.065 (-0.067,0.197)	-0.003 (-0.006,0.001)	0.012 (-0.026,0.050)	0.000 (-0.003,0.003)
	≥10 years	0.048 (-0.244,0.340)	-0.158 (-0.318,0.002)	0.048 (-0.106,0.201)	-0.004 (-0.008,0.000)	**0.066 (0.017,0.115)**	0.003 (0.000,0.007)
Testosterone	**<10 years**	**-0.255 (-0.495,-0.016)**	**-0.161 (-0.309,-0.012)**	**0.188 (0.032,0.345)**	**-0.006 (-0.009,-0.002)**	0.023 (-0.026,0.072)	-0.001 (-0.004,0.002)
	≥10 years	0.012 (-0.340,0.365)	**-0.159 (-0.315,-0.004)**	**0.237 (0.089,0.386)**	**-0.006 (-0.010,-0.001)**	0.006 (-0.048,0.059)	-0.001 (-0.005,0.003)
SHBG	**<10 years**	0.239 (-0.022,0.500)	**0.166 (0.025,0.308)**	**-0.213 (-0.368,-0.059)**	**0.005 (0.001,0.009)**	**-0.049 (-0.090,-0.007)**	-0.002 (-0.005,0.001)
	≥10 years	0.201 (-0.192,0.593)	**0.239 (0.070,0.407)**	**-0.202 (-0.364,-0.040)**	**0.007 (0.002,0.011)**	**-0.073 (-0.123,-0.023)**	-0.003 (-0.007,0.000)
FAI	**<10 years**	**-0.247 (-0.0446,-0.047)**	**-0.165 (-0.281,-0.049)**	**0.202 (0.078,0.325)**	**-0.005 (-0.008,-0.002)**	**0.037 (0.000,0.073)**	0.000 (-0.002,0.003)
	≥10 years	-0.083 (-0.394,0.228)	**-0.199 (-0.323,-0.076)**	**0.223 (0.102,0.345)**	**-0.006 (-0.009,-0.003)**	0.037 (-0.004,0.077)	0.001 (-0.002,0.004)

FAI, Free androgen ratio; SHBG, Sex-hormone binding globulin

Model 3: Age, ethnicity, time, body mass index (trans_BMI) (for fat mass percentage, lean mass percentage, android/gynoid ratio as outcome), height (for weight as outcome), chronic disease, hormone replacement therapy, lipid lowering medication use, systolic blood pressure, diastolic blood pressure, age at menopause, alcohol, smoking status, Dutch Healthy Diet Index, serum total cholesterol, physical activity, glucose, insulin, C-reactive protein; total testosterone + total testosterone*time + SHBG + SHBG*time (for estradiol), SHBG + SHBG*time (for testosterone), total testosterone + total testosterone*time + estradiol + estradiol*time (for SHBG), estradiol + estradiol*time (for FAI)

Supplementary Table 3. Endogenous sex hormone levels and changes in body composition in postmenopausal women, stratified by BMI: the Rotterdam Study.

Sex hormones		MODEL 3 β (95% CI)					
	BMI	Weight	Fat mass %	Lean mass %	Fat mass/ lean mass ratio	Android fat %	Android/ Gynoid ratio
Estradiol	**<25**	0.186 (-0.039,0.411)	0.067 (-0.144,0.279)	-0.124 (-0.355,0.107)	0.002 (-0.003,0.008)	0.014 (-0.042,0.069)	0.000 (-0.004,0.004)
	≥25	-0.011 (-0.244,0.222)	-0.131 (-0.269,0.007)	0.050 (-0.099,0.199)	-0.004 (-0.008,0.001)	0.025 (-0.011,0.062)	0.001 (-0.001,0.004)
Testosterone	**<25**	-0.155 (-0.403,0.093)	-0.115 (-0.364,0.134)	0.110 (-0.138,0.359)	-0.003 (-0.009,0.003)	-0.046 (-0.119,0.027)	-0.003 (-0.008,0.002)
	≥25	-0.125 (-0.404,0.155)	**-0.187 (-0.356,-0.017)**	**0.260 (0.087,0.434)**	**-0.007 (-0.013,-0.002)**	0.003 (-0.037,0.042)	-0.002 (-0.005,0.001)
SHBG	**<25**	0.143 (0.000,0.286)	0.089 (-0.175,0.352)	-0.006 (-0.297,0.285)	0.001 (-0.006,0.007)	0.022 (-0.051,0.096)	0.001 (-0.004,0.007)
	≥25	0.243 (-0.072,0.558)	0.115 (-0.036,0.266)	-0.163 (-0.328,0.002)	**0.006 (0.000,0.011)**	0.002 (-0.037,0.041)	0.000 (-0.003,0.003)
FAI	**<25**	-0.146 (-0.358,0.065)	-0.101 (-0.303,0.102)	0.065 (-0.146,0.277)	-0.002 (-0.007,0.003)	-0.035 (-0.094,0.024)	-0.002 (-0.006,0.002)
	≥25	-0.177 (-0.417,0.063)	**-0.155 (-0.279,-0.030)**	**0.213 (0.082,0.345)**	**-0.007 (-0.011,-0.003)**	0.001 (-0.028,0.030)	-0.001 (-0.003,0.001)

FAI, Free androgen ratio; SHBG, Sex-hormone binding globulin

Model 3: Age, ethnicity, time, chronic disease, hormone replacement therapy, lipid lowering medication use, systolic blood pressure, diastolic blood pressure, age at menopause, alcohol, smoking status, Dutch Healthy Diet Index, serum total cholesterol, physical activity, glucose, insulin, C-reactive protein; total testosterone + total testosterone*time + SHBG + SHBG*time (for estradiol), SHBG + SHBG*time (for testosterone), total testosterone + total testosterone*time + estradiol + estradiol*time (for SHBG), estradiol + estradiol*time (for FAI)

SUPPLEMENTARY REFERENCES

1. van Lee L, Geelen A, van Huysduynen EJ, de Vries JH, van't Veer P, Feskens EJ: The Dutch Healthy Diet index (DHD-index): an instrument to measure adherence to the Dutch Guidelines for a Healthy Diet. Nutr J 2012;11:49
2. Leening MJ, Kavousi M, Heeringa J, van Rooij FJ, Verkroost-van Heemst J, Deckers JW, Mattace-Raso FU, Ziere G, Hofman A, Stricker BH, Witteman JC: Methods of data collection and definitions of cardiac outcomes in the Rotterdam Study. Eur J Epidemiol 2012;27:173-185
3. World Health Organisation . Definition and diagnosis of diabetes mellitus and intermediate hyperglicemia: report of a WHO/IDF consultation. . World Health Organisation, Geneva, 2006, p. 1-50
4. Stel VS, Smit JH, Pluijm SM, Visser M, Deeg DJ, Lips P: Comparison of the LASA Physical Activity Questionnaire with a 7-day diary and pedometer. J Clin Epidemiol 2004;57:252-258

Chapter 5

General discussion

GENERAL DISCUSSION

Type 2 diabetes is a serious and common chronic disease with a complex etiology involving genetics and lifestyle factors. According to American Diabetes association, diabetes causes more deaths than breast cancer and AIDS combined (1). Type 2 diabetes and its complications lower the quality of people's lives and generate enormous economic and social burdens, constituting a global public health problem (2). Knowing its importance, the diversity of its etiology (3) and the fact that type 2 diabetes can be prevented or delayed (4), a further elucidation of its risk factors and pathophysiology is required. Susceptibility to type 2 diabetes is determined by a complex interaction of multiple risk factors. To further understand the disease pathophysiology, a combination of basic, clinical, and population-based scientific approaches are warranted. Chronic low-grade inflammation, body fat composition and sex hormones are among the most important factors that enhance the risk of type 2 diabetes. The aim of this thesis was to further explore the role of pathways that link the above mentioned risk factors to development of type 2 diabetes. Moreover, we checked for potential gender differences in the relation of the newly identified risk markers with type 2 diabetes.

In this general discussion, the main findings of this thesis will be summarized, after which methodological considerations will be addressed. This chapter will be concluded with reflections upon clinical implications and directions for future research.

MAIN FINDINGS

Inflammation and type 2 diabetes

Inflammation is an essential component of immunosurveillance and host defense, yet a chronic low-grade inflammatory state is a pathological component of a wide range of chronic conditions, such as the metabolic syndrome , non-alcoholic fatty liver disease (NAFLD), cardiovascular diseases as well as type 2 diabetes (5; 6).

In **chapter 2.1** we investigated a wide range of inflammatory markers, in a phase-specific approach, regarding progression from normoglycemia to prediabetes, type 2 DM and initiation of insulin therapy. We identified EN-RAGE, IL13 and IL17 as novel inflammatory markers that are related to risk of type 2 DM. Higher EN-RAGE levels were associated with an increased risk of incident pre-diabetes, whereas higher IL13 levels were associated with a decreased risk of pre-diabetes, incident type 2 DM and need for insulin therapy. Higher IL17 levels were associated with a decreased risk of incident type 2 DM. In addition, we reconfirmed the previously reported associations between high CRP levels and the increased risk for type 2 diabetes (7-9)

Obesity and diabetes are generally preluded by disequilibrium in the redox balance, implicated as one of the sources of systemic low-grade chronic inflammation (10). Mainly, previous literature provides evidence on the role of individual antioxidants on inflammation, which neglects the interactive effect among nutrients (11). The link between the overall capacity of diet and inflammation, which is likely to be more informative, is not established yet. Hence, in **chapter 2.2** we evaluated whether total dietary antioxidant capacity (assessed by dietary ferric reducing

antioxidant potential (FRAP)), serum uric acid (UA) and gamma glutamyltransferase (GGT) were associated with low-grade chronic inflammation and circulating adipocytokines. We found no consistent association between FRAP and CRP levels, while both UA and GGT were associated with low CRP. Furthermore, high overall dietary antioxidant capacity and lower levels of UA were associated with lower levels of pro-inflammatory adipocytokines and higher levels of anti-inflammatory adipocytokines. This chapter strengthened the evidence regarding the protective role of an antioxidant rich diet against low-grade chronic inflammation that might consequently be linked to its associated diseases such as type 2 diabetes (12; 13). Hyperuricemia, which is increasing worldwide, has been thought to be part of the cluster of metabolic abnormalities together with glucose intolerance and dyslipidemia (14) and therefore a potential predictor of type 2 diabetes (15). Moreover, uric acid produces an inflammatory response through activation of NF-κB in the Hypothalamus, having implications for the pathogenesis of metabolic disorders (16). Limited evidence is available about the association between serum uric acid and different stages of the spectrum from normoglycaemia to type 2 diabetes mellitus. Thus, in **chapter 2.3** we investigated the association between serum uric acid and risk of prediabetes and type 2 diabetes mellitus. Our findings supported uric acid as a marker more closely related to early-phase mechanisms in the development of type 2 diabetes mellitus than late-phase mechanisms.

Lipids, body fat and type 2 diabetes

Increasing evidence show that body composition components, mainly body fat (17; 18)and specifically visceral fat (19) as well as lipid profiles (20; 21) are responsible for increased type 2 diabetes risk. However, the so-called metabolically unhealthy lean phenotypes, such as lean diabetics, point towards the complexity of the relation between body fat and lipid profile (22). Therefore, optimizing risk markers for type 2 diabetes requires exploration both in lipid profile and body fat level. A low level of HDL cholesterol is a known risk factor for type 2 diabetes that precedes the onset of the disease (23). In **chapter 3.1** we investigated the associations of serum levels of apolipoproteins (apoA1, apoCIII, apoD, apoE), HDL-C, and the ratios of the apolipoproteins to apoA1 and their apolipoproteinic score with the risk of type 2 diabetes. We found that serum levels of apoCIII and the apoCIII-to-apoA1 ratio were associated with risk of type 2 diabetes independent of known risk factors and were stronger biomarkers than HDL-C level. Aside from the observed association between HDL-C and type 2 diabetes, these findings highlight the role of variation in composition of HDL particles and other lipoproteins in relation to the disease. In regards to body composition, the latest evidence highlights visceral fat as the most risky fat portion for type 2 diabetes development (24). Although expensive methods such as computed tomography (CT) and magnetic resonance imaging (MRI) (25) are the golden standard in measurement of visceral fat, a reliable cost-effective indicator (26; 27) may also be useful and contribute to risk prediction for type 2 diabetes. In **chapter 3.2** we investigated the associations of different novel metabolic indices, which combine both anthropometric and laboratory measures, and their components with incident type 2 diabetes, among women and men. We further assessed the associations of truncal fat depot measured by DXA with incident type 2 diabetes. We found that among women, novel combined metabolic indices were stronger risk markers for type 2 diabetes than the traditional anthropometric and laboratory measures and were comparable to DXA measures. Neither

combined metabolic indices nor DXA measures were superior to traditional anthropometric and lipid measures in association with type 2 diabetes among men. Furthermore, in **chapter 3.3** we investigated the associations of CT measured epicardial fat volume (a portion of visceral fat) with incident events of type 2 diabetes, coronary heart disease and stroke among women and men. Our findings suggest that epicardial fat volume may be associated with excessive risk for type 2 diabetes, coronary heart disease and stroke among women and men, independent of traditional diabetes and cardiovascular risk factors.

Sex hormones and type 2 diabetes

While previous studies indicate that endogenous sex hormones may differentially modulate glycemic status and risk of type 2 diabetes in women and men (28), it remains unclear whether the effects of DHEA on risk of type 2 diabetes are different in women and men. Therefore, in **chapter 4.1** we prospectively examined the associations between
DHEA and its main derivatives DHEAS and androstenedione, as well as the ratio of DHEAS to DHEA with incident type 2 diabetes among healthy middle-aged and elderly men and women. We found that DHEA serum levels might be an independent marker of type 2 diabetes in healthy populations of both men and postmenopausal women. Available evidence suggests a stronger association between adiposity and risk of type 2 diabetes among women compared with men (29). In **chapter 4.2** we investigated the association between endogenous estradiol, androgens and SHBG levels and changes in body composition in postmenopausal women, and whether these associations depend on the number of post-menopausal years or BMI. The results suggested that endogenous sex steroid levels and SHBG were associated with 5 year changes in body composition in postmenopausal women, indirectly affecting (30) the risk for type 2 diabetes development.

METHODOLOGICAL CONSIDERATIONS

Prospective cohort design: promises and pitfalls

The studies in this thesis were performed in the Rotterdam Study, a prospective population-based cohort study (31). Cohort design is a standard robust design in observational epidemiological studies with common health outcomes (32; 33). The design of the prospective cohort study allows for various outcomes to be studied in relation to the exposures. However, the efficiency of prospective cohort studies decreases with lower incidence of the outcome since a large population should be followed for a long time to observe cases of the disease (34). As such, the prospective design of the Rotterdam Study provides an appropriate framework to study outcomes such as prediabetes, type 2 diabetes and initiation of insulin therapy. The distinguishing feature of a prospective cohort study is that at the time of enrollment of subjects and collection of baseline exposure information, none of the subjects has developed any of the outcomes of interest. Therefore, measurement of the exposure is not affected by occurrence of the disease. This issue causes recall bias in case-control design. According to Bradford Hill`s criteria, the temporal relationship is the only absolutely essential condition for causality (35). To believe that an exposure causes

a certain disease, it must always precede the occurrence of the disease. Cross-sectional and case-control designs could not provide strong evidence for temporal relation. Prospective design allows for assessment of the temporality of the studied associations due to the fact that exposure and lack of the disease is investigated in the beginning of the study.

The prospective nature of cohort studies further minimizes the chance of reverse causation, given that the disease always occurs after a certain time under exposure (follow-up time). In reverse causality, the exposure is affected by early stages of the disease. Cohort design allows the researcher to assess the participants for the disease and observe any early sign of it. Nonetheless, reverse causation cannot be totally eliminated, since not all diseases could be assessed in early stages and it is challenging to set an optimal criteria for early stage disease. Finally, when the studied disease includes the interaction of many diverse pathways, which is the case for type 2 diabetes, choosing early stage disease is more complex (36). Due to its complex etiology, for type 2 diabetes research, both reverse causality and simultaneity are important issues to be considered. For instance, when we study the association of an inflammatory marker serum level with risk of type 2 diabetes, thanks to the prospective design of our study, we can be more confident that the measurements indicate the biomarker levels before the occurrence of the disease. However, knowing that inflammation can be both a trigger and a feature of type 2 diabetes, we cannot totally exclude the possibility of the reverse causation. Depending on the related pathway of the investigated marker, its serum level might be affected by presence of prediabetes or type 2 diabetes. A potential scenario could be the vicious circle: a certain inflammatory marker leads to type 2 diabetes and once the disease is present, a certain pathway activates, which increases the exposure serum level. The same logic applies also when associations between lipid profiles or obesity and type 2 diabetes are studied. Thus, despite the broad view of the risk factors for type 2 diabetes provided by observational epidemiology, a final conclusion on causation is disputed. Alternative approaches such as Mendelian randomization studies have gained a lot of popularity in recent years that offer new opportunities to investigate casual relation (37).

Considerations regarding the studied inflammatory markers and apolipoproteins

Most of the studied inflammatory markers and apolipoproteins in this thesis were measured for the first time to date through expensive innovative methods. Given the relatively long follow-up of our study, it would have been more interesting if a final measurement close to the end of the observation period would have been available for comparison. Due to limited available resources, we did not have repeated measurements of these biomarkers during the follow-up. However, blood levels of these biomarkers are stable over time (38; 39) and therefore studying the association of a single time point measurement with future clinical events is yet of value and unlikely to be subject to information bias. For the same reason of the limited resources, the markers were measured in a small random sample of 971 subjects in the third visit of the first cohort of the Rotterdam Study. Nevertheless, the sample size was large enough to find a number of significant associations (40; 41). As a sensitivity analysis, to identify the most robust findings in every analysis, we applied a conservative Bonferroni corrected p value of 1.9×10^{-3} (0.05/26 markers).

Measurement of the apolipoproteins, studied in **chapter 3.1**, is less common than inflammatory markers. Few previous studies report measures of apolipoproteins A1 and B and their ability to enable identification of individuals at increased risk of cardiovascular disease or type 2 diabetes (42; 43). However, lack of standardized methods to measure these risk markers has so far resulted in the non-comparability of values and often a conflicting interpretation of clinical studies. Reference values for apolipoproteins depend on the choice of assay technique and therefore the chosen technique should be taken into account when comparing the values (44).

Pros and cons of using combined metabolic indices

The recently introduced combined metabolic indices including VAI, LAP and TyG, mainly combine different anthropometric and laboratory measures, each of them providing information from a different source somehow related to type 2 diabetes, and therefore, they carry more predictive or prognostic information. Furthermore, they are not invasive, easily accessible, harmless and relatively cost-effective in comparison to imaging modalities considered as standard measures of the exposures for which the novel indices are hypothesized as good surrogate indicators (for instance VAI vs CT measures on visceral fat) (25). However, the complexity of the metabolic indices makes it difficult to distinguish whether they are indicators of visceral fat, insulin resistance, glucotoxicity, lipotoxicity or all together. Moreover, the values are not specific, meaning that two people with the same value for one combined index, might have totally different values for each simple component. Also, the nature of these combined indices makes it difficult to establish a cut-off value to distinguish normal range from pathological values. Each index captures different aspects of body size or laboratory profile, depending on the included subcomponents and we need to determine which combination best identifies those at specific risk for type 2 diabetes, prior to establish the useful cut-offs (45). Unlike TyG, VAI and LAP have sex specific mathematical formulas, which on the one hand make them gender-tailored risk markers and on the other hand difficult to generalize or implement in total population risk scores for type 2 diabetes.

Adjustment for confounders and potential residual confounding

In observation studies, such as the ones presented in this thesis, associations between exposure (e.g. inflammatory markers) and outcome (type 2 diabetes) could be biased because of confounding. A confounding factor is an extraneous variable that is not an intermediate in the causal pathway between the exposure and the outcome, but correlates with both, causing a spurious association. Residual confounding is the distortion that remains after controlling for confounding in the design and/or analysis of a study. When the confounder is not considered in the analysis or cannot directly and accurately be measured in the study, it will lead to residual confounding (46). Depending on how the confounding factor is related to both the exposure and outcome, residual confounding can lead to either overestimation or underestimation of the observed effect estimate.

The wealth of the available data in the Rotterdam Study enabled us to adjust for various possible confounders in different associations studied in this thesis. Yet, the possibility of residual confounding cannot be completely ruled out. For instance, in **chapter 2.2** physical activity was measured at the third visit of the Rotterdam Study, while dietary intake questionnaire was completed

in the first visit of the first Rotterdam Study cohort. Therefore, we cannot fully exclude residual confounding by physical activity levels. Moreover, there are many suggested type 2 diabetes risk models and scores and not yet a unique widely-recognized universally applicable model, which make the adjustment more flexible in different studies (47). Some confounding factors we considered for the analysis in almost every chapter of this thesis, such as smoking status and alcohol consumption, were self-reported and therefore measurement error of the confounding variable might have occurred.

Selection bias

Selection bias is about who is and who is not in the study population. Each study included in this thesis was restricted to a particular subgroup of the Rotterdam Study. In each study, we did our best to ensure achievement of proper study population and avoid selection bias.

The set of markers including inflammatory markers and apolipoproteins, studied in chapter 2.1 and chapter 3.1, was first designed specifically to investigate their associations with dementia. Given the random sampling, these persons could be considered representative of the source population. However, to avoid any potential bias, we excluded from the analyses the subjects with dementia at baseline, resulting in a subset of 971 subjects (41; 48). Similarly, in both **chapter 4.1** and **chapter 4.2** analyses were restricted to postmenopausal women with a natural menopause, given that characteristics of natural menopausal versus surgical menopausal women might be different (49). In natural menopause ovarian function decreases slowly over several years until menstruation ceases, but in surgical menopause circulating levels of estrogen, progesterone, and androgen decrease abruptly, which could lead to different associations with certain diseases rates (49; 50).

In **chapter 2.2**, we used a subpopulation for the analysis regarding adiponectin, resistin, leptin and PAI-1 as outcome. As this subpopulation was different with respect to some health characteristics such as higher levels of C-reactive protein, body mass index, systolic blood pressure and higher prevalence of chronic disease, the possibility of selection bias cannot be ruled out. However, it has been shown that using a restricted source population for a cohort study usually does not appear to compromise validity of exposure-outcome associations (51).

Information bias

Information bias (also called misclassification, observation bias or measurement bias) happens when key information is either measured, collected, or interpreted inaccurately. Misclassification happens for a variety of reasons and can be either differential or non-differential misclassification. Differential misclassification happens when the information errors differ between groups. In other words, the bias is different for exposed and non-exposed, or between those who have the disease and those without it. Non-differential misclassification happens when the information is incorrect, but is the same across groups (46). The total antioxidant capacity of diet presented in **chapter 2.2** was assessed through a food frequency questionnaire (FFQ). FFQs and other self-report measures can be limited by errors in reporting, recall and by incomplete assessment of all sources of micro and antioxidant intake. This may introduce non-differential misclassification and would bias the results toward the null. Moreover, assessment of diet was done at baseline and

there may have been changes in antioxidant consumption over time. However, it has been shown that dietary habits change very little over time in middle-aged adults (52).
Information bias may be present also due to measurement error, both in laboratory measures or imaging modalities used in this thesis. The possibility of measurement errors with blood biomarkers such as the inflammatory, uric acid, apolipoproteins, laboratory components of combined metabolic indices and sex hormones investigated in this thesis, cannot be totally excluded, despite the qualitative techniques of measurements. However, the golden standard imaging measures such as DXA for android and gynoid fat studied in **chapter 3.2** or CT scans for epicardial fat studied in **chapter 3.3**, represent more robustly measured markers and less prone to information bias related to measurement errors.

CLINICAL IMPLICATIONS AND FUTURE DIRECTIONS

Clinical implications

Much work has been done to develop diabetes risk models and scores, but most are rarely used because they require tests not routinely available or they were developed without being specifically designed for a certain clinical use (47). In other words, it is hard for doctors to know how to use the diabetes risk scores, which one and when. There is no universal ideal risk score, as the utility of any score depends not merely on its statistical properties but also on its context of use. Despite there being vast numbers of risk prediction models, hardly any of them are in use in clinical practice. The balance of research effort is now shifting from devising new risk scores to exploring how best to use those that we already have. There is a steadily increasing focus on the usability, impact, comparison and improvement of different introduced type 2 diabetes risk scores to identify people at risk as early as possible and in the most cost-effective manner. To this end, a better understanding of type 2 diabetes pathophysiology is required. Moreover, new insights on type 2 diabetes pathophysiology would bring novelties regarding the optimal management for each phase during the course of the disease. It is important to update not only the therapeutic treatment, but also to renew the different preventive measures aiming for the highest efficacy (53; 54).
In **chapter 2.1**, the identification of EN-RAGE and IL13 as novel risk markers associated with the risk of type 2 diabetes suggests new pathways involved in the pathophysiology of the disease that might eventually help clinicians to target individuals at highest risk. Beyond the identification of novel inflammatory markers for type 2 diabetes risk, our findings relate them specifically to different stages of the disease. Thus, therapeutic approaches may target different inflammatory markers in patients at different phases of the disease course.
Our findings presented in **chapter 3.1** highlight the role of variation in composition of
HDL particles and other lipoproteins in relation to type 2 diabetes. Based on our results, we suggest that therapeutic approaches for type 2 diabetes should aim for the normalization of both quantity and composition of HDL particles and other lipoproteins. In **chapter 3.2**, we showed that among women, novel combined metabolic indices were stronger risk markers for type 2 diabetes than the traditional anthropometric and laboratory measures and were comparable

to DXA measures. Neither combined metabolic indices nor DXA measures were superior to traditional anthropometric and lipid measures in association with type 2 diabetes among men. These findings carry the potential to contribute to improvement of type 2 diabetes risk prediction among women and men.

The identification of DHEA as a protective marker against type 2 diabetes risk in **chapter 4.1**, suggests its potential to be used in preventive interventions. Our results are in the same line with previous studies suggesting that treatment with DHEA induces significant decreases in abdominal fat and an increase in insulin sensitivity (55). Also, it has been previously shown that 6 months of DHEA replacement improved insulin action in elderly individuals (56). Our findings provide further evidence on the positive effect of DHEA in type 2 diabetes, suggesting that DHEA hormone replacement therapy might be used for treatment of type 2 diabetes. However, the decision of implementing DHEA in prevention or treatment of type 2 diabetes remains difficult for the clinicians, given that there are also studies that argue strongly against the use of DHEA for this purpose (57).

Previous data have shown that lifestyle interventions targeting diet, exercise, and behavior modification can reduce the risk for type 2 diabetes and have demonstrated the applicability of these interventions in young, elderly or culturally diverse population (58). Besides the clinical implications, this thesis confirms practical suggestions for a healthier lifestyle. In particular, our results suggest that inclusion of antioxidants foods in our diet may prevent low-grade chronic inflammation and its associated diseases such as type 2 diabetes and their complications.

Future research directions

Our results in **chapter 2.1** indicated new inflammatory pathophysiological pathways associated with type 2 diabetes, emphasizing the need for further studies to establish the role of EN-RAGE, IL13 and IL17 in the development of the disease. Further research should reconfirm the suggested phase-specific reported associations and explore the possibilities for specific anti-inflammatory agents to postpone or halt disease development and to prevent or delay its complications. Despite the large evidence on the role of inflammation in type 2 diabetes, there are many unanswered questions to be addressed in the near future: What is the relative contribution of inflammation to the development of type 2 diabetes? How efficacious are the anti-inflammatory approaches at improving glycaemia and type 2 diabetes complications? What could be the side effects of anti-inflammatory approaches, given that immune system is a very sensitive system to be modified? Do anti-inflammatory strategies target the underlying mechanisms of the disease, and if so, would starting these therapies early prevent progression or even the overt manifestation of the disease?

In **chapter 2.3** we indicated that serum uric acid might be more closely associated with early-phase pathogenic mechanisms that contribute to the development of type 2 diabetes rather than late-phase mechanisms. Further research should explore the details how uric acid is involved in early phase type 2 diabetes development. Also, it remains uncertain whether uric acid has any potential implication in prevention, treatment or prognosis of type 2 diabetes. Further research should determine whether it is effective to utilize uric acid level as a predictor of type 2 diabetes for its primary prevention (59). In **chapter 3.1** our results call for more data to determine the

importance of levels of apoCIII in specific lipoproteins for type 2 diabetes risk assessment and management and to elucidate the interaction between triglycerides and apoCIII in relation to type 2 diabetes risk. Previous research support the concept that the apolipoproteinic profile plays a remarkable role in type 2 diabetes etiology (60) and prognosis (61). We did not have available measurements of apolipoprotein amounts in every lipoprotein particle separately and specifically in HDL (only total serum levels), so we could not study the diabetes risk apolipoproteinic profile within HDL or other lipoprotein particles compounded by the studied apolipoproteins, which requires further research. Also, based on the **chapter 3.2** of this thesis, further longitudinal data are needed to validate the predictive performance of the novel combined indices (VAI, LAP, TyG) for type 2 diabetes risk. Furthermore, a comparison of the predictive performance of the novel indices with DXA, CT and MRI specific measures of visceral fat is warranted. Our results suggest that some non-invasive indicators of the visceral fat might have the potential to reliably substitute the expensive, invasive imaging modalities in clinical practice for the earliest detection of subjects at high risk for type 2 diabetes.

In **chapter 3.3** the use of an innovative fully automatic method of non-enhanced CT-scans (62; 63) for measuring epicardial fat volume represents a golden standard measure. Given that the association of epicardial fat with type 2 diabetes is unknown and we are the first to investigate it in our population based Rotterdam Study, the use of a golden standard measure such as CT

Table 1. Inflammatory markers investigated in chapter 2.1.

Novel Markers	Already known markers
CD40, ng/mL	CRP
CD40 ligand, ng/mL	Complement 3
EN-RAGE, ng/mL	IL18
Eotaxin, pg/mL	MCP1
FAS, ng/mL	RANTES
HCC4, ng/mL	Resistin
IL13, pg/mL	TNFR-II
IL16, pg/mL	MIF
IL17, pg/mL	IL1ra
IL8, pg/mL	
MDC, pg/mL	
MIP1 alpha, pg/mL	
MIP1 beta, pg/mL	
PARC, ng/mL	
sRAGE, ng/mL	
TRAILR3, ng/mL	
CFH, ug/mL	

CD40, cluster of differentiation 40; CD40 ligand, cluster of differentiation 40 ligand ; EN-RAGE, Extracellular Newly identified Receptor for Advanced Glycation End-products binding protein; FAS, Fas Cell Surface Death Receptor; HCC4, Human CC chemokine-4; IL13, interleukin 13; IL16, interleukin 16; IL17, interleukin 17; IL8, interleukin 8; MDC, Monocyte Derived Chemokine; MIP1alpha, Macrophage Inflammatory Protein 1 alpha; MIP1beta, Macrophage Inflammatory Protein 1 beta; PARC, Pulmonary and Activation-Regulated Chemokine; sRage, Soluble Receptor of Advanced Glycation End-products; TRAILR3, Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptor 3; CFH, Complement Factor; IL18, interleukin 18; MCP1, Monocyte Chemotactic Protein 1; RANTES, Regulated Upon Activation, Normally T-Expressed, And Presumably Secreted; TNFR-II, Tumor Necrosis Factor Receptor 2; IL1ra, Interleukin 1 Receptor Antagonist; CRP, C-Reactive Protein.

is a great strength, providing high precision of the exposure measurement. However, once the association is established from further evidence, other surrogate and more convenient indicators of the exposures need to be explored for more cost-effective risk estimation.

Moreover, we suggest with our results in **chapter 3.3** that epicardial fat should be further explored in relation to unfavorable metabolic profiles.

In conclusion, the different risk markers for type 2 diabetes studied in this thesis give new insights regarding the disease pathophysiology and its course. Awaiting further research, these findings carry the potential to contribute to improvements in disease prevention, treatment and prognosis.

REFERENCES

1. ADA: Fast Facts. Data and Statistics about Diabetes. American Diabetes Association 2017;
2. Bommer C, Heesemann E, Sagalova V, Manne-Goehler J, Atun R, Barnighausen T, Vollmer S: The global economic burden of diabetes in adults aged 20-79 years: a cost-of-illness study. Lancet Diabetes Endocrinol 2017;5:423-430
3. Ling C, Groop L: Epigenetics: a molecular link between environmental factors and type 2 diabetes. Diabetes 2009;58:2718-2725
4. Iqbal N: The burden of type 2 diabetes: strategies to prevent or delay onset. Vasc Health Risk Manag 2007;3:511-520
5. Hotamisligil GS: Inflammation and metabolic disorders. Nature 2006;444:860-867
6. Stern MP: Diabetes and cardiovascular disease. The "common soil" hypothesis. Diabetes 1995;44:369-374
7. Dehghan A, Kardys I, de Maat MP, Uitterlinden AG, Sijbrands EJ, Bootsma AH, Stijnen T, Hofman A, Schram MT, Witteman JC: Genetic variation, C-reactive protein levels, and incidence of diabetes. Diabetes 2007;56:872-878
8. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM: C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. Jama 2001;286:327-334
9. Wang X, Bao W, Liu J, Ouyang YY, Wang D, Rong S, Xiao X, Shan ZL, Zhang Y, Yao P, Liu LG: Inflammatory markers and risk of type 2 diabetes: a systematic review and meta-analysis. Diabetes Care 2013;36:166-175
10. Savini I, Catani MV, Evangelista D, Gasperi V, Avigliano L: Obesity-associated oxidative stress: strategies finalized to improve redox state. Int J Mol Sci 2013;14:10497-10538
11. Alissa EM, Ferns GA: Dietary fruits and vegetables and cardiovascular diseases risk. Crit Rev Food Sci Nutr 2017;57:1950-1962
12. Martinez-Gonzalez MA, de la Fuente-Arrillaga C, Nunez-Cordoba JM, Basterra-Gortari FJ, Beunza JJ, Vazquez Z, Benito S, Tortosa A, Bes-Rastrollo M: Adherence to Mediterranean diet and risk of developing diabetes: prospective cohort study. BMJ 2008;336:1348-1351
13. Dal S, Sigrist S: The Protective Effect of Antioxidants Consumption on Diabetes and Vascular Complications. Diseases 2016;4
14. Moller DE, Kaufman KD: Metabolic syndrome: a clinical and molecular perspective. Annu Rev Med 2005;56:45-62
15. Johnson RJ, Perez-Pozo SE, Sautin YY, Manitius J, Sanchez-Lozada LG, Feig DI, Shafiu M, Segal M, Glassock RJ, Shimada M, Roncal C, Nakagawa T: Hypothesis: could excessive fructose intake and uric acid cause type 2 diabetes? Endocr Rev 2009;30:96-116
16. Lu W, Xu Y, Shao X, Gao F, Li Y, Hu J, Zuo Z, Shao X, Zhou L, Zhao Y, Cen X: Uric Acid Produces an Inflammatory Response through Activation of NF-kappaB in the Hypothalamus: Implications for the Pathogenesis of Metabolic Disorders. Sci Rep 2015;5:12144
17. Kahn SE, Hull RL, Utzschneider KM: Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006;444:840-846
18. Neeland IJ, Turer AT, Ayers CR, Powell-Wiley TM, Vega GL, Farzaneh-Far R, Grundy SM, Khera A, McGuire DK, de Lemos JA: Dysfunctional adiposity and the risk of prediabetes and type 2 diabetes in obese adults. JAMA 2012;308:1150-1159
19. Boyko EJ, Fujimoto WY, Leonetti DL, Newell-Morris L: Visceral adiposity and risk of type 2 diabetes: a prospective study among Japanese Americans. Diabetes Care 2000;23:465-471
20. Carmena R: Type 2 diabetes, dyslipidemia, and vascular risk: rationale and evidence for correcting the lipid imbalance. Am Heart J 2005;150:859-870
21. Qi Q, Liang L, Doria A, Hu FB, Qi L: Genetic predisposition to dyslipidemia and type 2 diabetes risk in two prospective cohorts. Diabetes 2012;61:745-752
22. Coleman NJ, Miernik J, Philipson L, Fogelfeld L: Lean versus obese diabetes mellitus patients in the United States minority population. J Diabetes Complications 2014;28:500-505
23. Haffner SM, Stern MP, Hazuda HP, Mitchell BD, Patterson JK: Cardiovascular risk factors in confirmed prediabetic individuals. Does the clock for coronary heart disease start ticking before the onset of clinical diabetes? JAMA 1990;263:2893-2898
24. Levelt E, Pavlides M, Banerjee R, Mahmod M, Kelly C, Sellwood J, Ariga R, Thomas S, Francis J, Rodgers C, Clarke W, Sabharwal N, Antoniades C, Schneider J, Robson M, Clarke K, Karamitsos T, Rider O, Neubauer S: Ectopic and Visceral Fat Deposition in Lean and Obese Patients With Type 2 Diabetes. J Am Coll Cardiol 2016;68:53-63

25. Shah RV, Murthy VL, Abbasi SA, Blankstein R, Kwong RY, Goldfine AB, Jerosch-Herold M, Lima JA, Ding J, Allison MA: Visceral adiposity and the risk of metabolic syndrome across body mass index: the MESA Study. JACC Cardiovasc Imaging 2014;7:1221-1235
26. Amato MC, Giordano C, Galia M, Criscimanna A, Vitabile S, Midiri M, Galluzzo A, AlkaMeSy Study G: Visceral Adiposity Index: a reliable indicator of visceral fat function associated with cardiometabolic risk. Diabetes Care 2010;33:920-922
27. Amato MC, Giordano C: Visceral adiposity index: an indicator of adipose tissue dysfunction. Int J Endocrinol 2014;2014:730827
28. Ding EL, Song Y, Malik VS, Liu S: Sex differences of endogenous sex hormones and risk of type 2 diabetes: a systematic review and meta-analysis. JAMA 2006;295:1288-1299
29. Wang SL, Pan WH, Hwu CM, Ho LT, Lo CH, Lin SL, Jong YS: Incidence of NIDDM and the effects of gender, obesity and hyperinsulinaemia in Taiwan. Diabetologia 1997;40:1431-1438
30. Kim CH, Kim HK, Kim EH, Bae SJ, Park JY: Association between changes in body composition and risk of developing Type 2 diabetes in Koreans. Diabet Med 2014;31:1393-1398
31. Ikram MA, Brusselle GGO, Murad SD, van Duijn CM, Franco OH, Goedegebure A, Klaver CCW, Nijsten TEC, Peeters RP, Stricker BH, Tiemeier H, Uitterlinden AG, Vernooij MW, Hofman A: The Rotterdam Study: 2018 update on objectives, design and main results. Eur J Epidemiol 2017;32:807-850
32. Hulley SB CS, Browner WS: Designing Clinical Research: An Epidemiologic Approach. 2001;2nd Ed. Lippincott Williams & Wilkins; Philadelphia: p. 1-336
33. M E: Critical Appraisal of Epidemiological Studies and Clinical Trials. 2007;3rd Ed. Oxford
p. 1-570
34. Laurens Holmes Jr FO: Concise Epidemiologic Principles and Concepts. Guidelines for Clinicians and Biomedical Researchers.
35. Morabia A: On the origin of Hill's causal criteria. Epidemiology 1991;2:367-369
36. Sattar N, Preiss D: Reverse Causality in Cardiovascular Epidemiological Research: More Common Than Imagined? Circulation 2017;135:2369-2372
37. Swerdlow DI: Mendelian Randomization and Type 2 Diabetes. Cardiovasc Drugs Ther 2016;30:51-57
38. Ho GY, Xue XN, Burk RD, Kaplan RC, Cornell E, Cushman M: Variability of serum levels of tumor necrosis factor-alpha, interleukin 6, and soluble interleukin 6 receptor over 2 years in young women. Cytokine 2005;30:1-6
39. Ockene IS, Matthews CE, Rifai N, Ridker PM, Reed G, Stanek E: Variability and classification accuracy of serial high-sensitivity C-reactive protein measurements in healthy adults. Clin Chem 2001;47:444-450
40. Brahimaj A, Ligthart S, Ikram MA, Hofman A, Franco OH, Sijbrands EJ, Kavousi M, Dehghan A: Serum Levels of Apolipoproteins and Incident Type 2 Diabetes: A Prospective Cohort Study. Diabetes Care 2017;40:346-351
41. Brahimaj A, Ligthart S, Ghanbari M, Ikram MA, Hofman A, Franco OH, Kavousi M, Dehghan A: Novel inflammatory markers for incident pre-diabetes and type 2 diabetes: the Rotterdam Study. Eur J Epidemiol 2017;32:217-226
42. Onat A, Hergenc G, Bulur S, Ugur M, Kucukdurmaz Z, Can G: The paradox of high apolipoprotein A-I levels independently predicting incident type-2 diabetes among Turks. Int J Cardiol 2010;142:72-79
43. Sasongko MB, Wong TY, Nguyen TT, Kawasaki R, Jenkins A, Shaw J, Wang JJ: Serum apolipoprotein AI and B are stronger biomarkers of diabetic retinopathy than traditional lipids. Diabetes Care 2011;34:474-479
44. Boerma GJ, de Bruijn AM, van Teunenbroek A: Reference values for apolipoprotein A-I and apolipoprotein B in serum still depend on choice of assay techniques. Eur J Clin Chem Clin Biochem 1994;32:923-927
45. Amato MC, Giordano C, Pitrone M, Galluzzo A: Cut-off points of the visceral adiposity index (VAI) identifying a visceral adipose dysfunction associated with cardiometabolic risk in a Caucasian Sicilian population. Lipids Health Dis 2011;10:183
46. Kenneth J. Rothman SG, Timothy L. Lash: Modern Epidemiology.
47. Noble D, Mathur R, Dent T, Meads C, Greenhalgh T: Risk models and scores for type 2 diabetes: systematic review. BMJ 2011;343:d7163
48. Ligthart S, Sedaghat S, Ikram MA, Hofman A, Franco OH, Dehghan A: EN-RAGE: a novel inflammatory marker for incident coronary heart disease. Arterioscler Thromb Vasc Biol 2014;34:2695-2699
49. Davison SL, Bell R, Donath S, Montalto JG, Davis SR: Androgen levels in adult females: changes with age, menopause, and oophorectomy. J Clin Endocrinol Metab 2005;90:3847-3853
50. Ozdemir S, Celik C, Gorkemli H, Kiyici A, Kaya B: Compared effects of surgical and natural menopause on climacteric symptoms, osteoporosis, and metabolic syndrome. Int J Gynaecol Obstet 2009;106:57-61

51. Nilsen RM, Suren P, Gunnes N, Alsaker ER, Bresnahan M, Hirtz D, Hornig M, Lie KK, Lipkin WI, Reichborn-Kjennerud T, Roth C, Schjolberg S, Smith GD, Susser E, Vollset SE, Oyen AS, Magnus P, Stoltenberg C: Analysis of self-selection bias in a population-based cohort study of autism spectrum disorders. Paediatr Perinat Epidemiol 2013;27:553-563
52. Goldbohm RA, van 't Veer P, van den Brandt PA, van 't Hof MA, Brants HA, Sturmans F, Hermus RJ: Reproducibility of a food frequency questionnaire and stability of dietary habits determined from five annually repeated measurements. Eur J Clin Nutr 1995;49:420-429
53. Nyenwe EA, Jerkins TW, Umpierrez GE, Kitabchi AE: Management of type 2 diabetes: evolving strategies for the treatment of patients with type 2 diabetes. Metabolism 2011;60:1-23
54. Kahn SE, Cooper ME, Del Prato S: Pathophysiology and treatment of type 2 diabetes: perspectives on the past, present, and future. Lancet 2014;383:1068-1083
55. Villareal DT, Holloszy JO: Effect of DHEA on abdominal fat and insulin action in elderly women and men: a randomized controlled trial. JAMA 2004;292:2243-2248
56. Weiss EP, Villareal DT, Fontana L, Han DH, Holloszy JO: Dehydroepiandrosterone (DHEA) replacement decreases insulin resistance and lowers inflammatory cytokines in aging humans. Aging (Albany NY) 2011;3:533-542
57. Basu R, Dalla Man C, Campioni M, Basu A, Nair KS, Jensen MD, Khosla S, Klee G, Toffolo G, Cobelli C, Rizza RA: Two years of treatment with dehydroepiandrosterone does not improve insulin secretion, insulin action, or postprandial glucose turnover in elderly men or women. Diabetes 2007;56:753-766
58. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM, Diabetes Prevention Program Research G: Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N Engl J Med 2002;346:393-403
59. Kodama S, Saito K, Yachi Y, Asumi M, Sugawara A, Totsuka K, Saito A, Sone H: Association between serum uric acid and development of type 2 diabetes. Diabetes Care 2009;32:1737-1742
60. van Hoek M, van Herpt TW, Dehghan A, Hofman A, Lieverse AG, van Duijn CM, Witteman JC, Sijbrands EJ: Association of an APOC3 promoter variant with type 2 diabetes risk and need for insulin treatment in lean persons. Diabetologia 2011;54:1360-1367
61. Barbagallo CM, Averna MR, Amato S, Marino G, Labisi M, Rao AC, Barbagallo M, Cupidi GF, Notarbartolo A: Apolipoprotein profile in type II diabetic patients with and without coronary heart disease. Acta Diabetol Lat 1990;27:371-377
62. Mathur KS, Kashyap SK, Kumar V: Correlation of the extent and severity of atherosclerosis in the coronary and cerebral arteries. Circulation 1963;27:929-934
63. Bos D, Vernooij MW, Shahzad R, Kavousi M, Hofman A, van Walsum T, Deckers JW, Ikram MA, Heeringa J, Franco OH, van der Lugt A, Leening MJ: Epicardial Fat Volume and the Risk of Atrial Fibrillation in the General Population Free of Cardiovascular Disease. JACC Cardiovasc Imaging 2017;

Chapter 6

Summary and samenvatting

SUMMARY

In this thesis, we aimed to investigate associations between novel risk markers and the risk for prediabetes, type 2 diabetes, insulin therapy initiation in general population as well as among women and men from the population-based Rotterdam Study. Our main focus points were markers of inflammation, lipids, body fat distribution and sex hormones.

Chapter 1 introduces readers with the epidemiology, burden and pathophysiology of type 2 diabetes as well as describes the role of inflammation, lipids, body fat and sex hormones in the development of the disease. It also presents objectives and the outline of this thesis.

Chapter 2 contains three studies in which mainly the role of different inflammatory markers in the risk for type 2 diabetes is examined. Chapter 2.1 studied the association of a set of inflammatory markers with progression from normoglycemia to pre-diabetes, type 2 DM and finally to insulin therapy. Our findings suggest that various inflammatory markers may be associated with progression from normoglycemia to pre-diabetes (IL13, ENRAGE, CRP), T2D (IL13, IL17, CRP) or insulin therapy start (IL13). Among them, EN-RAGE is a novel inflammatory marker for pre-diabetes, IL17 for incident T2D and IL13 for pre-diabetes, incident T2D and insulin therapy start. Chapter 2.2 investigated the role of dietary antioxidants and plasma oxidant-antioxidant status in low-grade chronic inflammation and adipocytokine levels. Our findings suggest that high overall dietary antioxidant capacity of diet and lower levels of UA were associated with lower levels of pro-inflammatory adipocytokines and higher levels of anti-inflammatory adipocytokines. In chapter 2.3, we aimed to investigate the association between serum uric acid and risk of prediabetes and type 2 diabetes mellitus. Our findings agree with the notion that serum uric acid is more closely related to early-phase mechanisms in the development of type 2 diabetes mellitus than late-phase mechanisms.

In chapter 3 we focused on the role of lipids and body fat in the risk for type 2 diabetes. Chapter 3.1 investigated the role of serum levels of various apolipoproteins on the risk for type 2 diabetes. We found that serum apoCIII levels as well as apoCIII-to-apoA1 ratio are associated with incident T2D, independent of known risk factors and stronger than HDL-C levels. Chapter 3.2 investigated the associations of several novel metabolic indices, combining anthropometric and lipid measures (VAI, LAP, TyG), and DXA measurements on body fat with incident type 2 diabetes among women and men from the large population-based Rotterdam Study. We found that among women, novel combined metabolic indices were stronger risk markers for T2D than the traditional anthropometric and laboratory measures and were comparable to DXA measures. Neither combined metabolic indices nor DXA measures were superior to traditional anthropometric and lipid measures in association with T2D among men. Chapter 3.3 studied the associations of epicardial fat volume with incident events of type 2 diabetes, coronary heart disease (myocardial infarction and cardiovascular mortality) as well as stroke, in total population and among women and men. Our results suggested that increased amount of epicardial fat volume may be associated with excessive risk for T2D, hard CHD and stroke in the total population independent of traditional diabetes and cardiovascular risk factors. Chapter 4.1 assessed the associations between serum levels of dehydroepiandrosterone (DHEA) and its main derivatives DHEA sulphate (DHEAS) and androstenedione, aswell as the ratio of DHEAS to DHEA, and

risk of type 2 diabetes. Our results suggested that DHEA may play a positive role in the pathogenesis of type 2 diabetes, which may have important implications for preventive interventions. Chapter 4.2 investigated the association between endogenous sex steroid levels and changes in body composition in postmenopausal women. Our findings support the concept that changes in endogenous sex steroid levels are closely associated with changes in body composition in postmenopausal women.

The main findings and methodological considerations of this thesis are discussed in the general discussion in Chapter 5. Chapter 5 ends with clinical implication of our findings and future research in type 2 diabetes.

SAMENVATTING

In dit proefschrift hebben we ons gericht op het onderzoeken van associaties tussen nieuwe risicomarkers en het risico op prediabetes, diabetes type 2 en het starten van insulinetherapie in de algemene populatie en in vrouwen en mannen apart in de Rotterdam Studie. Onze belangrijkste focus punten waren ontsteking markers, lipiden, distributie van lichaamsvet en geslachtshormonen.

Hoofdstuk 1 introduceert de lezer met de epidemiologie, belasting, pathofysiologie van diabetes type 2 en beschrijft de rol van ontstekingen, lipiden, lichaamsvet en geslachtshormonen in de ontwikkeling van de ziekte. Dit hoofdstuk bevat ook de doelstellingen en de hoofdlijnen van dit proefschrift.

Hoofdstuk 2 bevat drie studies waarin voornamelijk de rol van verschillende inflammatoire markers in het risico op type 2 diabetes werd onderzocht. In hoofdstuk 2.1 werd de associatie van een set inflammatoire markers met de progressie van normoglycemie naar pre-diabetes, diabetes type 2 (T2D) en uiteindelijk naar insulinetherapie onderzocht. Onze bevindingen suggereren dat verschillende inflammatoire markers geassocieerd kunnen zijn met progressie van normoglycemie naar pre-diabetes (IL13, ENRAGE, CRP), T2D (IL13, IL17, CRP) of de start van insulinetherapie (IL13). Onder hen is EN-RAGE een nieuwe inflammatoire marker voor pre-diabetes, IL17 voor incident T2D en IL13 voor pre-diabetes, incident T2D en de start van insulinetherapie. Hoofdstuk 2.2 onderzocht de rol van antioxidanten in het dieet en de plasma oxidant – antioxidant status in laaggradige chronische ontstekingen en adipocytokinen waardes. Onze bevindingen suggereren dat een hoog algeheel antioxidantvermogen van het dieet en lagere waardes van UA geassocieerd zijn met lagere waardes van pro-inflammatoire adipocytokines en hogere waardes van anti-inflammatoire adipocytokines. In hoofdstuk 2.3 was onze doelstelling om de associatie tussen serumurinezuur en het risico op prediabetes en diabetes type 2 te onderzoeken. Onze bevindingen komen overeen met de notie dat serumurinezuur nauwer gerelateerd is aan vroege fase-mechanismes bij de ontwikkeling van diabetes type 2 dan met mechanismes in de late fase.

In hoofdstuk 3 hebben we ons gericht op de rol van lipiden en lichaamsvet in het risico op diabetes type 2. Hoofdstuk 3.1 onderzocht de rol van serumwaardes van verschillende apolipoproteïnen op het risico voor diabetes type 2. We ontdekten dat serum-apoCIII waardes en apoCIII-tot-apoA1-ratio geassocieerd zijn met incidenteel T2D, onafhankelijk van bekende risicofactoren en sterker geassocieerd dan HDL-C waardes. Hoofdstuk 3.2 onderzocht de associatie tussen verschillende nieuwe metabole indices, gecombineerd met antropometrische en lipide waardes (VAI, LAP, TyG) en DXA-metingen voor lichaamsvet met diabetes type 2 in vrouwen en mannen uit de grote op populatie-gebaseerde Rotterdam Studie. We vonden dat in vrouwen de nieuwe gecombineerde metabole indices sterkere risicomarkers zijn voor T2D dan de traditionele antropometrische en laboratoriummetingen en vergelijkbaar waren met DXA-metingen. Noch gecombineerde metabole indices, noch DXA-metingen waren superieur aan traditionele antropometrische en lipidemetingen in associatie met T2D in mannen.

Hoofdstuk 3.3 bestudeerde de associaties van epicardiaal vetvolume met incident diabetes type 2, coronaire hartziekten (hartinfarct en cardiovasculaire mortaliteit) en beroertes, in de totale bevolking en bij vrouwen en mannen. Onze resultaten suggereerden dat een verhoogde hoeveel-

heid epicardiaal vetvolume kan worden geassocieerd met verhoogd risico op T2D, harde CHD en beroerte in de totale populatie, onafhankelijk van de traditionele diabetes en cardiovasculaire risicofactoren.
In hoofdstuk 4.1 werden de associaties tussen de serumwaardes van dehydroepiandrosteron (DHEA) en de belangrijkste derivaten ervan, DHEA-sulfaat (DHEAS) en androsteendion, en de verhouding van DHEAS tot DHEA en het risico van diabetes type 2 onderzocht. Onze resultaten suggereerden dat DHEA een positieve rol kan spelen in de pathogenese van diabetes type 2, dit kan belangrijke implicaties hebben voor preventieve interventies. Hoofdstuk 4.2 onderzocht de associatie tussen endogene geslachtshormonen en veranderingen in de lichaamssamenstelling bij postmenopauzale vrouwen. Onze bevindingen ondersteunen het concept dat veranderingen in endogene geslachtshormoonwaardes nauw verbonden zijn met veranderingen in de lichaamssamenstelling bij postmenopauzale vrouwen.
De belangrijkste bevindingen en methodologische overwegingen van dit proefschrift worden besproken in de algemene discussie in hoofdstuk 5. Hoofdstuk 5 eindigt met klinische implicaties van onze bevindingen en toekomstig onderzoek naar diabetes type 2.

Appendices

Author's affiliations
Publications and manuscripts
About the author
PhD portfolio
Words of gratitude

AUTHOR'S AFFILIATIONS

Adela Brahimaj, Symen Ligthart, Najada Stringa, Niels van der Schaft, Mohsen Ghanbari, Taulant Muka, Jessica C. Kiefte-de Jong, Asija Zaciragic, Ke-xin Wen , Maryam Kavousi, Abbas Dehghan, Oscar H. Franco, Meike Vernooij, M. Arfan Ikram, Albert Hofman
Department of Epidemiology, Erasmus University Medical, Rotterdam, the Netherlands

M. Arfan Ikram
Department of Neurology, Erasmus University Medical Center, Rotterdam, the Netherlands

M. Arfan Ikram, Daniel Bos, Meike Vernooij
Department of Radiology, Erasmus University Medical Center, Rotterdam, the Netherlands

Katerina Trajanoska, Fernando Rivadeneira, Eric. J. G. Sijbrands
Department of Internal Medicine, Erasmus University Medical Center, Rotterdam, the Netherlands

Joop S. E. Laven
Division of Reproductive Medicine, Department of Obstetrics and Gynaecology, Erasmus Medical Center, Rotterdam, the Netherlands

Jessica C. Kiefte-de Jong
Department of Global Public Health, Leiden University College, The Hague, the Netherlands

Asija Zaciragic
Department of Physiology, University of Sarajevo, Sarajevo, Bosnia and Herzegovina

Najada Stringa
Department of Epidemiology and Biostatistics, VUmc, Amsterdam, the Netherlands

Abbas Dehghan
Department of Biostatistics and Epidemiology, MRC-PHE Centre for Environment and Health, School of Public Health, Imperial College London

Daniel Bos, Albert Hofman
Harvard School of Public Health, Boston, MA, USA

LIST OF PUBLICATIONS

1. **Brahimaj A**, Ligthart S, Ikram MA, Hofman A, Franco OH, Sijbrands EJ, Kavousi M, Dehghan A: Serum Levels of Apolipoproteins and Incident Type 2 Diabetes: A Prospective Cohort Study. Diabetes Care 2017;40:346-351
2. **Brahimaj A**, Muka T, Kavousi M, Laven JS, Dehghan A, Franco OH: Serum dehydroepiandrosterone levels are associated with lower risk of type 2 diabetes: the Rotterdam Study. Diabetologia 2017;60:98-106
3. **Brahimaj A**, Ligthart S, Ghanbari M, Ikram MA, Hofman A, Franco OH, Kavousi M, Dehghan A: Novel inflammatory markers for incident pre-diabetes and type 2 diabetes: the Rotterdam Study. Eur J Epidemiol 2017;32:217-226
4. van der Schaft N, **Brahimaj A**, Wen KX, Franco OH, Dehghan A: The association between serum uric acid and the incidence of prediabetes and type 2 diabetes mellitus: The Rotterdam Study. PLoS One 2017;12:e0179482
5. Stringa N, **Brahimaj A**, Zaciragic A, Dehghan A, Ikram MA, Hofman A, Muka T, Kiefte-de Jong JC, Franco OH: Relation of antioxidant capacity of diet and markers of oxidative status with C-reactive protein and adipocytokines: a prospective study. Metabolism 2017;71:171-181
6. Nano J, Muka T, Cepeda M, Voortman T, Dhana K, **Brahimaj A**, Dehghan A, Franco OH: Association of circulating total bilirubin with the metabolic syndrome and type 2 diabetes: A systematic review and meta-analysis of observational evidence. Diabetes Metab 2016;42:389-397
7. Muka T, Vargas KG, Jaspers L, Wen KX, Dhana K, Vitezova A, Nano J, **Brahimaj A**, Colpani V, Bano A, Kraja B, Zaciragic A, Bramer WM, van Dijk GM, Kavousi M, Franco OH: Estrogen receptor beta actions in the female cardiovascular system: A systematic review of animal and human studies. Maturitas 2016;86:28-43

ABOUT THE AUTHOR

Adela Brahimaj was born the 3rd of February 1988 in Vlorë, Albania. She followed primary education and high school in her hometown. In 2006, she started her training as a medical doctor in the Faculty of Medicine at the University of Tirana, Albania. She graduated in October 2012 and was licensed as general physician in February 2013. At the same time, she was awarded an EU funded scholarship to follow a Master of Science program in Epidemiology at the Netherlands Institute for Health Sciences. In August 2014, she obtained her master degree and started her PhD at the Department of Epidemiology, Cardiovascular Group at the Erasmus University Medical Center in Rotterdam under the supervision of Prof. dr. Oscar H. Franco, Dr. Maryam Kavousi and Dr. Abbas Dehghan. During her research activity, she focused on novel risk markers for type 2 diabetes. Within the first year of her PhD, she followed also a Doctor of Science program in Epidemiology. In the last year of her PhD, Adela was awarded the Albert Renold Fellowship grant by the European Foundation for the Study of Diabetes (EFSD) for a research visit at the Department of Epidemiology and Biostatistics, School of Public Health, Imperial College London, UK.

PHD PORTFOLIO

Name PhD student	Adela Brahimaj
Departments	Epidemiology, Erasmus Medical Center Rotterdam Cardiovascular Epidemiology
Research school	Netherlands Institute for Health Sciences (NIHES), Rotterdam
PhD period	September 2014-April 2018
Promotor	Prof. dr. Oscar H Franco
Co-promotors	Dr. Maryam Kavousi and Dr. Abbas Dehghan

	Year	Workload (ECTS)
Training		
Master of Science in General Epidemiology, NIHES, Rotterdam, the Netherlands	2013-2014	
Doctor of Science in General Epidemiology, NIHES, Rotterdam, the Netherlands	2014-2015	
Erasmus summer Program		
Principles of Research in Medicine	2013	0.7
Methods of Public Health Research	2013	0.7
Introduction to Global Public Health	2013	0.7
Genomics in Molecular Medicine	2013	1.4
Social Epidemiology	2013	0.7
Primary and Secondary Prevention Research	2013	0.7
Topics in Meta-analysis	2014	0.7
Cohort Studies	2014	0.7
Case-control Studies	2014	0.7
Methods of Health Services Research	2014	0.7
Markers and Prognostic Research	2014	0.7
Conceptual Foundation of Epidemiologic Study Design	2015	0.7
Causal Inference	2015	0.7
History of Epidemiologic Ideas	2015	0.7
Advances in Epidemiologic Analysis	2015	0.7
Causal Mediation Analysis	2015	0.7
Core curriculum		
Study Design	2013	4.3
Biostatistical Methods I: Basic Principles	2013	5.7
Methodologic Topics in Epidemiologic Research	2013	1.4
Biostatistical Methods II: Classical Regression Models	2013	4.3
Public Health Research Methods	2013	5.7
Advanced courses		
Women's Health	2014	0.9
Quality of Life Measurement	2014	0.9
From Problem to Solution in Public Health	2014	1.1
Public Health in Low and Middle Income Countries	2014	3.0
Nutrition & Physical Activity, Public Health Institute of Cambridge University	2014	1.4
Planning and Evaluation of Screening	2014	1.4

	Year	Workload (ECTS)
Bayesian Statistics	2015	1.4
Advanced Topics in Clinical Trials	2015	1.9
Principles of Epidemiologic Data-analysis	2015	0.7
Maternal and Child Health	2015	0.9
Introduction to Medical Psychology	2015	1.0
General academic courses		
English Language	2013	1.4
Courses for the Quantitative Researcher	2013	1.4
Introduction to Medical Writing	2014	1.1
Endnote, Medical Library, Erasmus MC	2014	0.3
Research Integrity, Erasmus MC	2016	2.0
Attended Seminars		
Seminars of the Department of Epidemiology	2013-2017	2.0
2020 Meetings	2013-2017	2.0
Cardiovascular Group Meetings	2013-2017	2.0
Inter(national) conferences		
Poster presentation at the European Congress of Epidemiology in June 2015, Maastricht, the Netherlands.	2015	
Oral presentation at European Diabetes Epidemiology Group (EDEG), April 2016, Dublin.	2016	
Poster presentation at European Society of Cardiology (ESC) 2016, Rome, Italy	2016	
Oral presentation at European Association of the Study for Diabetes (EASD) 2017, Lisbon, Portugal	2017	
Scholarships and grants		
ERAWEB Master Student Grant	2013	
ERAWEB PhD Student Grant	2014	
Verenging Trustfunds Erasmus Universiteit Rotterdam Grants	2015-2017	
Albert Renold Travel Fellowship Programme, granted by European Foundation for the Study of Diabetes (EFSD).	2017	
Teaching activities		
MSc Thesis of Niels van der Schaft "The association between serum uric acid and the incidence of prediabetes and type 2 diabetes mellitus: The Rotterdam Study", Supervisor.	2016-2017	4.0
Other		
Peer review of articles for scientific journals	2016-2018	
Research visit at School of Public Health, Imperial College, London	October 2017	
Working with BBMRI consortium	2016-2018	

1 ECTS (European Credit Transfer System) is equal to a workload of 28 hours

WORDS OF GRATITUDE

Though it will not be enough to express my gratitude in words to all the people who helped me to make this thesis possible, I will still try to give my many, many thanks to them.

Dear Prof. Albert Hofman, your vision to establish ERAWEB at NIHES cultivated many young minds in clinical research and gave me the opportunity to study and work in such a great environment. Thank you also for your wonderful lectures and for being an inspirational example!

Especially, I would like to thank my promotor, Prof. Oscar H. Franco and my co-promotors, Dr. Maryam Kavousi and Dr. Abbas Dehghan.
Dear Oscar, thank you for the trust, continuous support and your positive energy.
Dear Maryam, you are a great researcher and I have learned a lot from you.
Dear Abbas, It was an honour to be your student. Your warm personality has always been encouraging in times of hardship and I would like to especially thank you for welcoming me at Imperial.

Dear co-authors, thank you for the collaboration, your input and contribution in the manuscripts of my thesis.

Dear members of the reading committee: Prof. Eric Sijbrands, Prof. Liesbeth van Rossum and Prof. Coen Stehouwer, thank you for reviewing this thesis. Dear members of the plenary committee: Prof. Meike Vernooij, Prof. Cees J. Tack, Prof. Fernando Rivadeneira and Prof. M. Arfan Ikram, thank you for accepting the invitation. I would like to express my highest considerations for you all.

Dear colleagues at Erasmus MC and beyond: A special thank you for the great discussions and time shared during many meetings and congresses.

Dear current and past colleagues from the department of epidemiology, cardiovascular group and ErasmusAGE: You have been an invaluable part of the PhD experience. A special appreciation for Mirjam, Solange, Nano and Frank for their help and support.

Dear friends and best friends: You know who you are and what you mean to me. Thank you for your support, encouragement and the great times we have shared!

Finally and most importantly, I would like to thank my family.
A wonderful family and love: my greatest blessings, my biggest achievements.
Dear mom, dad, Arlind and best grandparents ever: Thank you for doing so much for me!
I love you more than paints, melodies and words can say! And I hope you will always feel that, even when I'm not around to tell you so.

Faleminderit përzemërsisht!

Adela