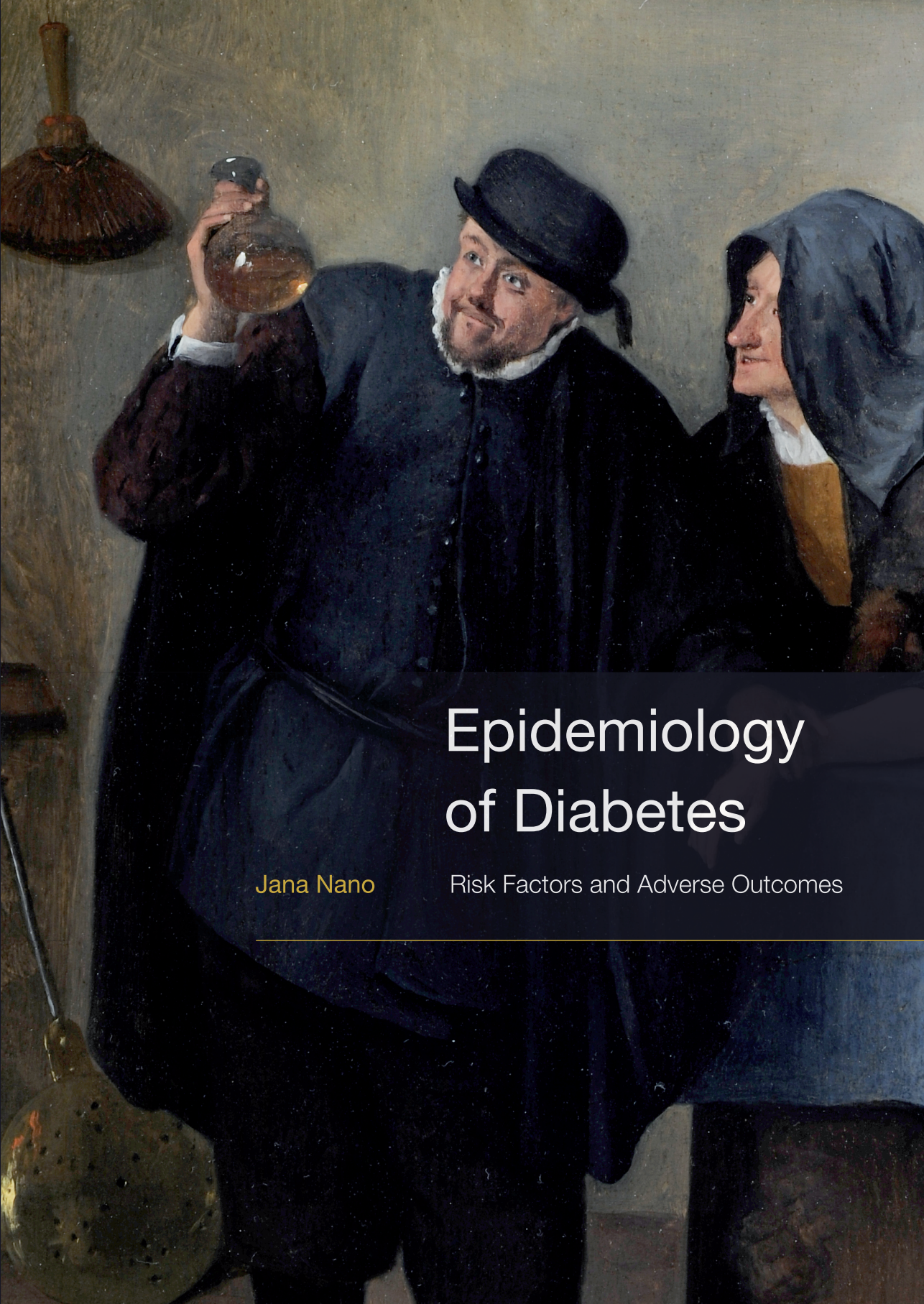

Epidemiology of Diabetes

Risk Factors and Adverse Outcomes

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Risk Factors and Adverse Outcomes

De epidemiologie van diabetes

Risicofactoren en nadelige gevolgen

Thesis

to obtain the degree of Doctor from the
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by

Jana Nano
born in Tirana, Albania

Erasmus University Rotterdam

Doctoral Committee

Promotors

Prof. dr. O. H. Franco

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Paranimphen

L. Jabbarian

E. Shevroja

The dedication of this book is split in fifth:
to Polikseni, to Nejva, to Luli, to Jani and to Martin

In loving memory of my grandfather Thoma Nano

CONTENTS

MANUSCRIPTS THAT FORM THE BASIS OF THIS THESIS

*denotes equal contribution

Chapter 2

Dhana K*, **Nano J*,** Ligthart S, Peeters A, Hofman A, Nusselder W, Dehghan A, Franco OH. Obesity and Life Expectancy with and without Diabetes in Adults Aged 55 Years and Older in the Netherlands: A Prospective Cohort Study. PLoS Med. 2016;13(7):e1002086.

Nano J*, Dhana K*, Asllani E, Sijbrands E, Ikram M.A, Dehghan A, Muka T*, Franco O.H*. Trajectories of Body Mass Index before the Diagnosis of Type 2 Diabetes: The Rotterdam Study (Submitted)

Chapter 3

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(3):577-86.

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Chapter 4

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CHAPTER 1

General Introduction

Diabetes mellitus is comprised of a group of metabolic disorders currently recognized and classified as a set of diseases characterized by chronic hyperglycemia. In the general population, type 2 diabetes is the most common form of diabetes which begins with the inability of cells to properly respond to insulin (insulin resistance) (1). Globally, the number of individuals with diabetes has more than doubled during the past 20 years, projecting an estimate of 642 million cases in 2040 (2). These numbers, partly fuelled by the accompanying increase in excess weight and adiposity (3, 4), pose alarming concerns on population health around the world and respective health care systems (2). Rather than diabetes *per se*, the management of adverse outcomes consequent to the disease remain one of the most important burdensome challenges. The World Health Organization estimates that diabetes mellitus is the 8th leading cause of death, largely attributable to high blood glucose and the increased risks of cardiovascular disease and other complications (e.g. chronic kidney disease, visual-related outcomes) (5). The need of diabetes primary prevention and associated complications is particularly pressing given the commitment to halt the rise in the prevalence and if disease is established, to achieve a 50% coverage of drug treatment and counselling in diabetes (6). Diabetes is also one of the four main non-communicable diseases for which there is a global target of 25% reduction in premature mortality by 2025 compared with 2010 (7). Until a decade ago, despite calls from the international diabetes community to address the prevention of diabetes as a global public health epidemic, many international health agencies and national governments had given fairly low priority to the increased frequency of diabetes (1, 8). The emerging threat of diabetes called for resolution and research efforts have been intensified in the last years to investigate new risk factors including biomarkers and lifestyle beyond traditional risk factors to expand our knowledge in the underlying pathophysiology of diabetes. Moreover, changing aspects of epidemiology of obesity, the most important modifiable risk factor for type 2 diabetes together with the burden of its consequences and related health outcomes, pose the necessity of new strategies for interventions in diabetes prevention (9).

According to the American Diabetes Association (ADA), diabetes diagnosis is defined as: fasting plasma glucose (FPG) ≥ 126 mg/dL (≥ 7.0 mmol/L) where fasting is defined as no caloric intake for at least 8 hours *or* 2-h PG ≥ 200 mg/dL (≥ 11.1 mmol/L) during a 75-g oral glucose tolerance test (OGTT, the test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water) *or* A1C ≥ 6.5% (≥ 48 mmol/mol) *or* in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥ 200 mg/dL (≥ 11.1 mmol/L) (10). These definitions are in line with the current World Health Organization (WHO) diagnostic criteria, except the HbA1C test (11). Prediabetes, a condition that precedes diabetes, is characterized by increased levels of blood glucose which are not high enough to be classified as diabetes. WHO and ADA have set different criteria for

prediabetes based on the upper limit of normal fasting plasma glucose (WHO: fasting plasma glucose level from 6.1 mmol/l (110 mg/dL); ADA: fasting plasma glucose level from 5.6 mmol/L (100 mg/dL)); 75% prediabetes people will eventually develop diabetes, whereas the rest hold the potential to reversibility (12). Therefore, efforts to tackle prediabetes has been intensified in the recent years.

OBESITY, TYPE 2 DIABETES AND MORTALITY

Obesity, the major modifiable risk factor for type 2 diabetes, has contributed to the dramatic increase in diabetes incidence worldwide (13). Many studies have attempted to quantify the effect of obesity on death, fuelling a sustained controversy about which levels of bodyweight can harm health (14). However, it has been argued that life expectancy does not capture the essence of the damage that obesity causes across a lifetime and that better long-term metrics are needed to convey risk, judge interventions and motivate behaviours (9, 15, 16). Previous estimates of the effect of obesity in diabetes have been limited to absolute risks or lifetime risk without combining information about quantity and quality of remaining years lived with or without the diabetes raising a gap in the intuitive understanding of risk and impact communicated among doctors and patients (17).

Although overweight and obese individuals are at higher risk for developing diabetes during their lifespan, a common assumption is that people who experienced recent weight gain are more likely to be diagnosed with diabetes. Also, it is well-known that individuals with type 2 diabetes vary greatly with respect to degree of BMI at time of diagnosis (18, 19). Therefore, a better understanding of the heterogeneity of diabetes is important for improving disease prevention and treatment. Well characterized strengths of an epidemiologic study (such as prospective design, repeated measurement for BMI, large sample size, long follow up detailed data on cardio-metabolic risk factors and medication) can facilitate the use of data-driven statistical methods, such as latent class trajectories (20-22).

NOVEL BIOMARKERS FOR TYPE 2 DIABETES

Novel biomarkers have been suggested for diabetes including sex hormones, bilirubin and gamma-glytammyltransferase (GGT). While the relation between sex-hormone binding globulin and diabetes has been recently established, literature on the associations of steroid sex hormones including endogenous estradiol and testosterone remain scarce, particularly in women (23, 24). On the other hand, bilirubin, the major end-product of heme catabolism has antioxidant properties and may compensate oxidative stress which in turn, has been shown to be an important factor in the pathophysiology

of diabetes (25). Another, pivotal actor of oxidative stress, GGT, a marker of alcohol consumption and liver disease, has been associated with hypertension, dyslipidaemia, diabetes, cardiovascular diseases and cancer (26-30). Given the consistent epidemiological evidence on increasing risk of diabetes, controversy exists whether GGT is causal to glycemic traits or diabetes (31). Moreover, GGT, together with waist circumference, body mass index and triglycerides levels comprise fatty liver index, a proxy for liver fat (32), that can be used in large epidemiological studies since it represents a non-invasive cheap technique as opposed to magnetic resonance, computed tomography and ultrasound. Given the emerging data showing that fatty liver is associated with increased risk of type 2 diabetes and cardiovascular disease (33), evidence is still indecisive whether FLI would potentially help to identify individuals of increased cardiometabolic risk and drive prevention strategies.

EPIGENETICS OF TYPE 2 DIABETES AND ITS RISK FACTORS

Genetic epidemiology studies the role of genetic factors in determining health and disease in families and in population and examines the interplay of such genetic factors with environmental factors (34). Following the completion of the Human Genome Project and the rapid improvement in genotyping technology, since the first decade of the 21st century, genetic epidemiology was revolutionized with a powerful approach, the genome-wide association study (GWAS) (35). This allowed to conduct analysis in large-scale population based settings and genotype thousands of genetic variants (single polymorphisms nucleotide-SNP) (36). The hypothesis free association studies led to the discovery of many common variants important to disease susceptibility.

An extension of GWAS, another more complex field emerged in the recent years: the epigenome-wide association study (EWAS). The role of epigenetic determinants is increasingly being recognized as a potential important link between environmental exposure and disease risk and thus may be a benchmark to capture both these influences. In contrast to genetic modifications which are in the majority of cases constant over individuals' time and are randomly assigned during birth, epigenetic changes are relatively susceptible to modifications by the environment as well as dysregulation over time. The epigenome encompass a series of chemical modifications that occur on the DNA or its associated proteins and are very important in gene function (37). DNA methylation, histone modification, and non-coding RNA are three major types of epigenetic marks (38). In this thesis, we perform analysis on DNA methylation and discuss histone modifications in some of the other projects.

DIABETES ADVERSE OUTCOMES

Diabetes represents one of the most important global disease burden (6, 39, 40). However, significant improvements have been made in halting this escalating trend mostly due to advances in treatment and well-management of complications. Mortality from diabetes and cardiovascular disease had shown a decrease trend in the past few decades (41-43). Consequently, because people are living longer, the number of elderly with dementia has been raising at the same time. Dementia is significantly higher in subjects with diabetes as compared to non-diabetic people, making this disease entity one of diabetes complications (44-46).

Despite overall improvement in diabetes prevention, significant variation in outcomes for people with diabetes still exists worldwide. For example, while there are several definitions for hypoglycaemia in clinical care, they have not been standardized among organization and there is inconsistency in the definitions used in different research studies; differences in rate of admission or length of stay for patients with diabetic emergencies are frequently observed (47, 48). The lack of standard outcome definitions can confuse their use in clinical practice, impedes development processes for new therapies, makes comparison of studies in the literature challenging and may lead to regulatory and reimbursement decision that fail to meet the needs of people with diabetes. Moreover, diabetes registries have been operating worldwide for several decades to identify the best management practices that lead to optimal outcomes and then to implement them across broad population, lessening the global burden of diabetes (49-51). And indeed, impressive gains in quality of care and outcomes have been made. However, the full potential impact of diabetes registries is currently constrained by the lack of two factors: international standard definitions and longer-term patient-centred outcomes. In the framework of value based medicine, outcome measurement, in contrast to more familiar measure of the care-delivery process has the potential to direct resources towards strategies with the highest value, (with value defined as the best possible health outcomes important to patients achieved for the lowest cost), which is particularly relevant for chronic disease that are major drivers of healthcare costs (52-54).

STUDY DESIGN

Systematic Reviews and meta-analysis

Some projects included in this thesis are systematic reviews and meta-analyses of the literature. Relevant research articles were identified using different electronic medical databases. Two independent reviewers screened the retrieved titles and abstracts and selected eligible studies. Discrepancies between the two reviewers were resolved through discussion and consensus with a third independent reviewer. We retrieved full texts for studies that satisfied all selection criteria. Further, reference lists of the included studies

were screened to identify additional relevant studies. Bias within each individual study was evaluated by two independent reviewers using the validated Newcastle-Ottawa Scale (NOS), a semi-quantitative scale designed to evaluate the quality of cohort studies (55). Study quality was judged on the selection criteria of participants, comparability of cases and controls, exposure and outcome assessment. Heterogeneity of study results was evaluated using Cochrane Q test and by the I^2 statistic, and was distinguished as low ($I^2 \leq 25\%$), moderate ($25\% < I^2 \geq 50\%$) or high ($I^2 \geq 75\%$) (56, 57). Begg funnel plots and Egger tests were used to assess the possibility of publication bias (58, 59).

Rotterdam Study

The studies described in this thesis are performed within a large population based cohort study, the Rotterdam Study (RS), also known in Dutch as "Erasmus Rotterdam Gezondheid Onderzoek (ERGO)". The study started in of Ommoord, a well-defined suburb of Rotterdam, the Netherlands. In 1989, all residents aged 55 years or older were invited to participate in the study (RS-I). Seventy-eight percent of the invitees agreed to participate (n= 7,983). In 1999, the Rotterdam Study was extended by including 3,011 participants from those who either moved to Ommoord or turned 55 (RS-II). The third cohort was formed in 2006 and included 3,932 participants 45 years and older (RS-III). There were no eligibility criteria to enter the Rotterdam Study cohorts except the minimum age and residential area based on postal codes. In total, the Rotterdam Study comprises 14,926 individuals.

All participants were examined in detail at baseline. In summary, a home interview was conducted (approximately 2 hours) and the subjects had an extensive set of examinations (~ 5hours) in a specially built research facility in the centre of their district. Participants have been re-examined every 3-5 years, and have been followed up for a variety of diseases. Genotyping was conducted, in self-reported white participants in all three cohorts using the Illumina Infinium HumanHap550K Beadchip in RS-I and RS-II and the Illumina Infinitum HumanHap 610 Quad chip in RS-III at the Genetic Laboratory of the Erasmus MC, Department of Internal Medicine, Rotterdam, the Netherlands. SNPs were imputed based on the 1000 Genomes cosmopolitan phase 1 version 3 reference. DNA methylation has been measured in peripheral blood taken by venepuncture. The DNA was extracted from the white blood cells (stored in EDTA tubes) by standardized salting out methods. Genome-wide DNA methylation levels were measured using the Illumina Human Methylation 450K array (60). An overview of baseline and follow-up visits is shown in Figure 1. The Rotterdam Study has been approved by the medical ethics committee according to the Population Screening Act: Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. All participants provided written informed consent to participate and to obtain information from their treating physicians.

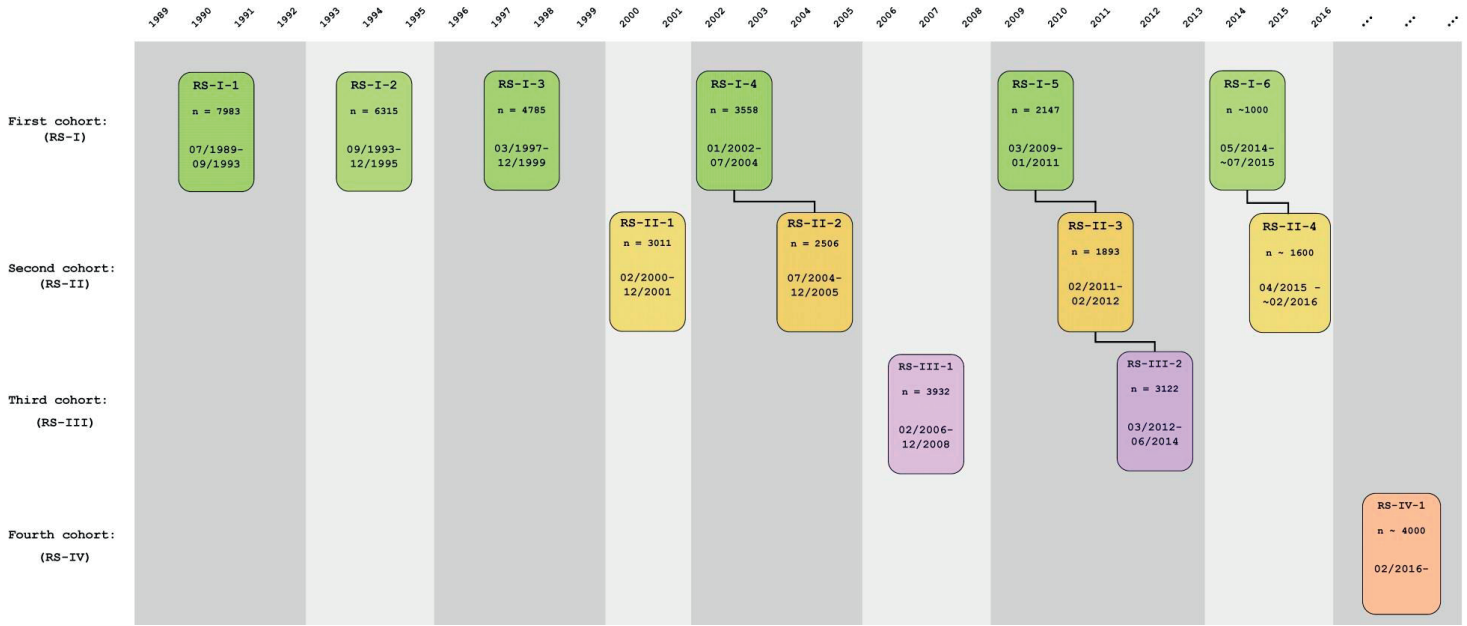

Figure 1. Overview of the Rotterdam Study cohorts and visits.

The Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium

The CHARGE consortium was formed to facilitate genome-wide association study meta-analyses and replication opportunities among multiple large and well-phenotyped longitudinal cohort studies (61). The working groups are divided in phenotype specific or method-specific. Gigantic efforts such as the CHARGE consortium have brought the development of genetic epidemiology research to another level. Meta-analyses of GWAS has enabled CHARGE to boost samples size thereby increasing the probability of identifying new genetic variants. With the emerging field of epigenetics, the consortia started new efforts to facilitate epigenome-wide association studies meta-analyses of different clinical traits. Among these projects, non-alcholic fatty liver disease working group was set up in late 2016 to run the first trans-ethnic EWAS in the field with the following participating cohorts: Framingham Heart Study (FHS) [including Offspring and Third Generation cohorts], Rotterdam Study (RS), Multi-Ethnic Study of Atherosclerosis (MESA), Genetic Epidemiology Network of Arteriopathy (GENOA) and The Coronary Artery Risk Development in Young Adults (CARDIA) Study (Figure 2).

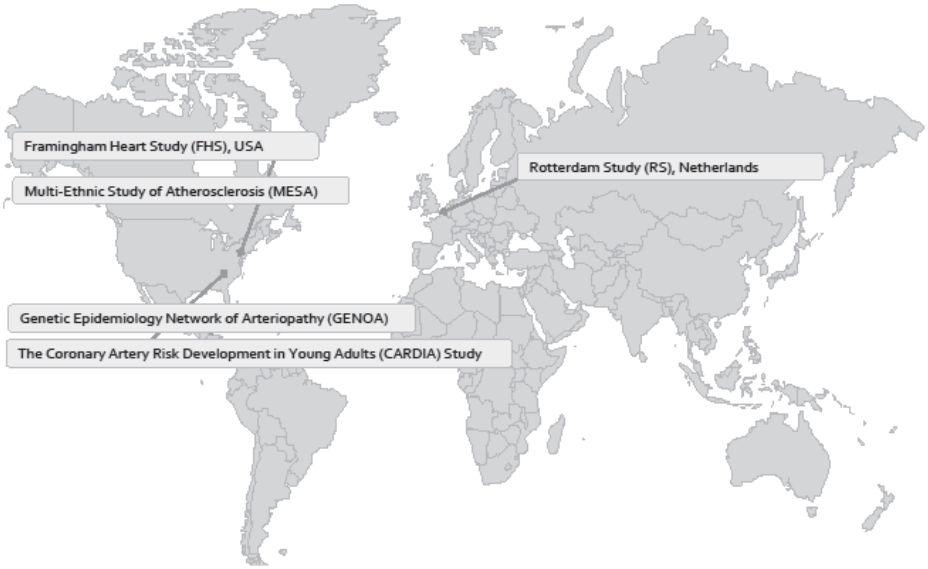

Figure 2. The CHARGE consortium: the participating cohort studies.

International Consortium for Health Outcomes Measurement (ICHOM)

ICHOM was founded in 2012 as a not-for-profit organization founded by Harvard Business School, Boston Consulting Group and the Karolinska Institute to develop Standard Sets of outcome measures for the world's medical conditions and then drive their

adoption by health care institutions. The systematic measurement of Standard Sets of outcomes by institutions around the world will enable for the first time, global outcome comparisons. This will catalyse a new wave of learning for health care professionals and enhance patient choice (62).

Since July 2017, ICHOM brought together an internationally recognised group of clinicians and non-clinicians leaders in the field of diabetes with expertise in clinical trials and registries, public and private health system management and patient-centred outcomes research, outcome measurement and quality improvement and patient advocacy with a total of 35 members from 6 continents. The aim is to develop a globally agreed Standard Set of outcomes for individuals with diabetes. Current metrics for care across these conditions tend to capture processes and costs, and do not measure whether they achieve the outcomes which matter most to patients. Developing a globally agreed set of outcomes for this population will enable us to measure important outcomes, and then compares in a consistent manner, with other countries around the world. This will enable the identification of those systems with the best outcomes and the subsequent ability to learn from the processes that they have in place.

AIM OF THIS THESIS AND OUTLINE

The overall aim of this thesis is to study (traditional and novel) risk markers of type 2 diabetes in light of new epidemiological trends and to further explore the relationship with adverse outcomes and standardize the latter to allow worldwide comparisons for the ultimate purpose: improve diabetes care.

In chapter 2 of this thesis, we calculate total life expectancy and life expectancy with and without type 2 diabetes for older adults with obesity, by comparing them to normal weight individuals (chapter 2.1). We further identify change of body mass index trajectories prior to diabetes development. Within these patterns, additional exploration of trajectories of other cardiometabolic risk factors including glycemic indices (such as glucose, insulin, insulin resistance, beta cell dysfunction), blood pressure and lipid profile are examined (chapter 2.2). In chapter 3, we investigate several novel biomarkers of type 2 diabetes. The association between steroid sex hormones and sex hormone-binding globulin and type 2 diabetes is investigated in chapter 3.1 comprising original data analysis within the Rotterdam Study and a meta-analysis of the current literature. In chapter 3.2, we quantitatively summarize current evidence on the relation between bilirubin, metabolic syndrome and diabetes risk. Chapter 3.3 describes the association between GGT levels and risk of prediabetes and type 2 diabetes and examines whether the observed association is causal. In chapter 3.4, the association between fatty liver index, a clinical-friendly proxy for fatty liver, with the risk of diabetes, cardiovascular disease and mortality is investigated. In chapter 4, we summarize some of the most up-to-

date findings in the field of epigenetics for diabetes and its risk factors and report three large meta-analyses using the new emerging approach of epigenome-wide association study to identify differentially methylated genes for liver enzymes, non-alcoholic fatty liver and obesity-related traits. We conduct a comprehensive systematic review of the current evidence on global and regional DNA methylation and histone modifications in glycemic traits and diabetes (chapter 4.1). Similarly, we performed a review on the role of epigenetics on circulatory inflammation markers (chapter 4.2). In chapter 4.3, we aim to identify DNA methylation signatures related to liver function and provide further experimental evidence on such associations. In chapter 4.4, we conducted a trans-ethnic epigenome-wide association study for non-alcoholic liver disease in combining data from Framingham Heart Study (FHS) and the Rotterdam Study (RS) and replicate the findings in the Multi-Ethnic Study of Atherosclerosis (MESA), Genetic Epidemiology Network of Arteriopathy (GENOA) and The Coronary Artery Risk Development in Young Adults (CARDIA) Study. In chapter 4.5, we performed a meta-analysis of epigenome-wide association studies for obesity related traits (body mass index and waist circumference) using Rotterdam Study as a discovery panel and the Atherosclerosis Risk in Communities (ARIC) Study as a replication panel. In chapter 5, we explore outcomes related to diabetes. In chapter 5.1, we aim to investigate the effect of diabetes on dementia risk within a causal inference framework. In chapter 5.2, we struggle to identify a consensus set of outcomes and risk adjustment variables with standard definitions for individuals with diabetes that could be tracked by health systems and clinical registries around the world. Finally, in chapter 6, we discuss the main findings of this thesis in a broader context and we further address the methodological considerations, potential clinical implications and directions for future research.

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CHAPTER 2

Obesity, Type 2 Diabetes and Mortality

Chapter 2.1

Obesity in older adults and life expectancy with and without diabetes: a prospective cohort study

Klodian Dhana[1¶], Jana Nano[1¶], Symen Ligthart[1], Anna Peeters[2], Albert Hofman[1,3], Wilma Nusselder[4], Abbas Dehghan[1], Oscar H Franco[1]

[¶] Both authors contributed equally to this work
[1] Department of Epidemiology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[2] Deakin University, Geelong, Victoria, Australia
[3] Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, Mass, USA
[4] Public Health Erasmus MC, University Medical Center Rotterdam, the Netherlands;

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ABSTRACT

Background

Overweight and obesity are associated with increased risk of type 2 diabetes. Limited evidence exists regarding the effect of excess weight on years lived with and without diabetes. We aimed to determine the association of overweight and obesity with the number of years lived with and without diabetes in a middle-aged and elderly population.

Methods and Findings

The study included 6,499 individuals (3,656 women) aged 55 years and older from the population-based Rotterdam Study. We developed a multistate life table to calculate the effect of normal weight, overweight and obesity on total life expectancy and life expectancy with and without diabetes. For life table calculations, we used prevalence, incidence rate and hazard ratios (HR) for 3 transitions (healthy-to-diabetes, healthy-to-death, and diabetes-to-death), stratifying by BMI at baseline and adjusting for confounders. During median follow-up of 11.1 years, we observed 697 incident diabetes events and 2,192 overall deaths. Obesity was associated with an increased risk of developing diabetes (HR, 2.13 for men and 3.54 for women). Overweight and obesity were not associated with mortality in men and women with or without diabetes. Total life expectancy remained unaffected by overweight and obesity. Nevertheless, men with obesity aged 55 years and older lived 2.8 (95% CI -6.1, -0.1) fewer years without diabetes than normal weight individuals, whereas, for women, the difference between obese and normal weight counterparts was 4.7 (95% CI -9.0, -0.6) years. Men and women with obesity lived 2.8 (95% CI 0.6, 6.2) and 5.3 (95% CI 1.6, 9.3) years longer with diabetes, respectively, compared to their normal weight counterparts. Since the implications of these findings could be limited to middle aged and older European Caucasian populations, our results need confirmation in other populations.

Conclusions

Obesity in the middle aged and elderly is associated with a reduction in the number of years lived free of diabetes and an increase in the number of years lived with diabetes. Those extra years lived with morbidity might place a high toll to individuals and healthcare systems.

INTRODUCTION

Overweight and obesity is one of today's highest public health concerns, which has contributed to the dramatic increase of type 2 diabetes [1, 2]. Previous estimates of the effect of obesity in diabetes have been limited to absolute risks or lifetime risk without combining information about quantity and quality of remaining years lived with or without the diabetes raising a gap in the intuitive understanding of risk and impact communicated among doctors and patients [3]. Complementing current knowledge with comparative measures of long-term dimension of disease such as life expectancy provide information on different scenarios including whether for example, years with disease are increasing, but the proportion of life spent free of disease is increasing or decreasing. Moreover, it has been extensively recommended to judge public health interventions [4].

Studies evaluating the association between obesity and life expectancy have shown that obesity in adulthood is associated with a decrease in life expectancy of approximately 6-13 years [5, 6]. Two US studies using data from National Health Surveys, showed that obesity in adulthood was associated not only with reduced life expectancy, but also with a reduced number of years lived free of diabetes and cardiovascular disease in men and women [7, 8]. Specifically, the study by Grover et al. showed that obesity in individuals aged 40-59 years was associated with a shorter life expectancy free of diabetes and cardiovascular disease by 5.9 years in men and 10.3 years in women [7]. Notably, this study did not distinguish between life expectancy with and without diabetes. The study performed by Narayan et al., which primarily focused on the effect of obesity on lifetime risk of diabetes, reported that individuals with obesity had an earlier onset of diabetes during their lifespan, and spent more years lived with diabetes [8]. Nevertheless, both studies do not provide a direct observation of a well-defined population as the results are obtained by modelling and simulation.

Therefore, we aimed to calculate the association of overweight and obesity with total life expectancy and years lived with and without diabetes at 55 years of age. We constructed multistate life tables using data collected from 1997-2001 and with over 14 years of follow up from the Rotterdam Study.

METHODS

Ethical Considerations

The Rotterdam Study has been approved by the medical ethics committee according to the Population Screening Act: Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. All participants in the present analysis provided written informed consent to participate and to obtain information from their treating physicians.

Study population

This study was embedded within the framework of the Rotterdam Study, a prospective cohort study of the community-dwelling population in Rotterdam, Netherlands. The objectives and design of the Rotterdam Study have been described in detail elsewhere [9]. In response to demographic changes leading acceleration of population aging, the Rotterdam Study was originally designed to investigate determinants of disease occurrence and progression in the elderly. In addition to contributing to the understanding of the etiology of geriatric illnesses, the study is expected to lead to specific recommendations for intervention. Following the pilot in 1989, recruitment started in January 1990 of all residents aged 55 years or older, of whom 7983 (78%) agreed to participate (RS-I). The study was extended in 2000, with a second cohort of individuals (RS-II) who had reached the age of 55 years or moved into the study area.

For the current study, we used data from the participants attending the third examination of the original cohort (RS-I-visit 3, 1997–1999; n=4797) and the participants attending the first examination of the extended cohort (RS-II-visit 1, 2000–2001; n=3011).

We excluded participants who did not visit the research center, did not have information on BMI (n= 1,051) or no information on smoking behavior (n=40). To account for disease-related weight loss, we excluded participants who had BMI <18.5 (n= 51). Individuals without informed consent (n=30) or those who did not have information of diabetes follow-up (n=137) were further excluded. Finally, 6,499 participants (3,656 women) were available for the current analysis.

Assessment of anthropometric measurements, health behaviors and laboratory measurements

Anthropometrics were measured in the research center by trained staff. Height and weight were measured with the participants standing without shoes and heavy outer garments. BMI was calculated as weight divided by height squared (kg/m2) [10]. According to the WHO cut-off criteria, we composed BMI as a categorical variable with three categories: normal weight (18.5≤ BMI<25), overweight (25≤ BMI <30) and obese (30≤ BMI).[10]. For our data analysis, obesity was grouped into a single category of BMI of 30.0 and higher because of the small sample size in each obesity class (e.g., 30< BMI≤ 35 and 35< BMI< 40 and BMI≥ 40). Smoking status was categorized as current smoker, former smoker and never smoker, and additionally, for current smokers, we accounted for cigarettes smoked per day. Information on education was assessed according to the standard international classification of education and was composed into four categories: elementary education, lower secondary education, higher secondary education and tertiary education [11]. Marital status was divided in single, married, widowed or divorced/separated. Physical activity was measured by questionnaire and expressed in METhours/week. For analysis, we divided the population in 3 equal groups (tertile) [12].

Alcohol consumption was categorized as less than 1 glass/day, 1-4 glasses/day for men and 1-2 glasses/day for women, and > 4 glasses/day for men and > 2 glasses/day for women. Comorbidity was considered present when "non-obesity related cancers other than skin cancer" or chronic obstructive pulmonary disease was prevalent at baseline. From baseline comorbidities we excluded cancers associated with obesity [13], or cancers that are curable and not likely to be related to weight loss or mortality such as skin cancer [14]. Cancers induced by obesity are in the pathway between obesity and mortality, therefore we accounted them as mediators. Chronic obstructive pulmonary disease was defined as a type of obstructive lung disease characterized by airflow limitation not fully reversible [15].Chronic obstructive pulmonary disease has been shown to be accompanied with weight loss [16].

Hypertension, dyslipidemia and cardiovascular disease were also considered as mediators and therefore, we did not adjust for them in the main analyses. However, to investigate the independent effect of obesity on diabetes and mortality, we conducted additional sensitivity analysis by adjusting in the multivariable analysis for comorbidities including chronic obstructive pulmonary disease, all cancers and cardiovascular disease at baseline. The presence of hypertension and dyslipidemia was based on medication information, whereas cardiovascular disease was defined as the presence of one or more definite manifestation of coronary heart disease (coronary revascularization or non-fatal or fatal myocardial infarction or death due to coronary heart disease), stroke and heart failure [17-19].

Assessment of outcome

Participants were followed up from the date of baseline center visit onwards. At baseline and during follow-up, cases of diabetes were ascertained by use of general practioners' records (including laboratory glucose measurements), hospital discharge letters, and serum glucose measurements from Rotterdam Study visits, which take place roughly every 4 years [20]. Diabetes was defined according to recent WHO guidelines [21] as a fasting serum blood glucose ≥ 7.0 mmol/L, a non-fasting blood glucose ≥ 11.1 mmol/L (when fasting samples were not available), or the use of blood glucose lowering medication. Information regarding the use of blood glucose lowering medication was ascertained from both structured home interviews and linkage to pharmacy records [21]. All potential prevalent cases of diabetes were independently reviewed by two study physicians. In case of disagreement, consensus was reached with an endocrinologist.

Statistical analysis

We did not publish or pre-register a plan for this study. The analysis plan is described below, with any differences noted in S1 Text.To calculate the life expectancy with and without diabetes in normal weight, overweight and obese groups, we created a multistate life table, which is a demographic tool that allows to combine the experience of individuals

in different health states to calculate the total life expectancy and the amount of years that individuals could expect to live in the different health states [22]. We constructed three different health states: free of diabetes, diabetes and death. The possible transition directions were from free of diabetes to diabetes (incident diabetes), free of diabetes to death (mortality among non-diabetics) and from diabetes to death (mortality among diabetics). No backflows were allowed, and only first event into a state was considered.

To obtain transition rates, we calculated the overall age- and sex-specific rates for each transition. Next, we calculated the prevalence of normal weight, overweight and obesity by sex, 10-year age groups, and separately for subjects with and without diabetes. Subsequently, we computed gender specific hazard ratios comparing overweight and obese to normal weight individuals by using Poisson regression with "Gompertz" distribution in 2 models. Model 1 was adjusted for age; and Model 2 was adjusted for age, smoking status, cigarettes smoked per day (for current smokers), alcohol consumption, education, marital status, physical activity and comorbidities (non-obesity related cancers other than skin cancer or chronic obstructive pulmonary disease).

Finally, transition rates were calculated for each category of BMI separately using the (a) overall transition rates, the (b) adjusted hazard ratios (model 2) for diabetes and mortality, and the (c) prevalence of normal weight, overweight and obesity by sex and with and without diabetes. Similar calculations have been described previously [23, 24]. The multistate life table started at age 55 years and closed at age 100 years.

We used Monte Carlo simulation (parametric bootstrapping) with 10 000 runs to calculate the confidence intervals of our life expectancy estimates [25].

To exclude any potential bias caused by smoking or comorbidities at baseline, we repeated the analysis among those who were both nonsmokers and without comorbidities (n=5,018). Additionally, we estimated the life expectancy among participants without hypertension, dyslipidemia and cardiovascular disease at baseline (n=3,843). To account for possible reverse causation effects, we estimated the hazard ratios after excluding diabetes events (n=64) or deaths (n=186) during the first two years of follow-up. Moreover, as sensitivity analysis we excluded the individuals with BMI < 22 (n=448) to provide more conservative estimates of overweight and obesity in association with mortality.

To deal with missing values (less than 5%) for covariates including education, living situation, income, and alcohol, we used single imputation with the Expectation Maximization method in SPSS (IBM SPSS Statistical for Windows, Armonk, New York: IBM Corp). This method allows to impute the missing values as function of other variables by using regression methods. We used STATA version 13 for Windows (StataCorp, College Station) and R statistical software (A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria) for our analysis.

Note: Supplementary Material can be found in the website of the published journal or can be provided on request.

RESULTS

In total, we observed 697 (12.4%) incident diabetes events and 2,192 (33.7%) overall deaths over 14 years of follow-up. The mean age of the population was 69.2 and 3,656 (56.3%) were women. Compared to women, men at baseline were younger, consumed higher alcohol amounts and smoked more, but showed lower levels of BMI and physical activity. While more women were on treatment for hypertension, more men were treated for dyslipidemia. Furthermore, prevalence of cardiovascular disease and other comorbidities were higher among men (Table 1).

Diabetes events and death

Table 2 shows the hazard ratios of the association between BMI categories with risk of incident diabetes and mortality among men and women. In multivariable adjusted model, obesity (BMI higher than 30) was associated with an increased risk of incident diabetes in men (HR 2.13, 95%CI 1.48, 3.07) and women (HR 3.54, 95%CI 2.64, 4.75) comparing with normal weight individuals (Table 2).

The association between obesity and mortality among those without diabetes did not reach statistical significance among both men (HR 1.00, 95%CI 0.78, 1.28) and women (HR 0.89, 95%CI 0.74, 1.06). Similarly, we did not find significant associations between obesity and mortality among individuals with diabetes. The corresponding HRs and 95%CI for men are 0.79 (0.56, 1.11) and for women 0.70 (0.55, 1.01) (Table 2).

Total life expectancy and life expectancy with and without diabetes

The association between normal weight, overweight and obesity with the risk of each transition was translated into number of years lived with and without diabetes (Fig 1 and Table 3). Total life expectancy for men and women with overweight and obesity were not significantly different than normal weight counterparts. Compared to normal weight men, life expectancy of 55-year-old men in the obese group was 0.0 years (95% CI -1.3, 1.3). For women, these differences were: 0.7 (95% CI -0.3, 1.6) years (Table 3). For both men and women, obesity was associated with fewer years lived without diabetes and more years lived with diabetes than their normal weight counterparts. Men and women with obesity lived 2.8 (95% CI -6.1, -0.1) and 4.7 (95% CI -9.0, -0.6) fewer years without diabetes, respectively, than those in the normal weight group. Additionally, men and women with obesity lived more years with diabetes than their normal weight counterparts: 2.8 (95% CI 0.6, 6.2) years for men and 5.3 (95% CI 1.6, 9.3) years for women (Fig 1 and Table 3).

Total life expectancy, number of years lived with and without diabetes for normal weight, overweight and obese individuals who are non-smokers and without prevalent comorbidities ("non-obesity related cancers other than skin cancer" and chronic obstructive pulmonary disease) are presented in the S1 Fig, and for individuals without hypertension, dyslipidemia and cardiovascular disease are presented in the S2 Fig. As

Table 1. Baseline characteristics of study population (n=6,499)

Characteristics	Men	Women
Population		
n	2,843 (43.7)	3,656 (56.3)
Age at interview (years)	68.7 (7.9)	69.6 (8.4)
Anthropometry		
BMI, kg m-2	26.6 (3.2)	27.4 (4.4)
Normal (BMI 18.5-25)	927 (32.6%)	1,174 (32.1%)
Overweight (BMI 25-30)	1,525 (53.6%)	1,575 (43.1%)
Obese (BMI 30+)	391 (13.8%)	907 (24.8%)
Social economic status		
Marital status		
Single	83 (2.9%)	254 (6.8%)
Married	2,247 (79.0%)	1,958 (53.6%)
Widowed	306 (10.8%)	1,069 (29.2%)
Divorced/separated	207 (7.3%)	375 (10.3%)
Education		
Elementary	268 (9.4%)	599 (16.4%)
Lower secondary	853 (30.0%)	1,953 (53.4%)
Higher secondary	1,109 (39.0%)	863 (23.6%)
Tertiary	613 (21.6%)	241 (6.6%)
Lifestyle variables		
Smoking		
Never smoker	910 (32.0%)	2,266 (62.0%)
Former smoker	1,417 (49.8%)	780 (21.3%)
Current smoker	516 (18.1%)	610 (16.7)
Daily cigarettes smoked	2.8 (7.0)	2.3 (6.1)
Alcohol (drinks/day)		
< 1 glass/day	1,270 (44.7%)	2,601 (71.1%)
1-4 glasses/day (men); 1-2 glasses/day (women)	1,340 (47.1%)	658 (18.0%)
> 4 glasses/day (men); > 2 glasses/day (women)	233 (8.2%)	397 (10.9%)
Physical activity (METh)	74.0 (43.8)	92.6 (43.1)
Treatment for hypertension	604 (22.2%)	909 (26.1%)
Treatment for dyslipidemia	410 (14.4%)	456 (12.5%)
Comorbidities (cancer [a] and chronic obstructive pulmonary disease)	270 (9.5%)	204 (5.6%)
Prevalence of cardiovascular disease	573 (20.2)	303 (8.3%)

BMI, body mass index

Values are means (SDs) or numbers (percentages) or median (IQR).

[a] Cancer includes "non-obesity related cancers other than skin cancer"

Table 2. Hazard ratios for incidence diabetes and death in overweight and obese men and women

Transition	Categories	Men		
		Cases, No. / Person-Years	Model 1 HR (95% CI)[a]	Model 2 HR (95% CI)[b]
Incident T2D	Normal weight	297/23110	1.0 (Reference)	1.0 (Reference)
	Overweight		1.45 (1.10, 1.90)	1.52 (1.15, 2.00)
	Obese		2.00 (1.40, 2.87)	2.13 (1.48, 3.07)
Mortality among those without T2D	Normal weight	858/24527	1.0 (Reference)	1.0 (Reference)
	Overweight		0.97 (0.84, 1.13)	1.02 (0.88-1.18)
	Obese		0.96 (0.75, 1.22)	1.00 (0.78-1.28)
Mortality among those with T2D	Normal weight	335/5259	1.0 (Reference)	1.0 (Reference)
	Overweight		0.90 (0.70, 1.15)	0.99 (0.77, 1.28)
	Obese		0.77 (0.55, 1.07)	0.79 (0.56, 1.11)
Transition	**Categories**	**Women**		
		Cases, No. / Person-Years	**Model 1 HR (95% CI)[a]**	**Model 2 HR (95% CI)[b]**
Incident T2D	Normal weight	400/ 33152	1.0 (Reference)	1.0 (Reference)
	Overweight		2.27 (1.72, 3.00)	2.32 (1.76, 3.06)
	Obese		3.47 (2.60, 4.65)	3.54 (2.64, 4.75)
Mortality among those without T2D	Normal weight	837/35227	1.0 (Reference)	1.0 (Reference)
	Overweight		0.82 (0.71, 0.96)	0.85 (0.76, 0.99)
	Obese		0.86 (0.72, 1.03)	0.89 (0.74, 1.06)
Mortality among those with T2D	Normal weight	253/ 6237	1.0 (Reference)	1.0 (Reference)
	Overweight		0.75 (0.54, 1.04)	0.77 (0.55, 1.09)
	Obese		0.72 (0.51, 1.02)	0.70 (0.55, 1.01)

[a] Adjusted for age.

[b] Adjusted for age, smoking, cigarettes smoked per day for current smokers, education level, marital status, physical activity, alcohol use and comorbidities (COPD and cancer not caused by obesity).

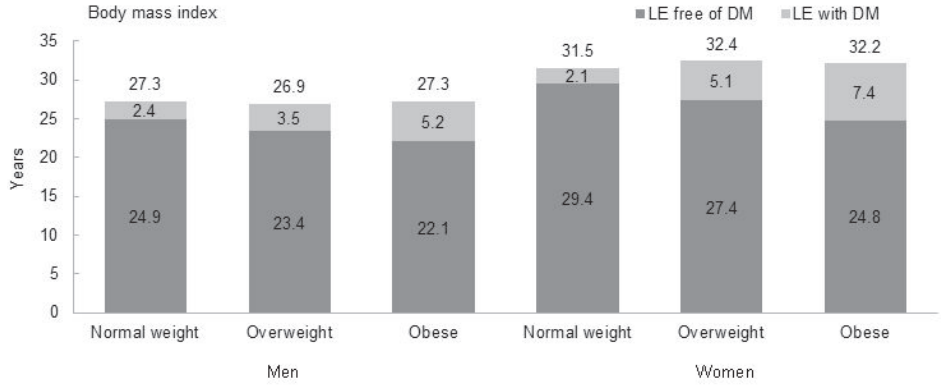
expected, compared to the overall population included in the main analyses, total life expectancy was higher for individuals who were nonsmokers and without comorbidities at baseline, or for individuals without cardiovascular disease, hypertension and dyslipidemia. However, differences in years lived with and without diabetes among normal weight, overweight and obese individuals were overall similar to those found in the total population. S1 Table shows the baseline characteristics of individuals who did not visit the research center or without information of BMI. This subgroup of individuals was older than individuals included in the study and were less physically active. Additionally, when we repeated the main analysis after excluding incident diabetes and deaths during the first 2 years of follow-up (S2 Table) or excluding individuals with BMI<22 (S3 Table) or adjusting for all comorbidities (all cancers, cardiovascular disease and chronic obstructive pulmonary disease) (S4 Table), we found generally similar results with main analyses.

Table 3. Differences in life expectancy, in years, at age 55 years for normal weight, overweight and obesity in men and women

	Total LE	Difference in total life expectancy	Life expectancy free of diabetes	Differences in number of years lived free of diabetes	Life expectancy with diabetes	Differences in number of years lived with diabetes
Men						
Normal weight	27.3 (26.7, 27.9)	Ref	24.9 (24.1, 25.7)	Ref	2.4 (1.9, 3.0)	Ref
Overweight	26.9 (26.5, 27.5)	-0.4 (-1.2, 0.5)	23.4 (22.6, 24.4)	-1.5 (-2.7, -0.1)	3.5 (2.8, 4.1)	1.1 (0.2, 2.2)
Obese	27.3 (26.0, 28.6)	0.0 (-1.3, 1.3)	22.1 (19.1, 24.7)	-2.8 (-6.1, -0.1)	5.2 (3.1, 7.9)	2.8 (0.6, 6.2)
Women						
Normal weight	31.5 (31.1, 32.1)	Ref	29.4 (28.4, 30.5)	Ref	2.1 (1.3, 2.9)	Ref
Overweight	32.4 (31.8, 33.1)	0.9 (0.1, 1.7)	27.4 (25.5, 29.6)	-2.1 (-4.3, 0.1)	5.1 (3.1, 6.7)	3.0 (1.1, 4.8)
Obese	32.2 (31.3, 33.0)	0.7 (-0.3, 1.6)	24.8 (21.1, 28.5)	-4.7 (-9.0, -0.6)	7.4 (4.0, 10.8)	5.3 (1.6, 9.3)

Ref, Reference; LE, life expectancy. We calculated the differences on total life expectancy and years lived with and without diabetes by subtracting the estimates of overweight and obesity to normal weight individuals.

Figure 1. Effect of obesity on life expectancy with and without diabetes at age 55 years.



Body mass index (BMI) categories: Normal weight BMI is <25kg/m2; Overweight BMI is 25-30kg/m2 and Obese BMI is ≥30kg/m2. LE, life expectancy; DM, type 2 diabetes mellitus.

DISCUSSION

Overweight and obesity at age 55 years and older represents not only a significant increase in the risk of developing diabetes, but also an important decrease in the number

of years lived free of diabetes and an extended number of years lived with diabetes when compared with normal weight counterparts. While total life expectancy remained unaffected, on average, obesity was associated with 2.8 fewer years lived free from diabetes in men and 4.7 fewer years in women. Additionally, obese men and women, respectively lived 2.8 and 5.3 years longer with diabetes compared to their normal weight counterparts.

Years lived free of diabetes are a result of two components: incidence of diabetes and mortality in those without diabetes. We observed a higher risk of incident diabetes in overweight and obese individuals when compared to their normal weight counterparts which could reflect an earlier diagnosis of diabetes across lifespan. Furthermore, a higher risk of mortality in those without diabetes will result in a decrease of total life expectancy, and consequently will shorten years lived free of diabetes. Number of years lived with diabetes are a consequence of incident diabetes risk, and mortality risk among those with diabetes. Higher incidence of diabetes would lead to an earlier occurrence of diabetes, whereas, lower risk of mortality among those with diabetes would lead to greater number of years lived with diabetes.

Our analysis indicated that overweight and obesity increased the risk of diabetes for both men and women and the hazard ratios were comparable with other studies [26, 27]. Additionally, we showed that overweight and obesity were not associated with mortality in individuals with and without diabetes. A recent meta-analysis including diabetic populations revealed a lower risk of mortality among overweight and obese subjects than normal weight counterparts [28]. Although our effect estimates of mortality risk among diabetic patients are similar to the meta-analysis, we cannot support the protective effect of obesity on mortality until further research is done.

In our study, total life expectancy in individuals aged 55 and over for both men and women remained unaffected by overweight and obesity. In contrast, an earlier study using Framingham Study data has showed that at the age of 40 years, obesity was associated with large decreases in total life expectancy [6]. This discrepancy could be explained by the difference of participants' age (55 vs 40) in life expectancy calculations and differences in calendar time of baseline measurements (1997 vs 1948). Given the improvements in prevention and treatment of cardio-metabolic risk factors in the last decade, the effect of obesity on mortality has diminished substantially [29, 30]. Consistent with our findings, recent data among middle-aged and elderly has demonstrated that overweight and obesity are not associated with a reduction in life expectancy [31, 32]. Nevertheless, our study extended the previous evidence by calculating the association of obesity in life expectancy with and without diabetes. We demonstrated that obesity increases the risk of developing diabetes earlier in life and further extends years lived with diabetes. Those findings support the previous results from Narayan et al.,[8] which used data from US National Health Survey. However, our study is unique

regarding the used approach for estimating life expectancy with and without diabetes. While Narayan et al obtained the estimates by modelling and simulation, we calculated the life expectancy with and without diabetes from direct observation of a well-defined population using multi-state life tables. Moreover, the study used self-reported data for the diagnosis of diabetes and information on height and weight, whereas in our study we had well-ascertained diabetes diagnosis obtained from physicians and linkage to pharmacy data and weight and height were measured by trained research assistants at the study center.

In our study, we noted a difference in the number of years lived with diabetes among men and women. Compared to men, women had an increased risk of diabetes by BMI, indicating an earlier occurrence of diabetes during their life. Moreover, women with diabetes had a lower risk of mortality compared to men. Taken these results together, we could explain why women spend more years with diabetes than men. This is in accordance with previous research conducted in US concluding that women spend more years living with diabetes than men [8], possibly due to larger differences in probabilities of death between males and females observed for patients with diabetes relative to those without diabetes [33].

Strengths of the current study include the use of data from a prospective, well-organized study with long-term follow-up. 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. Height and weight are measured in the research center by trained staff. Nevertheless, some limitations of this study should be addressed. In our analysis, we excluded individuals with missing information on weight and height since the BMI is our main exposure. This subgroup was generally similar with those included in the analyses, however they were older and less physically active. Furthermore, since the generalizability of these findings could be limited to middle aged and older European Caucasian populations, our results need confirmation in other populations. Additionally, studies evaluating the association of obesity with mortality could be prone to incorrect adjustment for confounders such as smoking or weight loss related to comorbidities. In our study, we adjusted for smoking status and the cigarettes smoked per day and comorbidities. Moreover, we conducted a sensitivity analysis to take into account reverse causation by excluding events during the first 2 years of follow-up.

The added value of this study is the combination of the observed effects of obesity on diabetes incidence and mortality translated into population measures such as life expectancy with and without diabetes that might be important to clinicians, patients and policy makers to tackle the next stages of obesity epidemics. Our study showed that among middle age and elderly individuals overweight and obesity do not seem to have an effect on total life expectancy, but are associated with earlier and extended

periods lived with diabetes. Those extra years of life will be filled with an expansion of accompanying comorbidities placing a higher toll to clinicians and health care systems and challenging the new global strategies for obesity and diabetes prevention.

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Chapter 2.2

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

Jana Nano[1, 2*], Klodian Dhana[3*], Eralda Asllani[1], Eric Sijbrands[4], M. Arfan Ikram[1], Abbas Dehghan[1,5], Taulant Muka[1, 6*], Oscar H. Franco[1,7*]

[1] Department of Epidemiology, Erasmus MC, Rotterdam, The Netherlands
[2] Institute of Epidemiology, Helmholtz Zentrum Munich, German Research Center for Environmental Health, Germany
[3] Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, USA.
[4] Department of Internal Medicine, Erasmus University Medical Center, Rotterdam, the Netherlands
[5] Department of Biostatistics and Epidemiology, MRC-PHE Centre for Environment and Health, School of Public Health, Imperial College London, UK
[6] Novordisk, Copenhagen, Denmark
[7] Institute of Social and Preventive Medicine (ISPM), University of Bern, Bern, Switzerland
* These authors contribute equally

(Submitted)

ABSTRACT

Background

Although obesity remains one of the most important risk factors for type 2 diabetes, the complex pathophysiology of diabetes among individuals with different BMI's suggests a potential of prevention strategies based on BMI trajectories. We investigated BMI trajectories and examined associated changes in other cardiometabolic risk factors prior to type 2 diabetes.

Methods

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

Results

During a mean follow-up of 13.7 years, 565 participants developed type 2 diabetes among whom we identified 3 distinct trajectories of BMI including the "progressive overweight" group (n= 481, 85.1%), "progressive weight loss" group (n= 59, 10.4%), and "persistently obese" group (n=25, 4.4%). The majority, the "progressive overweight" group, was characterized by a steady increase of BMI in the overweight range starting 10-years before diabetes diagnosis. Moreover, they experienced a constant increase of insulin levels and insulin resistance during the 5 years prior to diabetes. The second group of "progressive weight loss" had constant insulin levels and a slight increase of insulin resistance, but exhibited increased fluctuations of glucose levels during the follow-up as well as marked beta cell function loss. The "persistently obese" was severely obese throughout the follow-up time before diabetes diagnosis. They were characterized by a slight increase in insulin levels and sharp increase of insulin resistance accompanied by a rapid decrease of beta cell function.

Conclusion

We found heterogeneity of BMI changes prior to type 2 diabetes in a middle-aged and elderly white population. The majority of people had small incremental changes of body weight during follow-up. Therefore, we recommend to apply diabetes prevention strategies for weight loss to the whole overweight population rather than to focus on high-risk (obese) individuals.

INTRODUCTION

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

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

METHODS

Study population

The study was performed among participants of the prospective population-based Rotterdam Study (RS). In 1989, all residents aged 55 years or older in Ommoord, a suburb of Rotterdam, the Netherlands, were invited to participate in the study (RS-I). Seventy-eight percent of the invitees agreed to participate (n= 7,983). In 1999, the Rotterdam Study was extended by including 3,011 participants from those who either moved to Ommoord or turned 55 (RS-II). The third cohort was formed in 2006 and included 3,932 participants 45 years and older (RS-III). There were no eligibility criteria to enter the Rotterdam Study cohorts except the minimum age and residential area based on postal codes. The participants of the Rotterdam Study have been followed-up for more than 22 years for a variety of diseases and clinical data have been collected across five subsequent phases every 3-4 years. A more detailed description of the Rotterdam Study can be found elsewhere (9). The Rotterdam Study has been approved by the medical ethics

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

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

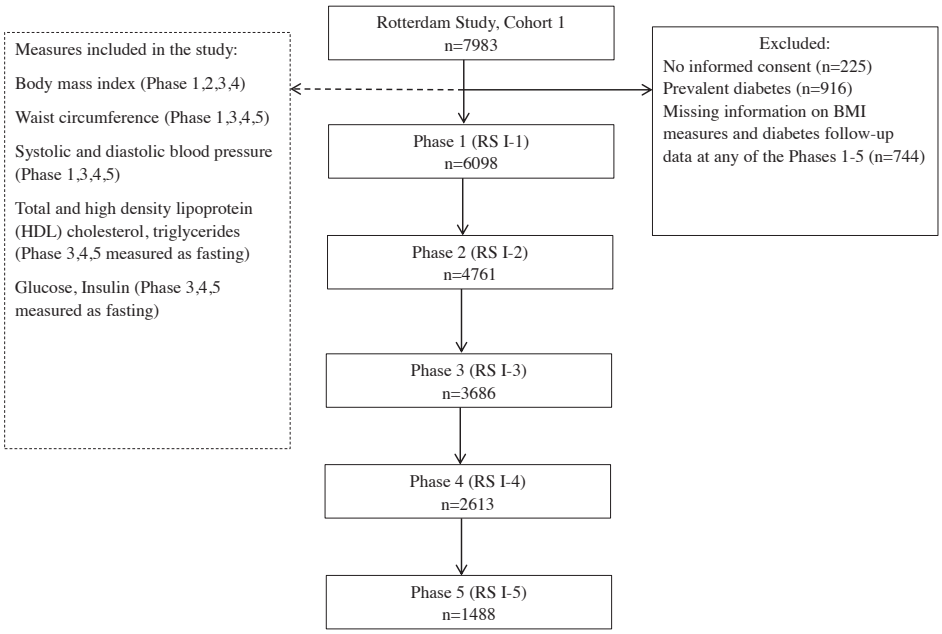

Figure 1. Flow diagram of the study participants for each phase.

Assessment of cardio-metabolic risk factors

Information on cardio-metabolic risk factors were obtained through home interviews or measured at the study center, as described previously (10, 11). Height and weight were measured in all five phases, whereas systolic and diastolic blood pressure and waist circumference were measured in phases 1, 3, 4 and 5, and fasting total cholesterol, high-density lipoprotein (HDL) cholesterol and fasting plasma glucose were measured in phases 3, 4 and 5 (Figure 1). Height and weight were measured with the participants standing without shoes and heavy outer garments. BMI was calculated as weight divided by height squared (kg/m2). Waist circumference was measured at the level midway between the lower rib margin and the iliac crest with participants in standing position without heavy outer garments and with emptied pockets, breathing out gently.

Serum total cholesterol, HDL cholesterol, and glucose were measured using standard laboratory techniques. Blood pressure was measured at the right brachial artery with a random-zero sphygmomanometer with the participant in sitting position, and the mean of two consecutive measurements was used. Smoking status was classified as current smoking or others (former and never) in all phases. We assessed medication use for hypertension, hyperlipidemia and diabetes through interview data.

Clinical outcome

The participants were followed from the date of baseline center visit onwards. At baseline and during follow-up, cases of T2D were ascertained by use of general practitioners' records (including laboratory glucose measurements), hospital discharge letters, and serum glucose measurements from Rotterdam Study visits, which take place roughly every

4 years. According to the WHO guidelines, type 2 diabetes was defined as a fasting blood glucose > 7.0 mmol/L, or the use of blood glucose lowering medication (12). Information regarding the use of blood glucose lowering medication was derived from both structured home interviews and linkage to pharmacy records. At baseline, more than 95% of the Rotterdam Study population was covered by the pharmacies in the study area. All potential events of prediabetes and type 2 diabetes were independently adjudicated by two study physicians. In case of disagreement, consensus was sought with a specialist. Follow-up data was complete until January 1st 2012 (13).

Statistical analysis

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

The analysis is conducted by taking in account information from the population retrospectively from the date of diagnosis with diabetes. The model used for latent class trajectories are linear mixed-effects model with BMI as the dependent variable and time before diagnosis (time 0), age, sex, and phase of study as independent variables. The variable "time before diabetes diagnosis" describes the shape of the trajectories of BMI and was entered in the model as a covariate in a cubic specification. To assign the number of classes in the analysis, the Bayesian information criterion (BIC) was used. The latent class trajectory model calculates a posterior probability of membership in each latent class for each participant, who is latter assigned to the class for which his/her posterior probability is the highest. To ensure that all obtained classes were of clinically

meaningful size, we imposed the condition that each class should include at least 5 % of participants and the mean posterior probability of each class should be higher than 75 %.

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

Because the aggregated effect of combined risk factors on diabetes might differ from each risk factor alone, we examined the trajectories of 8-years diabetes risk and 10-year diabetes risk in each group of BMI. The predicted 10-year CVD risk was calculated using the American College of Cardiology/American Heart Association (ACC/ AHA) Pooled Cohort Equation coefficients, which includes age, sex, total cholesterol, HDL cholesterol, systolic blood pressure, blood pressure lowering medication use, diabetes status, and smoking status in the prediction model [15]. These trajectories of cardio-metabolic risk factors were estimated using linear mixed-effects models controlling for follow-up time, age, sex, and study phase. Analyses of lipids were further adjusted for lipid-lowering treatment, analyses of blood pressure were further adjusted for antihypertensive treatment, and analyses of glucose were additionally adjusted for diabetes treatment. Quadratic and cubic terms for follow-up time were included in the BMI groups (latent classes) when significant ($p< 0.05$). For individuals not developing diabetes during follow-up (diabetes-free and non-diabetes mortality groups), year 0 is merely a time point in a normal life course, and we therefore fitted the trajectories by using linear models. Pair-wise differences in growth curves between BMI groups were tested using F-tests for each cardio-metabolic risk factor. Paired Chi square test (for categorical variables) was used to compare participant characteristics between the groups. To account for multiple testing due to comparing three pairs of BMI groups, we used a Bonferroni-adjusted significance level of $0.05/3 = 0.0167$ for the F-tests for each cardiometabolic risk factor. All other

statistical tests used a significance level of 0.05, and all statistical tests were two sided. Analyses were conducted using R statistical software, version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria), with the package "lcmm" [10].

RESULTS

Baseline characteristics of the study population are presented in Table 1. Overall, 6223 participants with a mean age of 68.8 years, mostly women (n=3681, 59.2%), and overweight (mean BMI= 26.24) were included in the study. The mean (SD) follow-up time was 13.7 (6.5) years during which 565 participants developed diabetes. Among individuals who did not develop diabetes, 1891 (30 %) remained alive until the end of follow-up and 3767 (60.5 %) died from non-diabetes causes. The baseline characteristics of these subgroups are presented in Table S1 in the Supplementary Material.

Table 1. Characteristics of study participants in the first clinical visit

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

Data are n (%), mean(SD)

Abbreviations

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

Patterns of BMI change over time

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

Among 1891 subjects who did not develop diabetes event and were alive until the end of follow-up, the "diabetes-free" group, the average BMI remained relatively stable (ranging from 25.9 to 27.3 during the follow-up). Among 3767 who died of other causes during the follow up, the "non-diabetes mortality" group, the average BMI at the start of the follow-up was in the overweight range (average BMI= 26.4) and reached the normal range just before. The analysis was performed in the total population but in order to plot the trajectories of change in BMI and other cardiometabolic risk factors, we assumed a hypothetical individual to be male with 65 years of age. Similar trajectories for a hypothetical woman of 65 years of age are shown in Figures S1-S4B in the Supplementary Material.

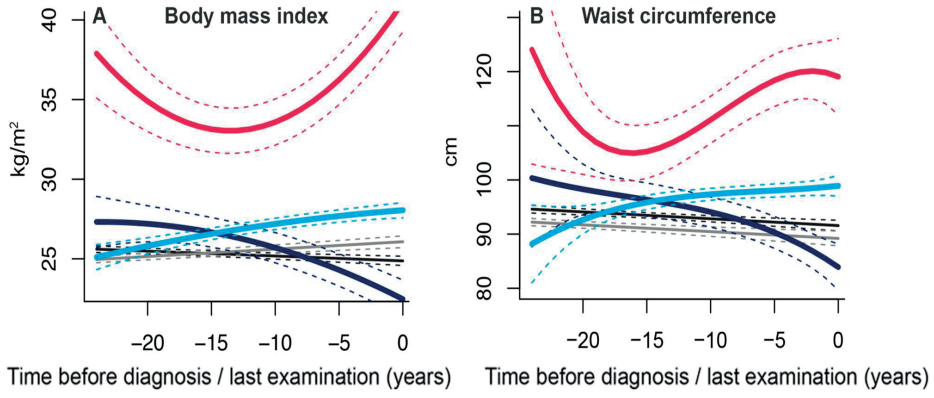

Figure 2. Trajectories of body mass index and waist circumference during 22 years of follow-up until diagnosis of type 2 diabetes, death or censoring from the study. The figures represent a hypothetical man of 65 years old. Light blue "progressive overweight" (including 85.1% of diabetes patients); red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

Table 2. Characteristics of study participants at the time of the diagnosis for the three groups with diabetes or at last visit for the groups without diabetes

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

Trajectories of waist circumference

Trajectories of waist circumferences differed significantly between the three groups ($p < 0.001$ for all pairwise comparisons) (Figure 2). The trajectories for the "progressive overweight", "persistently obese" and "progressive weight loss" groups broadly resembled the trajectories of BMI in these groups. The mean waist circumference in the "diabetes-free" and "non-diabetes mortality" groups decreased slightly during follow-up.

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

Trajectories between fating glucose levels differed between "progressive overweight" and "persistent obese" when compared to "progressive weight loss" group (Figure 3). The mean glucose levels of the latter were fluctuating for the whole follow-up time. For the "progressive overweight" and "persistent obese" groups, we observed an increase in mean levels of fasting glucose from 4.9 mmol/L to 9.4 mmol/L during follow up.

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

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

during follow up. The "progressive overweight" group showed a stable trend with an average of 1500 mmol/L, whereas the "progressive weight loss" group experienced lowered HOMA-%B levels from 1200 mmol/L to 700 mmol/L (Figure 3).

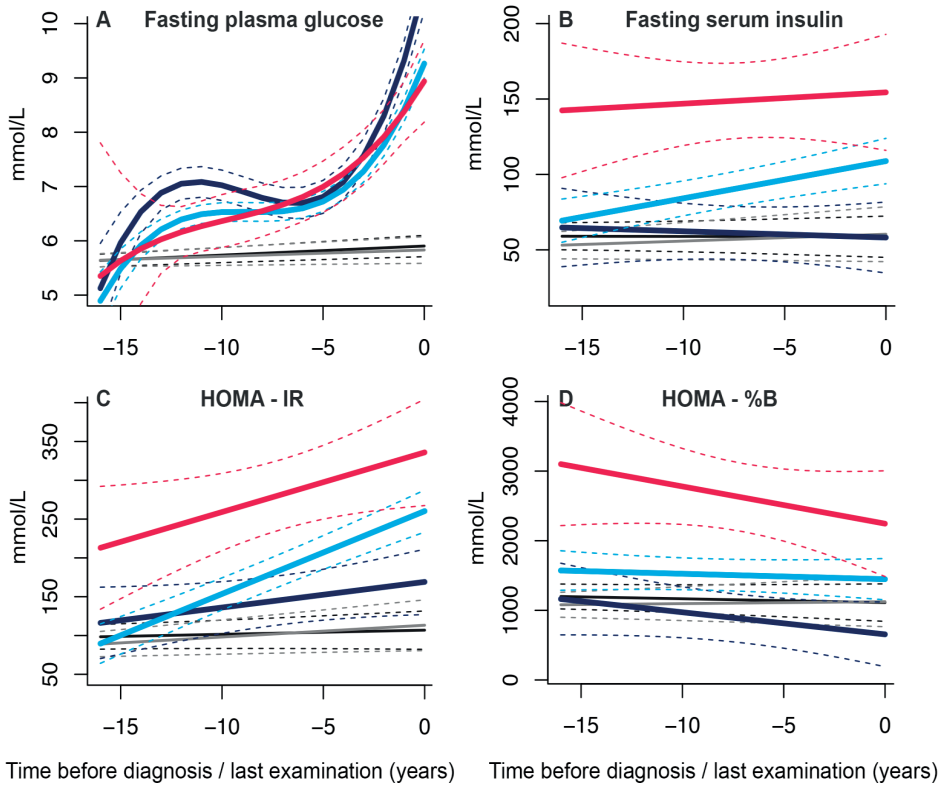

Figure 3. Trajectories of fasting plasma glucose, insulin, homeostatic model assessment –insulin resistance (HOMA-IR), homeostatic model assessment –beta cell function (HOMA-%B) during 14 years of follow-up until diagnosis of type 2 diabetes, death or censoring from the study. The figures represent a hypothetical man of 65 years on glucose-lowering treatment. Light blue "progressive overweight" (including 85.1% of diabetes patients); red "persistently obese" (n= 6.4%); dark blue "progressive weight loss" (n= 25, 5.8%); grey "diabetes-free"; black "non-diabetes mortality".

Trajectories of lipid profile and blood pressure

We found no differences in fasting total cholesterol levels HDL, LDL and triglycerides between the three groups of individuals who developed diabetes during follow-up (Figure 4). For total cholesterol, we evidenced a mark increase in the "persistent obese" group starting from 4.5 mmol/L. The other two groups kept a lowering trend throughout the study, with all groups having an average total cholesterol level within the reference range (< 5.5 mmol/L). On the other hand, decreasing levels of HDL were observed for both "progressive overweight" and "persistent obese" groups while the average levels

of "progressive weight loss" group remained stable throughout the follow-up. For LDL and triglycerides levels, "persistent obese" group exhibited an increasing trend before diabetes event. The average levels of LDL cholesterol demonstrated a modest decrease in the "progressive overweight" and "progressive weight loss" groups meanwhile, the trend was reversed in both groups for triglycerides levels during the follow-up.

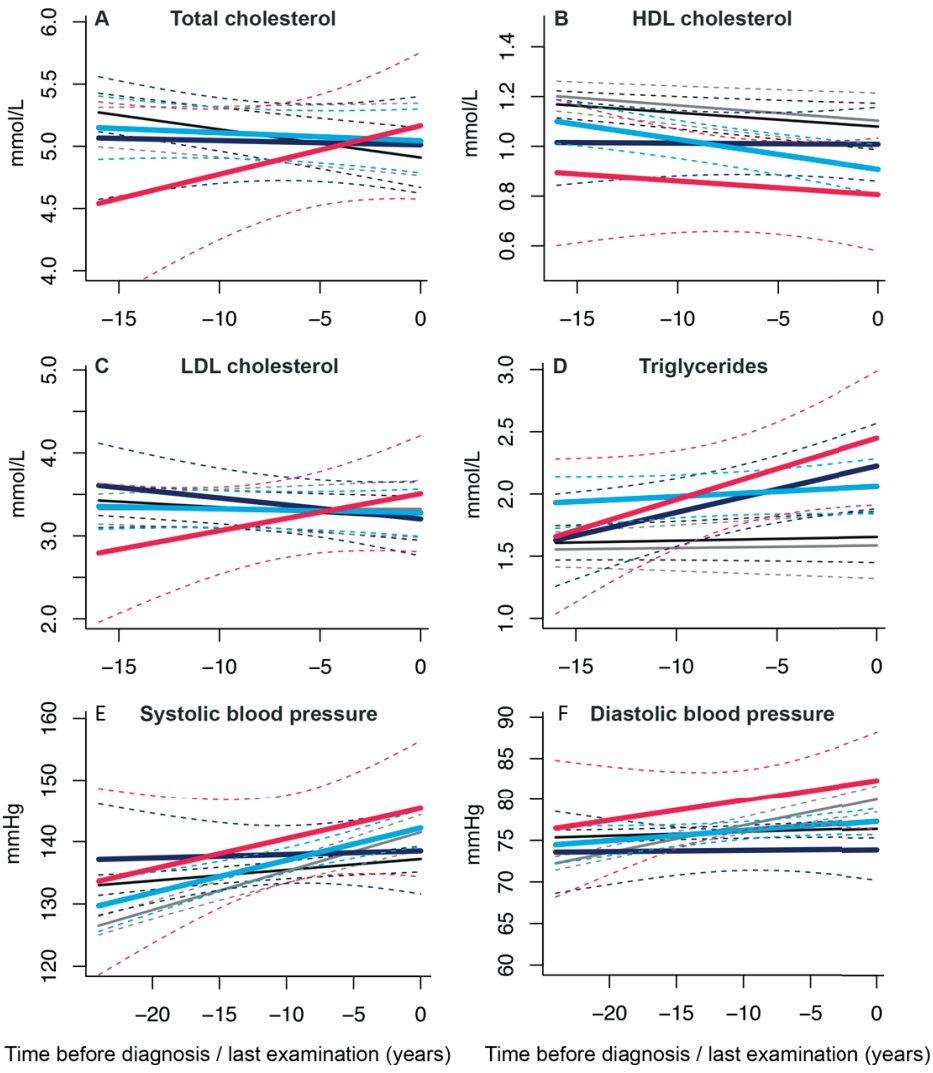

Figure 4. Trajectories of total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, triglycerides, systolic blood pressure and diastolic blood pressure. The figures represent a hypothetical man of 65 years on glucose-lowering treatment. Light blue "progressive overweight" (including 85.1% of diabetes patients); red "persistently obese" (n= 6.4%); dark blue "progressive weight loss" (n= 25, 5.8%); grey "diabetes-free"; black "non-diabetes mortality".

Trajectories of systolic and diastolic blood pressure differed significantly between all BMI groups. Both "progressive overweight" and "persistent obese" groups showed increasing trend before diagnosis of diabetes in both systolic and diastolic blood pressure levels whereas "progressive weight loss" group was relatively stable during the follow-up (Figure 4).

Trajectories of estimated 8-year Type 2 Diabetes risk

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

DISCUSSION

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

The majority of individuals in our study diagnosed with diabetes were progressively gaining weight within the overweight range. Development of diabetes was not preceded by a recent weight gain, as commonly believed, but rather by a continuous, weight gain over the years. While there were relatively stable HOMA beta cell function, they exhibit progressively increasing trends of insulin levels and HOMA-insulin resistance starting from the beginning of the follow-up, whereas glucose levels worsened approximately 5 years before the diagnosis. In the same line, the "persistent obese group" showed accentuated parameters patterns of glucose metabolism as compared with "progressive overweight" group. When we measured the Framingham 8-year diabetes risk, we observed a decreasing trend throughout the period of follow-up in the "progressive overweight" group, but the model was predicting well for the "persistently obese". This might indicate that prediction models do not perform well in the former group of individuals. The diagnosis of diabetes in the Rotterdam Study is done by active collection of information from general practitioners and screening at the research center based on clinical values

of glucose. Another interpretation of the result suggests that the diagnosis of diabetes might be bias towards enhanced screening efforts reserved to obese individuals rather than overweight. Similar findings are reported in an investigation of obesity trajectories prior to diabetes development in a UK cohort (8). The "stable overweight" group was less often diagnosed with diabetes from the general practitioners than the "persistently obese" group. This indicate an inclination of physicians to more effectively screen obese individuals in comparison to overweight individuals.

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

Despite the differences in BMI trajectories, most of the other cardiometabolic risk factors including blood pressure and lipid profile developed without substantial changes in the three groups. Moreover, we were able to assess medication data for all BMI subgroups and we found that antihypertensive medication and lipid lowering drugs were bearing the highest proportionality of use among the persistently obese individuals followed by progressive overweight and progressive weight lost group. This data showed that most probably, overweight individuals and those losing weight over time are less likely to receive medication. Notably, the progressive overweight group and progressive weight loss group constitute more than 95% of the middle aged and elderly population developing diabetes events. Therefore, treating in rightly manner these category of patients could have a big impact on decreasing the overall burden of diabetes and associated comorbidities in the total population.

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

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

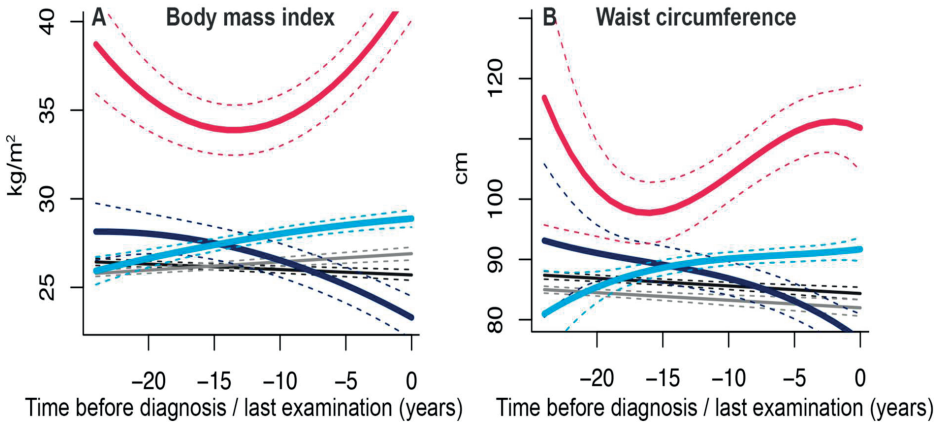

Figure S1. Trajectories of body mass index and waist circumference during 22 years of follow-up until diagnosis of type 2 diabetes, death or censoring from the study. The figures represent a hypothetical woman of 65 years on glucose-lowering treatment. Light blue "progressive overweight" (including 85.1% of diabetes patients); red "persistently obese" (n= 6.4%); dark blue "progressive weight loss" (n= 25, 5.8%); grey "diabetes-free"; black "non-diabetes mortality".

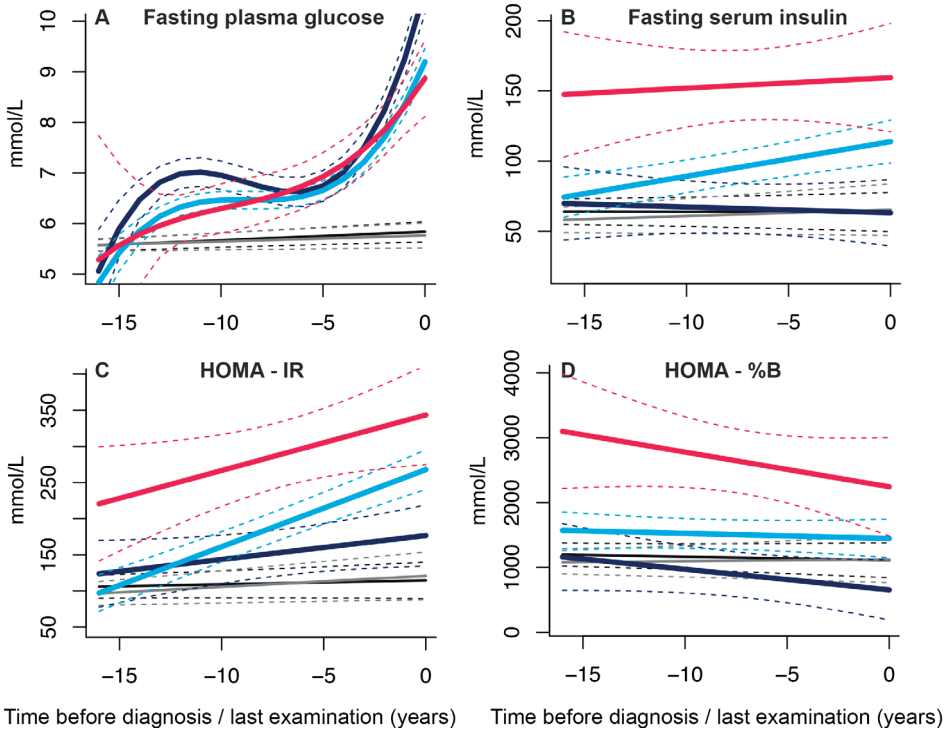

Figure S2. Trajectories of fasting plasma glucose, insulin, homeostatic model assessment –insulin resistance (HOMA-IR), homeostatic model assessment –beta cell function (HOMA-%B) during 14 years of follow-up until diagnosis of type 2 diabetes, death or censoring from the study. The figures represent a hypothetical woman of 65 years on glucose-lowering treatment. *Light blue* "progressive overweight" (including 85.1% of diabetes patients); *red* "persistently obese" (n= 6.4%); *dark blue* "progressive weight loss" (n= 25, 5.8%); *grey* "diabetes-free"; *black* "non-diabetes mortality".

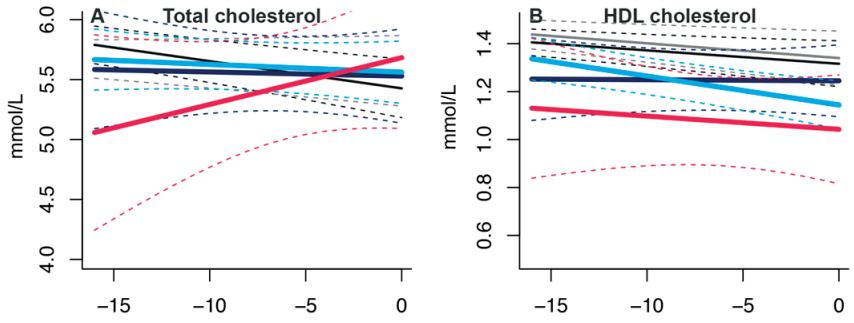

Figure S3. (continued on next page)

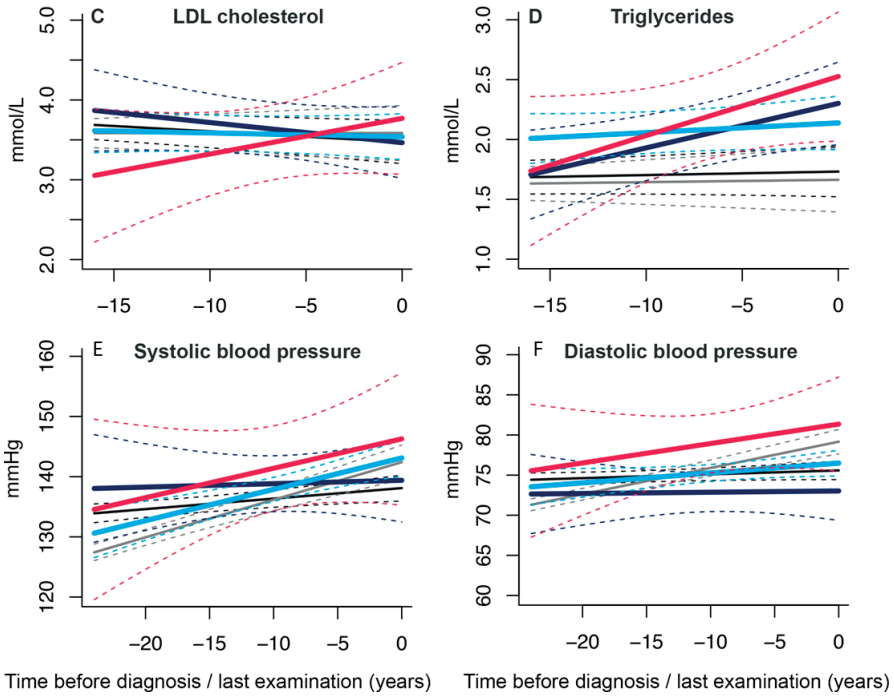

Figure S3. Trajectories of total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, triglycerides, systolic blood pressure and diastolic blood pressure. The figures represent a hypothetical woman of 65 years on glucose-lowering treatment. *Light blue* "progressive overweight" (including 85.1% of diabetes patients); *red* "persistently obese" (n= 6.4%); *dark blue* "progressive weight loss" (n= 25, 5.8%); *grey* "diabetes-free"; *black* "non-diabetes mortality".

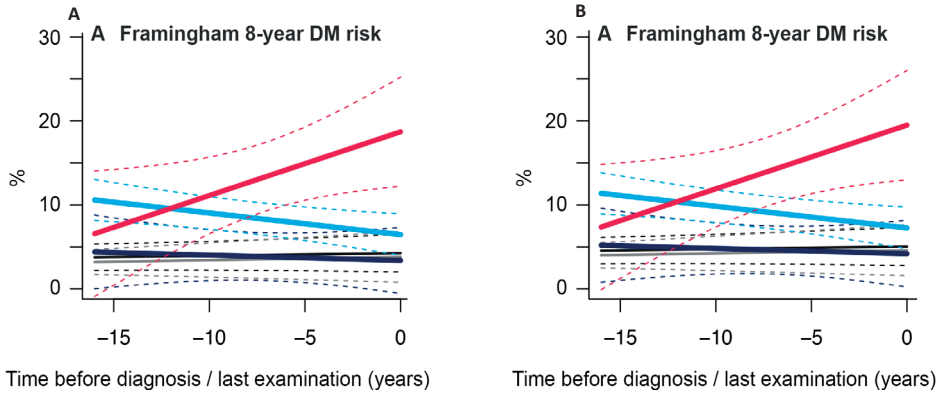

Figure S4. Trajectories of 8-year diabetes risk (ACC/AHA, American College of Cardiology/American Heart Association) during 22 years of follow-up until diagnosis of type 2 diabetes, death or censoring from the study. The figures represent a hypothetical man (Figure S4A)/woman (Figure SB) of 65 years on glucose-lowering treatment. *Light blue* "progressive overweight" (including 85.1% of diabetes patients); *red* "persistently obese" (n= 6.4%); *dark blue* "progressive weight loss" (n= 25, 5.8%); *grey* "diabetes-free"; *black* "non-diabetes mortality".

Table S1. Characteristics of study participants at their first clinical examination

	Individuals developing diabetes during follow-up (n = 565)			Individuals free of diabetes during follow-up (n = 5658)	
	Progressive Weight loss	Progressive weight gainers	Persistently obese	Diabetes-free	Non-diabetes mortality
	n = 59	n = 481	n = 25	n = 1891	n = 3767
Time before diagnosis/last visit, years	12.3 (5.4)	9.9 (5.1)	11.9 (4.9)	20.0 (1.9)	11.1 (6.0)
Age, years	67.2 (7.2)	66.4 (7.0)	64.5 (5.2)	62.2 (5.0)	72.4 (8.5)
Women (%)	30 (50.8)	282 (58.6)	20 (80.0)	1230 (65.0)	2119 (56.3)
Body mass index, kg/m^2	26.6 (3.1)	28.0 (3.2)	35.4 (5.8)	25.9 (3.3)	26.1 (3.7)
Waist circumference, cm	92.7 (10.0)	94.1 (10.0)	103.61(13.3)	87.3 (10.5)	91.0 (11.1)
Systolic blood pressure, mmHg	142.0 (25.2)	141.3 (20.1)	143.0 (22.3)	131.4 (19.8)	141.7 (22.3)
Diastolic blood pressure, mmHg	72.2 (11.4)	75.3 (11.1)	77.1 (9.9)	73.6 (10.4)	73.7 (11.9)
Antihypertensive treatment† (%)	11 (21.2)	107 (26.6)	7 (38.9)	171 (10.8)	598 (18.8)
Lipid lowering medication (%)	5 (10.6)	55 (21.2)	4 (21.1)	231 (14.6)	179 (11.3)
Current smoker (%)	17 (28.8)	109 (23.2)	4 (16.0)	345 (18.5)	918 (25.4)
Total cholesterol (mmol/L)	5.7 (1.0)	5.7 (0.9)	5.6 (0.8)	5.9 (0.9)	5.7 (1.0)
Glucose† (mmol/L)	7.5 (3.2)	6.7 (1.3)	6.7 (1.4)	5.5 (0.6)	5.6 (0.7)
Insulin† (pmol/L)	87.5 (68.5)	115.5 (117.0)	167.0 (98.6)	73.9 (57.6)	76.5 (47.8)
HDL cholesterol† (mmol/L)	1.2 (0.3)	1.2 (0.3)	1.1 (0.2)	1.4 (0.3)	1.4 (0.4)
LDL cholesterol† (mmol/L)	3.8 (0.9)	3.6 (0.8)	3.5 (0.9)	3.8 (0.8)	3.7 (0.9)
Triglycerides† (mmol/L)	1.7 (1.0)	1.8 (0.9)	2.0 (0.6)	1.4 (0.6)	1.4 (0.6)
HOMA-IR† (units)	183.9 (153.2)	216.1 (255.7)	297.5 (187.4)	112.2 (106.0)	117.0 (83.8)
HOMA-%B† (units)	1453.2 (1129.4)	2033.0 (1784.0)	3096.9 (1989.8)	1584.7 (1068.1)	1629.9 (976.8)

Values are mean ± SD, numbers (percentages)

Abbreviations

HDL, high density lipoprotein; HOMA-IR: homeostatic model assessment –insulin resistance; HOMA-%B: homeostatic model assessment –beta cell function; LDL, low density lipoprotein.

† Fasting measurements of lipids and glucose and treatment were available in the third, fourth and fifth visits of the original Rotterdam Study cohort.

The mean values of the characteristics of study participants in Table S1 are based on single measures at the baseline/first visit in the Rotterdam Study. This could explain the occasional observed differences with the predicted mean values in the figures for the latent class trajectory analyses.

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CHAPTER 3

Novel Biomarkers of Type 2 Diabetes

Chapter 3.1

Associations of steroid sex hormones and sex hormone-binding globulin with the risk of type 2 diabetes in women: a population-based cohort study and meta-analysis.

Taulant Muka[1,2*] , Jana Nano[1*], Loes Jaspers[1], Cindy Meun[3], Wichor M. Bramer[4], Albert Hofman[1,2], Abbas Dehghan[1], Maryam Kavousi[1*], Joop S.E. Laven[2*], Oscar H. Franco[1]

[1] Department of Epidemiology, Erasmus MC, Rotterdam, the Netherlands.
[2] Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, Mass, USA.
[3] Department of Obstetrics and Gynaecology, Erasmus MC, Rotterdam, the Netherlands.
[4] Medical Library, Erasmus MC, Rotterdam, the Netherlands.
* Authors contributed equally

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ABSTRACT

It remains unclear whether endogenous sex hormones (ESH) are associated with risk of type 2 diabetes (T2D) in women. Data of 3117 postmenopausal women participants of the Rotterdam Study (RS) were analysed to examine whether ESH and sex hormone-binding globulin (SHBG) were associated with the risk of incident T2D. Additionally, we performed a systematic review and meta-analysis of studies assessing the prospective association of ESH and SHBG with T2D in women. During a median follow-up of 11.1 years, we identified 384 incident cases of T2D in the RS. No association was observed between total (TT) or bioavailable testosterone (BT) with T2D. SHBG was inversely associated with the risk of T2D whereas total estradiol (TE) was associated with increased risk of T2D. Similarly, in the meta-analysis of 13 population-based prospective studies involving more than 1912 incident T2D cases, low levels of SHBG and high levels of TE were associated with increased risk of T2D, while no associations were found for other hormones. The association of SHBG with T2D did not change by menopause status, while the associations of ESH and T2D were based only in postmenopausal women. SHBG and TE are independent risk factors for the development of T2D in women.

INTRODUCTION

Menopause is an important transition in women's life, not only for marking the end of reproductive life, but also for being accompanied by an increased risk of cardiovascular disease and type 2 diabetes (T2D) (1; 2). Changes in hormonal patterns in menopause, including the decline in endogenous estradiol levels and the relative androgen excess, contribute to an increase in visceral adiposity that is associated with glycemic traits, and therefore may influence the risk of T2D (3; 4). Furthermore, polycystic ovary syndrome, a common disorder among women characterised by hyperandrogenism, has been identified as a significant non-modifiable risk factor associated with T2D (5).

While the relation between sex-hormone binding globulin (SHBG) and T2D has long been recognized (6; 7), literature on the associations of steroid sex hormones such as endogenous estradiol (E) and testosterone (T) with T2D is scarce. SHBG, T and E have been associated with glucose metabolism and development of insulin resistance (6-9). Few epidemiological studies investigating the relation between sex hormones and T2D have yielded conflicting results (10-12). These studies were limited by their cross-sectional design, selected samples, or insufficiently adjustment for diabetes risk factors. To date, no large prospective cohort study has examined the association of T2D with SHBG, T and E in healthy postmenopausal women. Thus, we aimed to investigate the association between SHBG, sex hormones and T2D in postmenopausal women. Furthermore, to clarify the contradictory results, we systematically reviewed and meta-analysed studies evaluating the association between SHBG, sex hormones and T2D in women.

METHODS

The Rotterdam Study

The Rotterdam Study is a prospective cohort study which started since 1990 in the Ommoord district, in the city of Rotterdam, The Netherlands. Details regarding the design, objectives, and methods of the Rotterdam Study have been described in detail elsewhere (13). In brief, in 1990 all inhabitants of a well-defined district of Rotterdam were invited, of whom 7,983 agreed (78.1%). In 2000, an additional 3011 participants were enrolled (RS-II), consisting of all persons living in the study district who had become 55 years of age. Follow up examinations were performed periodically, approximately every 3-5 years (13). There were no eligibility criteria to enter the Rotterdam Study cohorts except the minimum age and residential area based on ZIP codes. The Rotterdam Study has been approved by the medical ethics committee according to the Population Screening Act: Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. All participants in the present analysis provided written informed consent to participate and to obtain information from their treating physicians.

Ascertainment of type 2 diabetes

The participants were followed from the date of baseline center visit onwards. At baseline and during follow-up, cases of T2D were ascertained through active follow-up using general practitioners' records, glucose hospital discharge letters and glucose measurements from Rotterdam Study visits which take place approximately every 4 years (14). T2D was defined according to recent WHO guidelines, as a fasting blood glucose ≥ 7.0 mmol/L, a non-fasting blood glucose ≥ 11.1 mmol/L (when fasting samples were absent), or the use of blood glucose lowering medication (15). Information regarding the use of blood glucose lowering medication was derived from both structured home interviews and linkage to pharmacy records (14). At baseline, more than 95% of the Rotterdam Study population was covered by the pharmacies in the study area. All potential events of T2D were independently adjudicated by two study physicians. In case of disagreement, consensus was sought with an endocrinologist. Follow-up data was complete until January 1st 2012.

Sex steroid measurements

All blood samples were drawn in the morning (≤ 11:00 am) and were fasting.

Total estradiol (TE) levels were measured with a radioimmunoassay and SHBG with the Immulite platform (Diagnostics Products Corporation Breda, the Netherlands). The minimum detection limit for estradiol was 18.35 pmol/liter. Undetectable estradiol was scored as 18.35. Serum levels of total testosterone (TT) were measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS). The corresponding interassay coefficients of variations for TE, SHBG and TT are <7%, <5%, and <5%. Free androgen index (FAI), calculated as (T/SHBG) *100 is used as a surrogate measure of bioavailable testosterone (BT) (16).

Population of analysis

The present study used data from the third visit of the first cohort (RSI-3) and the baseline examinations of the second (RSII-1) cohort. Overall, there were 3,683 postmenopausal women eligible for blood measurements. Among them, 122 women did not come for a blood measurement at the research center and 32 did not have T2D follow-up data and were , excluded from the analysis. Furthermore, 412 women with prevalent T2D were excluded, leaving 3,117 for our final analysis. Potential confounding variables are described in detail in S1 Appendix.

Statistical analysis

Person years of follow-up were calculated from study entrance (March 1997- December 1999 for RSI-3, February 2000-December 2001 for RSII-1) to the date of diagnosis of T2D, death or the censor date (date of last contact of the living), whichever occurred

first. Follow-up was until January 1st 2012. Cox proportional hazard modelling was used to evaluate whether SHBG, TT, TE and BT were associated with T2D. Relative Risks (RR) and 95% confidence intervals (95% CIs) were reported. All sex hormones variables were assessed in separate models, continuously and in tertiles. For estradiol, first tertile included all women with levels of estradiol lower than the detection limit (n=992). To study the relations across increasing tertiles, trend tests were computed by entering the categorical variables as continuous variables in multivariable Cox's proportional hazard models. To achieve approximately normal distribution, skewed variables (SHBG, TT, BT, plasma triglyceride, low density lipoprotein cholesterol (LDL-C), C-reactive protein (CRP), thyroid-stimulating hormone (TSH) and insulin) were natural log transformed. In the base model (Model 1), we adjusted for age, cohort (1 and 2), fasting status (fasting sample vs. non-fasting sample). To examine whether the relations of sex hormones and SHBG with risk of T2D were independently of established risk factors for T2D, model 2 included terms of model 1, body mass index (BMI) (continuous), glucose (continuous) and insulin (continuous). BMI and waist circumference were highly correlated (Pearson's correlation coefficient = 0.81, P<0.001), so only BMI was used as a measure of adiposity, consistent with previous studies (10; 17). Model 3 included all covariates in model 2 and further potential intermediate factors including: metabolic risk factors (total cholesterol, systolic blood pressure (continuous), indication for hypertension (yes vs. no) and use of lipid-lowering medications (yes vs. no)), lifestyle factors (alcohol intake (continuous) and smoking status (current vs. former/never)), prevalent coronary heart disease (yes vs. no), age of menopause, hormone replacement therapy (yes vs. no), CRP (continues) and sex hormones for each other. Effect modifications of sex hormones by BMI and years since menopause were tested in the final multivariable model in addition to performing stratified analysis. We also performed a series of sensitivity analyses. Since waist circumference is a better measure of visceral adiposity, an important risk factor for diabetes and of sex hormone levels after menopause, we performed the analysis substituting it with BMI. To account for the specific effects of lipid particles on diabetes, we substituted total cholesterol with HDL-C, triglycerides and LDL-C. TSH, physical activity, number of pregnancies and type of menopause ((non-natural vs. natural) are associated with sex hormone levels and/or risk of T2D, therefore, the models were further adjusted for these factors. To explore potential reverse causation, we reran the analysis by excluding the first three years of follow up. Multiple imputation procedure was used (N= 5 imputations) to adjust for potential bias associated with missing data. Rubin's method was used for the pooled regression coefficients (β) and 95% Confidence Intervals (18). A p-value of less than 0.05 was considered as statistically significant. All analyses were done using SPSS statistical software (SPSS, version 21.0; SPSS Inc, Chicago, Illinois).

Systematic Review and Meta-Analysis

Data sources and search strategy

The review was conducted using a predefined protocol and in accordance with the PRISMA(19) and MOOSE(20) guidelines (S2 and S3 Appendix). Medline, Embase.com, Web of Science, the Cochrane Llibrary, PubMed and Google Scholar were searched from inception until November 2nd 2015 (date last searched) with assistance of an experience biomedical information specialist. The computer-based searches combined terms related to the exposure (eg, sex hormone binding globulin, testosterone, estradiol) with outcomes (eg, type 2 diabetes), without any language restriction. Details on the search strategy are provided in S4 Appendix.

Study selection and eligibility criteria

Studies were included if they (i) were observational cohort, case-cohort studies, or prospective nested case control studies; (ii) had reported on at least one of the sex hormones as exposures: SHBG, TT, BT, TE and bioavailable estradiol (BE); and (iii) had assessed associations with risk of T2D in women (pre and postmenopausal). Two independent reviewers screened the titles and abstracts of all initially identified studies according to the selection criteria. Full texts were retrieved from studies that satisfied all selection criteria. Data extraction, quality assessment and data synthesis and analysis are described in detail in S5 Appendix

Note: Supplementary Material/Appendeix can be found in the website of the published journal or can be provided on request.

RESULTS

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

Sex-hormones and the risk of developing T2D

In models adjusted for age, cohort effect and fasting status, lower SHBG levels (3rd tertile vs.1st tertile: RR=0.33, 95% CI=0.25 -0.43, p-trend<0.001) and higher levels of BT (3rd tertile vs.1st tertile: RR=2.01, 95% CI=1.55-2.60, p-trend<0.001) and TE (3rd tertile vs.1st tertile: RR=2.02, 95% CI=1.50 -2.70, p-trend<0.001) were associated with an increased risk of T2D (Table 2). Further adjustments for BMI, insulin and glucose attenuated but did not abolish the association between SHBG (3rd tertile vs.1st tertile: RR=0.56, 95% CI=0.41 -0.77, p-trend<0.001) or TE and incident T2D (3rd tertile vs.1st tertile: RR=1.39, 95% CI=1.004 -1.93, p-trend=0.07). On the other hand, adjustment for obesity and glycemic traits weakened the associations of BT with T2D such that they were not longer

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

	Women (N=3117)	% missing values
Age (years)	69.7 ± 8.7	0
Years since menopause (years)	20.9 ± 10.0	4.4
Age of menopause (years)	48.9 ± 5.2	4.4
Number of pregnancies of at least 6 months	2.3 ± 2	12.4
Natural menopause, n (%)	2433 (78.1)	0
Current smokers, n (%)	218 (9.2)	1.8
Alcohol intake g/day	1.3 (10)[a]	26.5
BMI (kg/m^2)	27.0 ± 4.3	2.3
Waist circumference (cm)	89.4 ± 11.6	5.8
Prevalent coronary heart disease, n (%)	86 (2.8)	0.06
Estradiol (pmol/l)	34.2 (41.62)[a]	0
Total testosteron (nmol/l)	0.8 (0.56) [a]	0
Sex-hormon binding globuline (nmol/l)	69.6 ± 33.0	0
Free androgen index	1.3 (1.1) [b]	0
Thyroid-stimulating hormone (mU/l)	1.95 (1.7)[a]	0.03
Hormone replacement therapy, n (%)	159 (5.3)	4.8
Insulin (pmol/l)	67 (47)[a]	0.26
Glucose (mmol/l)	5.5 ± 0.6	1.3
C-reactive protein (mg/ml)	1.7 (2.93)[a]	3.7
Total cholesterol (mmol/l)	6.0 ± 1.0	1.3
Low density lipoprotein cholesterol (mmol/l)	4.2 (1.22) [a]	2.5
High density lipoprotein cholesterol (mmol/l)	1.5 ± 0.4	2.3
Statin use, n (%)	681 (14)	4.8
Triglycerides (moml/l)	1.27 (0.74) [a]	0.26
Systolic Blood pressure (mm/Hg)	142.0 ± 21.1	1.03
Indication for hypertension, n (%)	794 (25.5)	1.03
Incident type 2 diabetes, n (%)	384 (12.3)	0

BMI, body mass index; HRT, hormone replacement therapy; NA, non-applicable. [a] Median (interquartile range)

statistically significant (Table 2). Controlling for metabolic risk factors, lifestyle factors, inflammatory markers and prevalent coronary heart disease did not materially affect these associations (Table 2). No association was found between TT and incident T2D in any of the models (Table 2).

Because associations of continues hormone variables with T2D in the Model 1 appeared linear, RRs stratified and sensitivity analyses were expressed per unit log or unit increase in hormone biomarkers. In the sensitivity analyses, substituting BMI with waist circumference as a measure of adiposity, substituting total cholesterol for other blood

Table 2. Associations of sex hormone-binding globulin, testosterone and estradiol with the risk of type 2 diabetes in postmenopausal women, the Rotterdam Study (N=3117)

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

Model 1: Adjusted for age, cohort, fasting status

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

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

lipids, adjusting further for serum TSH, physical activity, number of pregnancies of at least 6 months or menopause type, and excluding the first three years of follow up did not affect any of the associations (S1 Table). Also, in the stratification analysis, no significant interactions were found for SHBG and TE with BMI or years since menopause (S1 Table). Significant interaction terms were found for TT (p-interaction = 0.019) and FAI (p-interaction = 0.03) with years since menopause. However, no association was found between these hormones and T2D after stratification for time since menopause (S1 Table). Also, no effect modification by BMI was found for TT and BT (S1 Table).

Systematic Review and Meta-Analysis

Literature Search, Characteristics and Quality of Eligible Studies

Initial search identified 3209 potentially relevant citations. After screening and detailed assessment, 15 articles based on 12 unique studies were included (S1 Figure and S5 Appendix). Therefore, we meta-analysed estimates from 13 studies (including the current study) involving a total of 14,902 pre- and postmenopausal women with 1912 incident T2D cases reporting on the association between sex hormones and T2D risk. Detailed characteristics of these studies and quality assessment have been summarized in S2 Table. All studies were medium to high quality except one.

Sex hormones and T2D in Pooled Analysis

The meta-analyses for BT, TE and BE are based only on studies examining postmenopausal women; the meta-analysis for TT is based on 4 studies including postmenopausal women and 1 study including pre and postmenopausal women, whereas the findings for SHBG derive from studies including premenopausal women (n=2), postmenopausal women (n=4) and combined (n=3). The pooled RR for T2D adjusted for several metabolic risk factors comparing 3rd tertile vs. 1st tertile of SHBG, TT, BT, TE and BE were 0.44 (95%CI: 0.30-0.66, I^2=77,9%, p<0.001), 1.32 (95%CI: 0.79-2.21, I^2=53.8%, p=0.07), 1.75 (95%CI: 0.92-3.33, I^2=80.7%, p=0.001), 1.99 (95%CI: 1.21-3.27, I^2=55.1%, p=0.06) and 3.58

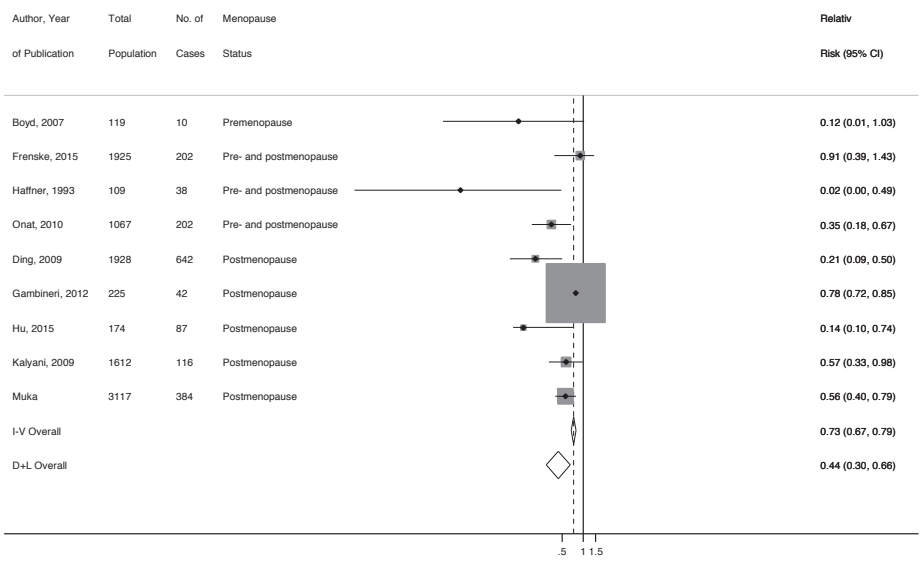
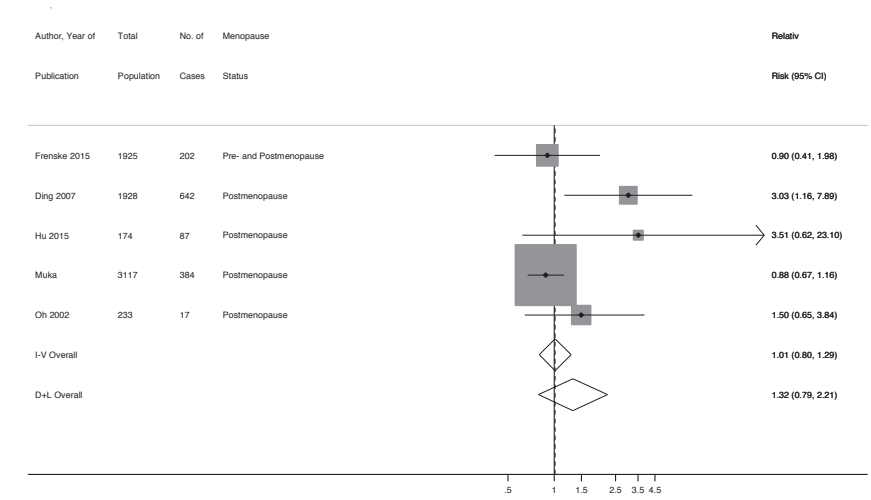


Figure 1. Relative risks of type 2 diabetes comparing top vs. bottom thirds of baseline plasma sex hormone-binding globulin. The summary estimates presented were calculated using random effects models (D+L) and fixed effects (I-V); Size of data markers are proportional to the inverse of the variance of the odds ratio; CI confidence interval (bars). X^2= 36.2, I^2=77.9%; P < 0.001

A) Top vs. bottom thirds of baseline plasma total testosterone levels



B) Top vs. bottom thirds of baseline plasma free testosterone levels

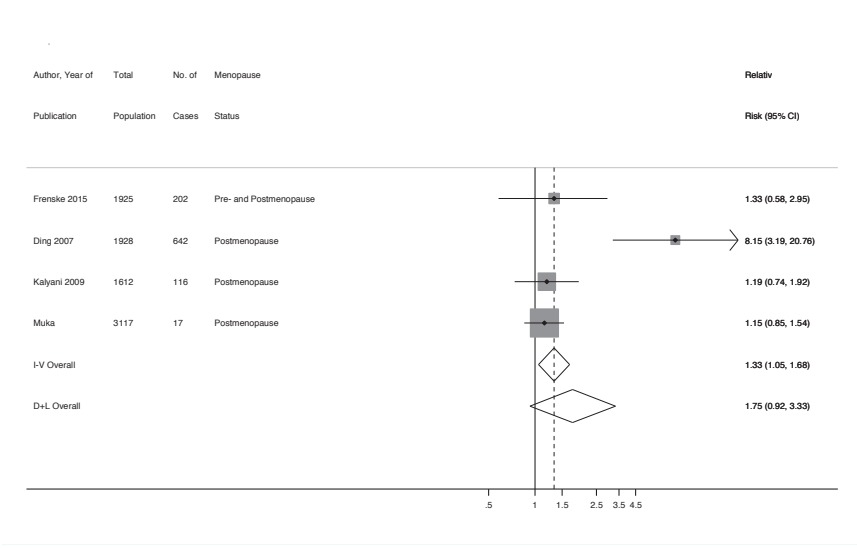
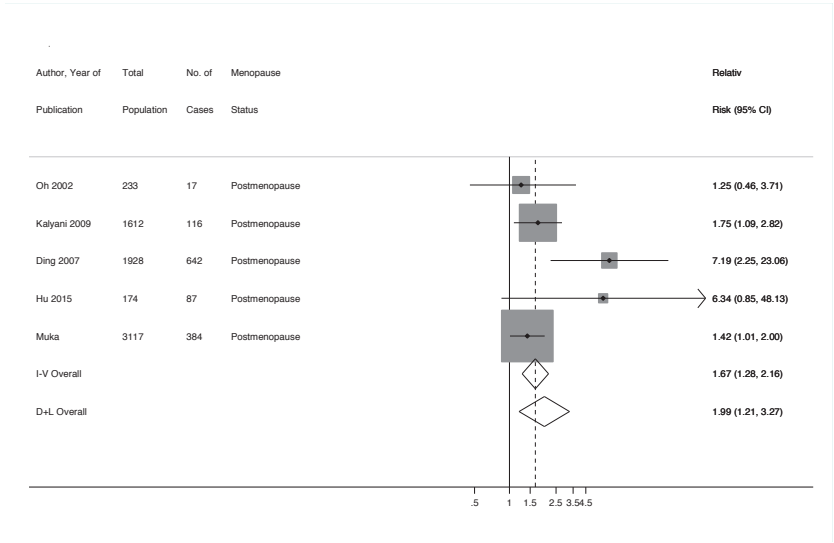


Figure 2. Relative risks of type 2 diabetes comparing top vs. bottom thirds of baseline plasma total and free testosterone levels. The summary estimates presented were calculated using random effects models (D+L) and fixed effects (I-V); Size of data markers are proportional to the inverse of the variance of the odds ratio; CI confidence interval (bars). A) X^2= 8.6, I^2=53.8%; *P* =0.07; B) X^2= 15.5, I^2=80.7%; *P* =0.001

A) Top vs. bottom thirds of baseline plasma total estradiol levels



B) Top vs. bottom thirds of baseline plasma free estradiol levels

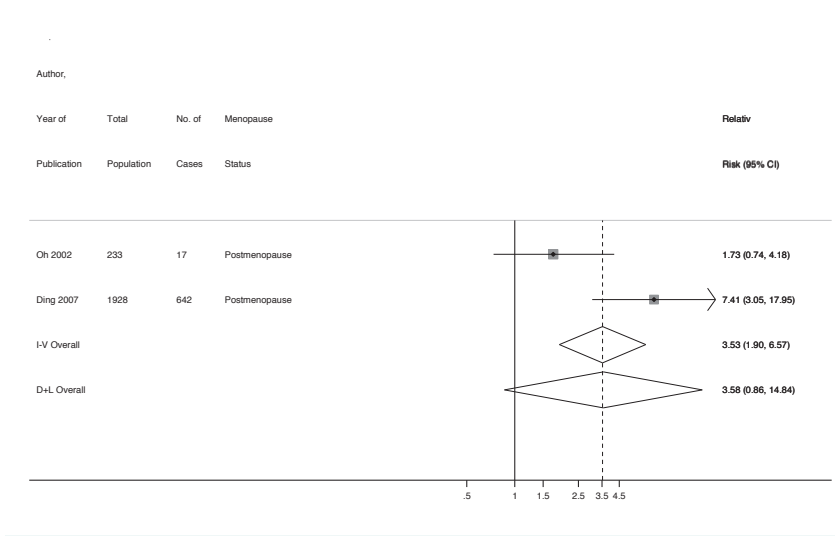


Figure 3. Relative risks of type 2 diabetes comparing top vs. bottom thirds of baseline plasma total and free estradiol levels. The summary estimates presented were calculated using random effects models (D+L) and fixed effects (I-V); Size of data markers are proportional to the inverse of the variance of the odds ratio; CI confidence interval (bars). A) X^2= 8.91, I^2=55.1%; *P* =0.06; B) X^2= 5.26, I^2=81.0%; *P* =0.02

(95%CI: 0.86-14.84, I^2=81.0%, p=0.02) (Figure 1-3). There was evidence of between-study heterogeneity for all these analyses with possible exception of the meta-analysis on the association between TE and the risk of T2D (Figure 1-3). For SHBG, heterogeneity was not explained by any of the study-level characteristics assessed such as menopause status, location and number of participants (S4 Table). For TT, the level of heterogeneity was largely explained by location (S4 Table). Five studies were not possible to include in the meta-analyses. Soriguer found that, in pre- and postmenopausal women, per one-unit log increase in SHBG, TT and BT, the corresponding RRs were 0.23 (0.1 to 0.53), 1.04 (0.59 to 1.83) and 1.12 (0.59 to 2.13) respectively. Boyd-Waschinko at al. reported a 5-fold increase in T2D incidence in the lowest quintile of SHBG. Similarly, Lindstedt et al. found that among patients in the low SHBG terile, 18% converted to T2D as compared with 5% in mid SHBG tertile and 2.5% in high SHBG tertile. Okubo et al reported lower levels of SHBG in T2D converters (59.7 ± 8.4 nmol/l) than non-converters (69.5 ± 2.5 nmol/l) during 3 years of follow-up but that was not significant different after adjusting for age, body mass index and waist to hip ratio (21). Sex steroids and SHBG were not associated with diabetes outcomes in pre and postmenopausal women in the study of Mather et al (22).

Publication bias

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

DISCUSSION

In this large population based study of postmenopausal women free of T2D at baseline, we showed that that lower levels of SHBG and higher levels of TE were associated with the risk of T2D, independently of established risk factors for T2D, including body mass index, glucose and insulin. In contrast, the association between testosterone and the risk of T2D was explained by body mass index, glucose and insulin. Pooled results from the systematic meta-analysis of 13 studies reinforce the validity and generalizability of our findings, suggesting that SHBG and TE are robust risk markers of T2D in women.

Unlike the previous meta-analysis by Ding EL et al, which was based mainly on studies with cross-sectional design and examined only mean differences between T2D cases and non T2D controls, our current pooled analysis is based on findings from 13 prospective studies (only two studies were included in the previous review were eligible),

including 10873 participants with 1623 T2D cases. Therefore, our meta-analysis provides a more detailed assessment of the nature and magnitude of the association between sex hormones and T2D in women.

SHBG levels have been associated with metabolic syndrome, glucose and insulin levels, established risk factors for T2D (7; 8; 23). Also, women with PCOS, a condition of anovulation and hyperandrogenism, are at increased risk of T2D, and levels of SHBG are decreased in these women (24). The complex biological mechanisms that explain the association between circulating SHBG levels and the risk for T2D are not fully understood. Classically, the primary function of SHBG was thought to be the binding of circulating hormones in order to regulate free sex hormone bioavailability to target tissues. Therefore, it has been hypothesized that the relation between SHBG and T2D may results from indirect influence of alterations in SHBG on sex hormone bioavailability. However, in our study, the association between SHBG and T2D risk remains significant after adjustment for TT, BE and TE, implicating SHBG levels as a risk factor for T2D independent of serum androgen levels. Additional evidence in support of an independent effect of SHBG on T2D comes from recent studies that have found several polymorphisms in the SHBG to associate with insulin resistance and T2D, suggesting that altered SHBG physiology may be a primarily defect in the pathogenesis of disease (25-28). Furthermore, a growing body of evidence show that SHBG may directly mediate cell-surface signalling, cellular delivery and biologic action of sex hormones via activation of a specific plasma receptor (29-31). At the target tissue level, the fraction of SHBG that is not bound to sex steroid has the ability to bind plasma membrane high-affinity receptors (RSHBG)(29). Sex steroids of variable biologic potency can activate the anchored SHBG- RSHBG complex and the activated complex can have either an agonist or antagonist effect. For example, SHBG-RSHBG complex can have direct cellular antagonistic properties against estrogen; SHBG may interact with cellular estrogen receptors which can trigger a biologic antiestrogenic response (29). Specific downstream effects of the SHBG-receptor complex merit further investigation since may help to clarify the underlying mechanisms linking SHBG to T2D.

Our result for a positive relation between estradiol and T2D are in contrast with the results from previous large randomized control trials of oral estrogen therapy, which showed a lower risk of T2D among postmenopausal women assigned to estrogen treatment (32-34). However, due to the observational design, our study does not provide causality. Mendelian Randomization experiments are warranted to investigate the potential causal implications of estradiol on T2D. Exogenous estrogen may have different physiological effects depending on type, route, duration and dose of estrogen therapy (35-38). For example, opposing effects of oral estrogen on fasting glucose vs glucose tolerance have been reported (35; 36). Also, in a randomized trial of postmenopausal women, oral estrogen elevated C-reactive protein levels up to 12 months of treatment but not transdermal estradoil (38). Moreover, a bimodal relationship of estrogen dose

may exist. In a clinical trial of postmenopausal women, lower dose of estrogen therapy increased insulin sensitivity whereas higher dose had the opposite effect (39).

In postmenopausal women, endogenous estradiol may be associated with diabetes risk through its relation to glucose, insulin, obesity and inflammation. Indeed, previous cross-sectional studies have linked both BE and TE with higher glucose and insulin resistance levels in postmenopausal women, independently of obesity (9; 40; 41). Also, while animal studies suggest estradiol regulate body composition, may studies in postmenopausal women have failed to show a consistent beneficial role of estradiol in weight loss and in the distribution of body fat (42). However, in our study, the association between TE and T2D, although attenuated, remained significant after adjustment for plasma levels of glucose and insulin, BMI and CRP, suggesting that estradiol may play a direct role in the pathophysiology of T2D in postmenopausal women. Furthermore, additional adjustment for TT did not affect this association, suggesting that estradiol may be more than just a marker of increased aromatase conversion. Explicit mechanisms of estrogen in relation to T2D require further study.

Our study showed no association between TT and the risk of T2D whereas a suggestive positive association was observed between BT and T2D. The lack of association between FT and the risk of T2D in our study might be due to lack of a direct measure of BT in the blood which could have biased our results toward the null. These findings are in line with previous studies reporting higher levels of insulin resistance with increasing levels of BT in postmenopausal women, while no association has been observed between TT and insulin resistance (14; 41). Similarly, BT has been related to increased odds of having impaired fasting glucose (14).

Strengths of our study include its prospective design; the long follow-up and adequate adjustments for a broad range of possible confounders. We also performed several sensitivity analyses such as excluding the first three years of follow up to avoid potential bias of undiagnosed disease at baseline. Furthermore, our study included in addition to analysis of primary data also a systematic review of all available published prospective cohorts, which is the first-ever quantitative synthesis of these associations thus far in women. Also, most of the studied included in our meta-analysis adjusted for potential confounding. However, there are several limitations that need to be taken into account. First, we did not have measures of bioavailable estradiol in the Rotterdam Study, which could have strengthened our results. Also, TE was measured using an immunoassay with a detection limit of 18.35 pmol/L, which is considered suboptimal particularly in postmenopausal women. However, the observed effect remained the same while analysing TE continuously and categorically. Second, free T levels were not measured directly in the blood and therefore have to be interpreted with caution. Nevertheless, free T levels in this study were derived from the ratio of T to SHBG, which is considered a precise proxy for bioavailable T (43). Third, we observed a moderate to high level of

heterogeneity across the included studies. Different assays (S3 Table) used to assess the levels of sex hormones and SHBG contributed to the observed heterogeneity. However, since the number of available studies included in each meta-analysis was generally small, it precluded our ability to conduct subgroup analyses involving various study-level characteristics (such as age). Fourth, there was evidence of publication bias for the association between SHBG and the risk of T2D, so it is possible that our results constitute an overestimation of the performance of the test. However, when we excluded small studies differences were not statistically significant and therefore, the effect of publication bias may be only minor. Fifth, except for SHBG, the other findings come from studies conducted mainly in postmenopausal women, and thus, these results cannot be extended to pre or perimenopause women. Finally, contrary to the results of random-effect models, the fixed-effect models showed a significant association of both BE and BT with the risk of T2D. The differences in random vs. fixed effects models might be explained by the substantial heterogeneity observed between-studies (for example, in the association of BT), which could be better captured under the random effects model (44). For BE, the small-size of the studies might undermine the precision of the estimate under a fixed effects model. However, in light of these observations, the overall results of this study should be interpreted with caution.

In conclusion, lower levels of SHBG and higher levels of TE are independently associated with risk of T2D risk in postmenopausal women. Further studies are needed to establish hormones thresholds at which diabetes risk is increased, because this may aid in identifying high-risk postmenopausal women in the clinical setting.

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Chapter 3.2

Association of circulating total bilirubin with metabolic syndrome and type 2 diabetes: systematic review and meta-analysis of observational evidence

Jana Nano MD[1], Taulant Muka MD, PhD[1], Magda Cepeda MD[1], Trudy Voortman PhD[1,2], Klodian Dhana MD[1], Adela Brahimaj MD[1], Abbas Dehghan MD, PhD[1], Oscar H. Franco MD, PhD[1]

[1] Department of Epidemiology, Erasmus University Medical Center, Rotterdam, the Netherlands, PO Box 2040, 3000 CA Rotterdam

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ABSTRACT

Objective

Emerging evidence suggests that bilirubin levels might be associated with metabolic syndrome (MetS) and type 2 diabetes (T2D), but the nature of the association remains unclear.

Data Sources

Relevant studies were identified using five databases (Embase.com, Medline (Ovid), Web-of-Science, PubMed, Cochrane Central and Google Scholar) last searched on October 21st 2015. Moreover, references of these studies were checked and authors were contacted to identify additional studies.

Study selection

We included randomized controlled trials, cohorts, case-control and cross-sectional studies in adult humans that examined the association between bilirubin levels in blood with MetS and T2D irrespective of language and date of publication. Abstracts and full text selection was done by two independent reviewers, with a third reviewer available for disagreements.

Results

Of the 2,313 searched references, we included 16 observational (11 cross-sectional, 2 prospective, 1 both cross-sectional and prospective, 2 retrospective and 1 national survey) studies that met our inclusion criteria. Overall, data were available on 175,911 non-overlapping participants, with a total of 7,414 MetS cases and approximately 9,406 T2D cases. In the meta-analysis of 7 cross-sectional studies, pooled odds ratio (95% Confidence Interval (CI)) for MetS in a comparison of extreme thirds of serum bilirubin levels was 0.70 (95% CI 0.62, 0.78), whereas, no significant association was found for pooled relative risk estimate between two prospective studies (0.57, 95% CI 0.11, 2.94). The corresponding estimate was 0.77 (95% CI 0.67, 0.87) for type 2 diabetes from four cross-sectional evidence.

Conclusions

Available evidence, mainly from cross-sectional studies, supports inverse association of bilirubin levels with adverse metabolic outcomes. Large-scale prospective studies are needed to establish whether bilirubin levels may be useful in the prevention of metabolic syndrome or type 2 diabetes.

INTRODUCTION

Metabolic syndrome (MetS) is characterized by a constellation of disorders, including high blood pressure, dyslipidaemia, hyperglycaemia, abdominal obesity, and has been consistently shown to be strongly associated with type 2 diabetes (T2D) [1, 2] and cardiovascular disease [1, 3]. MetS and T2D share the same risk factors including family history of diabetes [4-6]. However, the pathogenesis of T2D is still not fully established and appears to involve multiple factors.

Serum circulating total bilirubin, a breakdown product of normal heme catabolism, is useful for assessing liver function and several epidemiological studies have suggested inverse associations with MetS [7, 8], T2D [9] and its complications [10]. Experimental studies show that bilirubin might antagonize oxidative stress by acting as an antioxidant and cytoprotectant playing a role in scavenging excess reactive oxygen species [11-14]. Nonetheless, uncertainties remain about the magnitude, and consistency of the association. Furthermore, some of these studies were conducted in high vascular risk populations or with coexisting comorbidities such as Gilbert syndrome or chronic kidney disease [15-19]. An earlier review by Vitek in 2012 provided detailed information on the experimental and clinical evidence between the heme catabolic pathways and cardiometabolic outcomes [20]. However, the extent to which plasma bilirubin levels are associated with the risk of MetS and T2D in humans was not quantitatively addressed. With the ongoing debate on the potential value of total bilirubin levels as a biomarker and therapeutic target for both MetS and T2D [21-23], a comprehensive assessment of the association of baseline total bilirubin levels with MetS and T2D risk is needed. Therefore, we conducted a systematic review and meta-analysis to determine the associations of bilirubin levels with the risk of MetS and T2D.

METHODS

Literature Search

This review was conducted using a predefined protocol and reported in accordance with the PRISMA [24] and MOOSE [25] guidelines (Appendix 1 and 2). We used five databases (Embase.com, Medline, Web-of-Science, PubMed, Cochrane Central and Google Scholar search engines) to identify published studies. Last search was conducted on October 21th 2015. We aimed to identify studies that examined the association between circulating bilirubin levels and MetS and T2D in adult humans (≥18 years of age). The terms related to bilirubin (e.g. "hyperbilirubin") were combined with key terms related to these outcomes (such as e.g. "syndrome X" or "diabetes mellitus"). We did not apply any language restriction. The full search strategies of all databases are provided in Appendix 3. Reference lists of selected studies and experts were also used to identify further studies.

Study Selection and Inclusion Criteria

We included randomized controlled trials, cohorts, case-control and cross-sectional studies in humans that examined the association between bilirubin levels in blood (total bilirubin, direct or indirect bilirubin levels) with MetS and T2D. Two independent reviewers screened the retrieved titles and abstracts and selected eligible studies. Discrepancies between the two reviewers were resolved through discussion and consensus with a third independent reviewer. We retrieved full texts for studies that satisfied all selection criteria.

Data Extraction

Data extraction was done by two reviewers independently, using a predesigned form including study design, name of the study, publication date, geographical location, population source, time of the baseline survey, sample population, sample source (plasma/serum), nature of the sample (fresh or frozen and storage temperature), assay type, case definition, sample size, numbers of outcome events, mean age at baseline, sex, summary statistics (using a standardized abstraction form) and degree of adjustment for potential confounders. Adjustments were defined as "+" when risk estimates were adjusted for age and sex; "++" when further adjusted for potential risk factors such as blood pressure, body mass index, family history of diabetes, alcohol consumption; and "+++" when additionally adjusted for inflammatory markers such as C-reactive protein (CRP) or any of the liver enzymes. We extracted estimates reported for the greatest degree of adjustment. If risk estimates were not available in the published reports, we contacted the authors to request further data.

Quality Assessment

Bias within each individual study was evaluated by two independent reviewers using the validated Newcastle-Ottawa Scale (NOS), a semi-quantitative scale designed to evaluate the quality of cohort studies [26]. Study quality was judged on the selection criteria of participants, comparability of cases and controls, exposure and outcome assessment. The NOS assigns a maximum of four points for selection, two points for comparability and three points for outcome. Nine points on the NOS reflects the highest study quality. For cross-sectional studies, quality was evaluated using the NOS modified for cross-sectional studies [27], which was also modified for the purposes of our review question. A maximum of 8 reflected the highest study quality. Overall, a score of ≥5 indicated adequate quality for inclusion in the review.

Outcome Assessment and Statistical Methods

To uniformly evaluate the effects of top versus bottom third of the baseline distribution by bilirubin levels in all studies, we used previously described methods to transform

relative risk (RR) estimates [28], which were often differentially reported (example, per unit change, per one standard deviation change, or comparing quarters or thirds). Briefly, we transformed the log risk ratios by assuming a normal distribution, with the comparison between extreme thirds being equivalent to 2.18 times the log risk ratio for one standard deviation increase in exposure (or equivalently 2.18/2.54 times the log risk ratio for a comparison of extreme quarters of exposure). We calculated standard errors of the log risk ratios by using published confidence intervals and standardised them in the same way. Hazard ratios, relative risks, and odds ratios were assumed to approximate the same measure of relative risk [29].

Analyses were done using random effects models calculated from the logarithm of the RRs and corresponding 95% CI of the individual studies [30]. When risk estimates were reported separately for men and women within study, the overall estimate was pooled using fixed-effects meta-analysis. Summary RRs of studies were pooled using a random-effects model to minimize the effects of heterogeneity [31]. When studies reported risk ratios with varying degrees of adjustment, we used the maximally adjusted estimate. Heterogeneity of study results was evaluated using Cochrane Q test, and by the I^2 statistic [32, 33]; and was distinguished as low ($I^2 \leq 25\%$), moderate ($25\% < I^2 \geq 50\%$) or high ($I^2 \geq 75\%$) [33]. Begg funnel plots and Egger tests were used to assess the possibility of publication bias [34, 35]. All analyses were conducted using Stata, version 13 (StataCorp LP, College Station, Texas).

Note: Supplementary Material/Appendeix can be found in the website of the published journal or can be provided on request.

RESULTS

Our search identified 2,313 potentially relevant citations. After initial screening based on titles and abstracts, 48 articles remained for further evaluation of full text. Of the 48 articles retrieved, 32 were excluded for reasons shown in Fig 1. The remaining 16 unique studies met our inclusion criteria (Appendix Supplement 4 List of references). In all studies included, serum bilirubin levels were determined from fasting samples. Further assay characteristics are shown in Table S1.

Association of bilirubin levels with MetS

The association between circulating bilirubin levels and MetS was investigated in 9 unique studies compromising 7 cross-sectional analysis, 1 both cross-sectional and prospective analyses, 1 prospective analysis [36], involving 44,385 participants and 7,414 cases with MetS. The studies included men and women from Asian populations (Korea, Japan and China), Sweden and Slovenia with a mean age 50.7 years old. The pooled odds ratios for MetS when comparing participants in the top versus bottom thirds of

bilirubin distribution levels for all cross-sectional studies, adjusted for several potential risk factors, was 0.70 (95% CI 0.62, 0.78) (Fig. 2A). The pooled relative risk for two other studies that performed prospective analysis revealed the same direction of effect but not significant (0.57; 95% CI 0.11, 2.94). No evidence of heterogeneity in the cross-sectional analyses was found (I^2= 0%, P- value= 1.0), but substantial between-study heterogeneity was observed in the prospective studies (I^2= 96%, P- value <0.001). In a sex-stratified analyses, the pooled odds ratio for men and women in cross-sectional analysis was 0.76

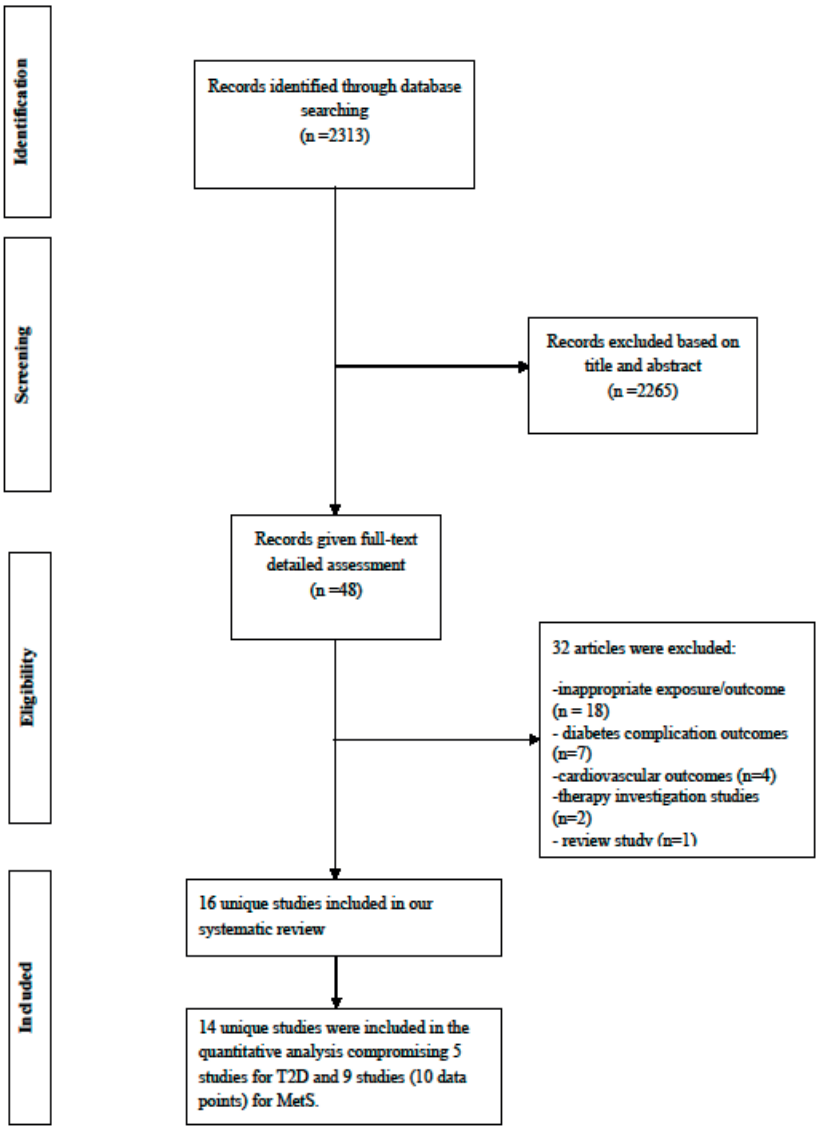

Figure 1. Flowchart of studies selected for the current review and meta-analysis.

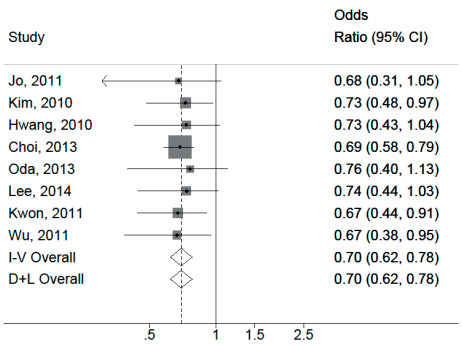

Figure 2A. Association of serum bilirubin levels with metabolic syndrome in cross-sectional studies. Study reference are provided in Appendix 5. The summary estimates presented were calculated using random effects models (D+L) and fixed effects (I-V); Size of data markers are proportional to the inverse of the variance of the odds ratio; CI confidence interval (bars)

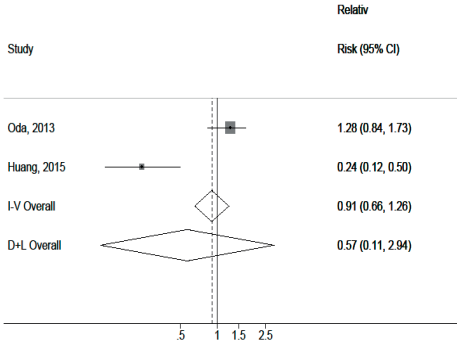

Figure 2B. Association of serum bilirubin levels with metabolic syndrome in prospective studies. Study reference are provided in Appendix 5. The summary estimates presented were calculated using random effects models (D+L) and fixed effects (I-V); Size of data markers are proportional to the inverse of the variance of the odds ratio; *CI* confidence interval (bars)

(95%CI 0.65, 0.89) and 0.67 (95% CI 0.56, 0.8), respectively (Fig.S1.A and Fig.S2). However, we were able to observe a non-significant pooled relative risk (0.75, 95% CI 0.54, 1.02) among men participants in the prospective studies (Fig. S1.B). No prospective study was available for women. Subgroup analyses by categories of degree of adjustment (++ or +++), mean age, diagnostic criteria (NCEP ATPIII or IDF) and sample size (< 2000 or >2000 participants) did not show substantial differences.

Association of bilirubin levels and T2D

Five studies (2 cross-sectional, 1 retrospective, 1 prospective and 1 from national survey) reported effects of circulating bilirubin levels on T2D odd ratio including a total of 131,526 participants and approximately 9,406 cases. All of them were cross-sectional analysis and were judged to be at low risk of bias. The studies included men and women from the Netherlands, USA, Korea and Japan.

The pooled odds ratio for T2D cross-sectional studies was 0.77 (95% CI 0.67, 0.87) comparing top versus bottom thirds of bilirubin levels, when adjusted for potential risk factors (Fig. 3). We found no evidence of heterogeneity in the results across studies (I^2 = 0%, P value 0.9). The study of Abbasi et al, that performed a prospective analysis reported an odds ratio of 0.75 (95% CI 0.62, 0.92) per one standard deviation increase in log-transformed bilirubin levels. Due to lack of gender- specific estimates in the studies included, it was not possible to perform gender-analysis. The overall effect did not vary significantly across age (> 50 or <50 years old), case definition of T2D (self-reported and questionnaire or else) and degree of adjustments.

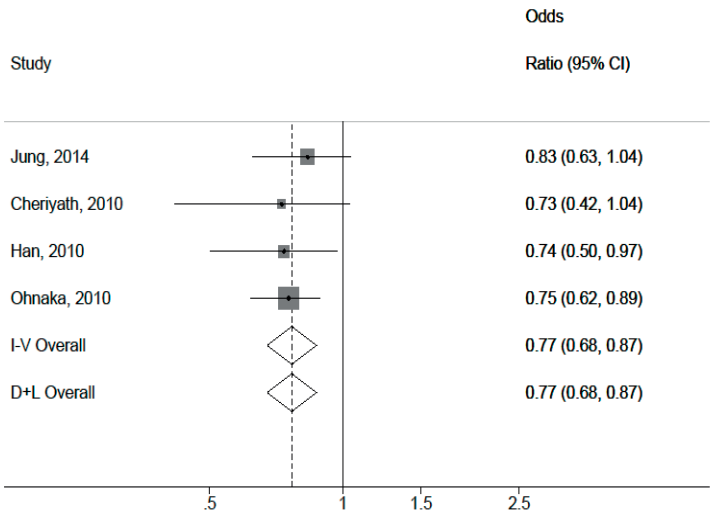

Figure 3. Association of serum bilirubin levels with type 2 diabetes in cross-sectional studies. Study reference are provided in Appendix 5. The summary estimates presented were calculated using random effects models (D+L) and fixed effects (I-V); Size of data markers are proportional to the inverse of the variance of the odds ratio; *CI* confidence interval (bars)

Assessment of publication bias

Results from Egger's test for small-study effects (MetS: P value = 0.12 and T2D: P value = 0.07) suggest that publication bias in unlikely (Fig. S2).

DISCUSSION

We have conducted the first study that systematically reviews and summaries through a meta-analytical approach, available observational studies that have assessed the associations of circulating total bilirubin levels with MetS and T2D. In analyses limited to mostly cross-sectional evidence suggested an inverse association between bilirubin levels and MetS odds ratios in fully adjusted models, but not significant association from prospective evidence (although limited number of studies) was demonstrated. In a comparison of extreme thirds of serum bilirubin for T2D, pooled odds ratio estimates of four studies suggested lower odds ratios by 23%.

Our results of an inverse association between bilirubin levels and MetS and T2D are in line with previous literature confirming a protective role of bilirubin and vascular disease outcomes such as coronary artery disease [37], peripheral artery disease [38], ischemic stroke [38], amputations events in T2D patients [39], diabetic retinopathy [40], diabetic nephropathy [41], and total mortality [42]. It has been shown that diabetic patients with Gilbert syndrome, which is the most common hereditary genetic disorder

Table 1. Characteristics of the studies enrolled in the meta-analysis

Lead Author, publication year	Name of study or source of participants	Location of study	Year (s) of study	Age range or mean (SD), years	Type of study/ Follow-up when prospective	Total no. of participants	% male	No. of cases	Covariates adjusted for	Definition outcome	Study quality
Metabolic Syndrome											
Jo, 2011 [72]	RHE	Korea	2006-2007	44.5 (6.7)	Cross sectional	5231	38	730	Age, ALT, GGT, UA, smoking, alcohol	NCEP/ATP III	7
Kim, 2010 [73]	HPC	Korea	2006-2007	58.6 (6.4)	Cross sectional	1229	0	378	Age, BMI, current smoking, alcohol use, CRP	NCEP/ATP III˜	8
Hwang, 2010 [7]	HPC	Korea	2006-2007	47 (5.1)	Cross sectional	5654	32	950	Age, BMI, smoker, alcohol, exercise, fatty liver	NCEP/ATP III˜	7
Choi, 2013 [8]	HPC	Korea	2009-2010	41.6 (9.4)	Cross sectional	12,342	55.8	989	Age, gender, BMI, smoking, alcohol exercise	NCEP/ATP III˜	7
Oda, 2013 [36]	RHE	Japan	2008-2009	51.75 (10.3)	Cross sectional	3871	62.9	489	Age, gender, smoking, drinking status, physical activity, history of CHD, stroke and use of HTA medication, antihyperlipidemic and antidiabetics medication	IDF	8

Table 1. Characteristics of the studies enrolled in the meta-analysis (continued)

Lead Author, publication year	Name of study or source of participants	Location of study	Year (s) of study	Age range or mean (SD), years	Type of study/ Follow-up when prospective	Total no. of participants	% male	No. of cases	Covariates adjusted for	Definition outcome	Study quality
Oda, 2013* [36]	RHE	Japan	2008-2009	51.75 (10.3)	Prospective/ 4 years	2558	62.7	248	Age, gender, smoking, drinking status, physical activity, history of CHD, stroke and use of HTA medication, antihyperlipidemic and antidiabetics medication	IDF	9
Lee, 2014 [74]	RHE	Korea	2007-2011	49.3 (7.2)	Retrospective longitudinal	6205	100	936	Age, energy intake, BMI, alcohol consumption, smoking status, menopausal status, CRP, and HOMA-IR	NCEP/ATP III˜	8
Kwon, 2011 [75]	KGRC	Korean	2005-2008	55.6 (8.2)	Cross sectional	5266	0	2053	Age, sex, BMI, current smoking, alcohol status, ALT, AST, GGT, albumin, CPR, HOMA-IR	NCEP/ATP III	8
Wu, 2011 [76]	Screening	China	2004-2005	62.3 (9.5)	Cross sectional	1423	34.2	575	Age, ALT, GGT, UA, smoking, alcohol	NCEP/ATP III˜	7

Table 1. Characteristics of the studies enrolled in the meta-analysis (continued)

Lead Author, publication year	Name of study or source of participants	Location of study	Year (s) of study	Age range or mean (SD), years	Type of study/ Follow-up when prospective	Total no. of participants	% male	No. of cases	Covariates adjusted for	Definition outcome	Study quality
Huang, 2015 [57]	RHE	Taiwan	2001-2009	45.5 (6.7)	Prospective	606	100	66	Age, smoking, bilirubin, fasting glucose, triglycerides, HDL, blood pressure, weight gain, waist circumference	IDF	8
Type 2 Diabetes											
Abbasi, 2015 [9]	PREVEND	Netherlands	1997-2007	49.4 (12.4)	Cohort/ 7.7 years	3381	50.6	210	Sex, alcohol use, family history of diabetes, BMI, systolic blood pressure, diastolic blood pressure, triglycerides, fasting insulin, AST, ALT, albumin, CRP	†	9
Ohnaka, 2010 [77]	Fukuoka Cohort Study	Japan	2004-2007	62.7 (6.8)	Cross sectional	12,400	41	907	Age, sex, smoking, alcohol use, BMI, job-related and leisure-time physical activity, ALT, GGT, CRP	¥	7

Table 1. Characteristics of the studies enrolled in the meta-analysis (continued)

Lead Author, publication year	Name of study or source of participants	Location of study	Year (s) of study	Age range or mean (SD), years	Type of study/ Follow-up when prospective	Total no. of participants	% male	No. of cases	Covariates adjusted for	Definition outcome	Study quality
Jung, 2014 [78]	HPC	Korea	2007-2011	49.3 (8.3)	Retrospective longitudinal/ 4 years	5960	100	409	Age, BMI, WC, SBP, DBP, drinking, smoking, exercise, family history of diabetes, FPG, uric acid, AST, ALT, GGT, TG, HDL, LDL, CRP, HOMA-IR	‡	9
Cheriyath, 2010 [79]	NHANES	USA	1999-2006	20 and older	National-wide survey	15,876	NR	1588 ¥	Age, sex, race, married status, education, BMI, smoking	‡	7
Han, 2010 [80]	population-based	Korean	2003-2009	48.7 (12.12)	Cross-sectional	93,909	53	6292	Age, BMI, HTA, cholesterol, triglycerides, HDL, AST, ALT, AP, GGT	¶	8

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; AP, alkaline phosphatase; BMI, body mass index; CRP, C-reactive protein; CHD, coronary heart disease; CVD, cardiovascular disease; FPG, fasting plasma glucose; GGT, γ-glutamyltransferase; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; HTA, hypertensive; KGRC, Korean Genomic Rural Cohort; HPC, health promotion center; NA, not available; NHANES, National Health And Nutrition Examination Survey; NR, not retrievable, PREVEND, The prevention of renal and vascular end-stage disease study; RHE, routine health examination; SBP, systolic blood pressure; T2D, type 2 diabetes; WC, waist circumference;

NCEP ATP III Any three of the five criteria below: waist circumference > 102 cm (M), >88 cm (F), fasting glucose ≥ 100 mg/dl, triglycerides ≥ 150 mg/dl, HDL cholesterol: < 40 mg/dl (M), <50 mg/dl (F), ≥130 mmHg systolic or ≥85 mmHg diastolic

IDF Insulin resistance (identified by 1 of the following: type 2 diabetes, impaired fasting glucose, impaired glucose tolerance, or for those with normal fasting glucose levels (<110 mg/dL), glucose uptake below the lowest quartile for background population under investigation under hyperinsulinemic, euglycemic conditions. Plus any

of 2 of the following (antihypertensive medication and/or high blood pressure (≥ 140 mmHg systolic or ≥ 90 mmHg diastolic, plasma triglycerides ≥ 150 mg/Dl, HDL cholesterol < 35 mg/dL (M) or < 39 mg/dL (F), BMI > 30 kg/m^2 and /or wasit:hip ratio >0.9 in men, > 0.85 in women, urinary albumin excretion rate ≥20 microgram/min or albumin: creatinine ration ≥ 30 mg/g

†Presence of the following fasting glucose ≥126 mg/dl (≥ 7.0 mmol/L), nonfasting glucose > 200 mg/dL and current use of hypoglycemic medication and self-report physician diagnosis

¥self-report physician diagnosis and supplemental questionnaire

‡ fasting glucose and supplemental questionnaire data

¶ fasting glucose and use of hypoglycemic medication

ˇwaist circumference cut off was assigned ≥ 85 cm

ɣ approximation of 10% prevalence of T2D in the U.S population. Exact no of cases could not be retrieved.

causing elevated serum bilirubin levels, have reduced markers of oxidative stress and a decreased risk of nephropathy and cardiovascular disease [43, 44]. Bilirubin has been found to be inversely associated with MetS components such as central obesity, hypertriglyceridemia, fasting insulin, hyperglycemia [45, 46] highlighting the potential role of bilirubin as an early biomarker to identify individuals at increased risk of developing MetS [47]. In our meta-analyses, the protective association between bilirubin and MetS was slightly stronger in men than in women. A study from Endler et al investigating the association of serum bilirubin levels between coronary heart disease (CHD) patients and healthy controls, reported an inverse relationship between serum bilirubin levels and CHD risk in men, but not in women [48]. Generally, mean bilirubin levels are slightly higher in men than those in women [7, 8]. Different effects of bilirubin on adverse metabolic risk according to gender have been attribute to gender-specific biology (e.g effect of estrogen levels, heme-oxygenase enzyme activity, higher stored iron in males compared with females) or lifestyle factors (smoking, alcohol drinking, consumption of antioxidant vitamins)[49, 50]. Further studies on the relationship between bilirubin and sex differences are necessary.

Plausible biological mechanisms by which lower bilirubin levels may contribute to reduced MetS and T2D risk include its antioxidant actions [15, 51] (through inhibition of low-density lipoprotein oxidation [51]), anti-inflammatory effects [52], antiatherogenic properties [53], or more recently reported, pathways associated with vascular structure and reactivity [54]. Serum bilirubin has been demonstrated to be a major contributor to the total antioxidant capacity of diet in blood plasma [55]. Besides the mechanisms already known, it has been reported that bilirubin also affects lipid metabolism by acting as a physiological hypolipidaemic agent [56].

We found and inverse association of bilirubin with MetS odds ratio in the meta-analysis of cross-sectional studies. These evaluations do not inform about the direction of the association: whether low bilirubin levels preceded or are a consequence of MetS. A better design to answer this question would be longitudinal prospective analysis. The two studies from Oda et al and Huang et al, who evaluated the association of bilirubin with incident MetS prospectively, reported different direction of effect estimates, reflecting the non-significance in the combined analysis [36, 57]. In our review, the only prospective study to examine the association between bilirubin and the risk of T2D observed a 25% lower risk [9]. However, a recent study reported an inverse association of total bilirubin levels and prevalent prediabetes in non-smokers, whereas no association was found in the prospective analysis with incident prediabetes cases [58]. The finding of an inverse and independent association between total bilirubin levels and MetS/T2D risk requires further evidence to investigate it in well-characterized prospective studies. Mendelian Randomization experiments are also warranted to investigate potential causal implications of bilirubin on metabolic outcomes. Abbasi et al has suggested a

causal relation between a common genetic variant (rs6742078) within the *UGT1A1* gene, robustly associated with increased circulating levels of total bilirubin, and T2D risk using a Mendelian Randomization approach [9]. Moreover, this variant has been used as an instrument variant for examining causal effect of total bilirubin on outcomes such as cardiovascular disease and gallstone disease as well [59, 60]. However, these results should be interpreted with caution given the large sample size needed for Mendelian Randomization analysis or the plausibility of the instrumental variable assumptions [61, 62].

Randomized trials of interventions that modify bilirubin levels could further confirm the role of bilirubin in T2D and its complications [63]. Novel therapeutic strategies (such as heme oxygenase-1 inducers or inhibitors of *UGT1A1* gene) and drugs that reduce hepatic glucuronidation activity or inhibit hepatocyte uptake that cause mild-to-moderate elevations in circulating levels of bilirubin have been proposed as future tools for the prevention of cardiometabolic-related outcomes [64-66]. Because of the variability in evidence regarding the role of bilirubin, more research is urgently needed in this field. There are numerous relations between bilirubin levels and other predictors of cardio-vascular risk including non-alcoholic fatty liver disease [67], smoking status [39], exercise [68], CRP [69], hemoglobin A1c [69], and albuminuria [18]. Of the five T2D studies and eight MetS studies, only four had comprehensive adjustments for each of the outcomes. Thus, large-scale prospective studies are needed to confirm the current available evidence and additional research is required to address existing gaps. Bilirubin remains a promising though unproven strategy in prevention or treatment of adverse metabolic outcomes such as type 2 diabetes.

The strengths and potential limitations of this analysis deserve to be mentioned. We implemented a comprehensive strategy across multiple databases yielding several published studies on the topic. Our review included studies that reported recruiting participants from approximately general populations and involved approximately 175,911 participants. This meta-analysis was robust as we were able to present standardized estimates to allow consistent comparisons from almost all available contributing studies. Because the present review was based on variably adjusted data reported by eligible studies, there might be a risk of residual confounding as with meta-analyses involving published data. An additional issue is that it was not possible to achieve a comparable outcome definition for both T2D and MetS. In several studies, type 2 diabetes was assessed by self-report; however, it has been shown in validation studies that self-report of type 2 diabetes is accurate according to medical record review. On the other hand, the NCEP-ATPIII and IDF definitions have been found to show good agreement in the diagnosis of MetS [70]. We could not conduct meta-regression or subgroup analyses to estimate the effect of these different covariates on bilirubin-MetS/T2D risk since the number of studies investigating subgroups was rather limited. The lack of fractionation

of total bilirubin into indirect and direct fractions limited the ability to evaluate which bilirubin fraction was associated with metabolic outcomes. However, it is reported a strong correlation between total bilirubin and unconjugated bilirubin, as well as between total bilirubin and conjugated direct bilirubin in healthy subjects [16]. The eligible studies for MetS were solely from Asian populations, which hamper the generalization of our findings.

In conclusion, available data-mainly from cross-sectional studies supports inverse associations with serum bilirubin levels with risk of adverse metabolic outcomes. The biology underlying the association between bilirubin and metabolic-related diseases deserves further investigation through new perspectives (such as for example metabolomics [71]). Therefore, our findings highlight important gaps in the existing literature, with large-scale prospective studies in particular needed to establish whether bilirubin levels may be useful in the prevention of adverse metabolic outcomes such as MetS or T2D.

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Contributors: The contributions of the authors were as follows: JN and OHF conceived and designed the study. TM, AB, MC, TV and JN screened title/abstract, obtained full text, determined eligibility of articles and participated in data extraction. JN and TM assessed the quality of the included studies. JN participated in data synthesis/analysis and interpretation of the data. JN, AB, TV, MC, AD and OHF drafted the final manuscript. All authors contributed to the critical revision of the manuscript and approved the final version.

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Chapter 3.3

Gamma-glutamyltrasnferase levels, prediabetes and type 2 diabetes: a mendelian randomization study

Jana Nano[1], Taulant Muka[1], Symen Ligthart[1], Albert Hofman[1, 2], Sarwa Darwish Murad[3], Harry L.A. Janssen[3], Oscar H. Franco[1], Abbas Dehghan[1]

[1] Department of Epidemiology, Erasmus University Medical Center, Rotterdam, the Netherlands.
[2] Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA.
[3] Department of gastroenterology and hepatology, Erasmus University Medical Center, Rotterdam, the Netherlands.

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ABSTRACT

Background

High levels of serum gamma-glutamyltransferase (GGT) are associated with increased risk of prediabetes and type 2 diabetes in observational studies. It is un-clear whether this relationship is causal, arises from residual confounding or is a consequence of reverse causation.

Methods

We used data from a prospective population-based cohort study, compromising 8611 individuals without diabetes at baseline. Cox proportional hazard models were used to study the association between serum GGT levels and incident prediabetes and diabetes. A Mendelian randomization (MR) study was performed using a genetic risk score consisting of 26 GGT-related variants, based on a genome-wide association study (GWAS) on liver enzymes. Association with diabetes and glycaemic traits were investigated within the Rotterdam Study and large-scale GWAS.

Results

During follow-up, 1125 cases of prediabetes (mean follow-up 5.7 years) and 811 cases of type 2 diabetes (6.9 years) were ascertained. The predicted hazard ratios per standard deviation (SD) change in GGT levels in the multivariable model were 1.10 for prediabetes [95% confidence interval (CI): 1.02–1.19] and 1.19 for type 2 diabetes (95% CI: 1.10–1.30). The genetic risk score associated with increased GGT levels (beta per SD log GGT : 0.41, 95% CI: 0.35–0.47), explaining 3.5% of the observed variation in GGT. MR analysis did not provide evidence for a causal role of GGT, with a causal relative risk for prediabetes and type 2 diabetes per SD of log GGT of 0.97 (95% CI: 0.91–1.04) and 0.96 (95% CI: 0.89–1.04), respectively. Multiple instrumental analysis using genetic associations with type 2 diabetes and glycaemic traits from previous GWA studies detected no causal effect of GGT.

Conclusions

MR analyses did not support a causal role of GGT on the risk of prediabetes or diabetes. The association of GGT with diabetes in observational studies is likely to be driven by reverse causation or confounding bias. As such, therapeutics targeted at lowering GGT levels are unlikely to be effective in preventing diabetes.

INTRODUCTION

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

In recent years, genetic information has been used to infer causality in the pathogenesis of complex diseases. The inference is based on the fact that alleles are allocated randomly during gamete formation; therefore, genetic variants are inherited independently of potential confounding. A previous study from Switzerland (4360 individuals) used a single nucleotide polymorphism (SNP) in the GGT1 gene to study the causal relation between GGT and fasting insulin levels employing a Mendelian randomization (MR) approach.[2] This SNP explains merely 1.2% of the variation in GGT levels in this study. It is known that a weak instrumental variable makes the association susceptible to false positive findings.[3] So far, 26 loci have been identified for serum GGT levels.[4] Thus, a genetic risk score (GRS) combining the effect of all these loci could provide a stronger instrument for the MR analysis to infer causality.[5] Additionally, GGT has not been previously investigated as a causal biomarker for diabetes, as reported from a recent systematic review from Abbasi.[6] In this study, we investigated the association between serum GGT levels and risk of incidence prediabetes and type 2 diabetes in a large prospective population based cohort study of participants aged 45 years. Using a MR approach, we created a GRS incorporating 26 variants that are identified for serum GGT levels in a genome-wide association study (GWAS) and examined its association with prediabetes and type 2 diabetes. Next, we investigated the causal effect of GGT on diabetes and glycaemic traits by using summary-level data from previous GWAS consortia.

METHODS

Study population

The study was performed among participants of the prospective population-based Rotterdam Study. In 1989, all residents aged 55 years or older in Ommoord, a suburb of Rotterdam, The Netherlands, were invited to participate in the study (RS-I). Seventy-eight per cent of the invitees agreed to participate (n = 7983). In 1999, the Rotterdam Study was extended by including 3011 participants from those who either moved to Ommoord or turned 55 (RS-II). The third cohort was formed in 2006 and included 3932 participants aged 45 years and older (RS-III). There were no eligibility criteria to enter the Rotterdam Study cohorts except the minimum age and residential area based on postal

codes. Participants have been reexamined every 3–4 years, and have been followed up for a variety of diseases. A more detailed description of the Rotterdam Study can be found elsewhere.[7] The Rotterdam Study has been approved by the medical ethics committee according to the Population Screening Act: Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. All participants in the present analysis provided written informed consent to participate and to obtain information from their treating physicians. We used the third visit of the first cohort (1997–99) and the first centre visit for both the second cohort (2000–01) and the third cohort (2006–08) as baseline. We excluded 314 participants with no informed consent, 1376 participants with no fasting glucose measurement and 219 other participants with no information on GGT. Next, we excluded individuals with prevalent type 2 diabetes or prediabetes depending on the studied outcome (Figure 1). To perform the genetic investigation, participants were further excluded due to lack of genotyping data, leaving the total number of participants per analysis 6236 for prediabetes and 7383 for diabetes.

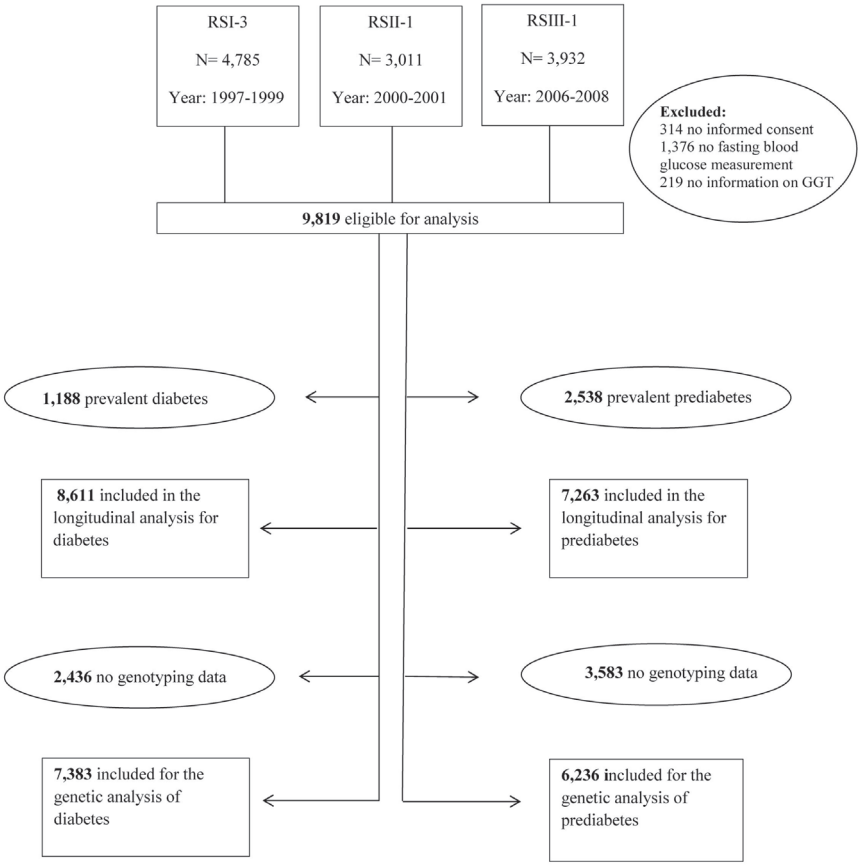

Figure 1. Study population sample from Rotterdam.

Measurement of gamma-glutamyltransferase

Fasting blood samples were collected by venipuncture, and immediately frozen at 20 C. Serum GGT was determined within 2 weeks using a Merck Diagnostica kit on an Elan Autoanalyzer Merck. All liver biochemistry measurements were obtained in the laboratory of the Department of Epidemiology, Erasmus University Medical Center.

Ascertainment of prediabetes and type 2 diabetes

The participants were followed from the date of baseline centre visit onwards. At baseline and during follow-up, cases of prediabetes and type 2 diabetes were ascertained through active follow-up using general practitioners' records, glucose hospital discharge letters and glucose measurements from the Rotterdam Study visits. According to the WHO guidelines, prediabetes was defined as a fasting blood glucose between 6.0 mmol/L and 7.0 mmol/L and type 2 diabetes was defined as a fasting blood glucose >7.0 mmol/L, or the use of blood-glucose-lowering medication.[8] Information regarding the use of blood-glucose-lowering medication was derived from both structured home interviews and linkage to pharmacy records. At baseline, more than 95% of the Rotterdam Study population was covered by the pharmacies in the study area. All potential events of prediabetes and type 2 diabetes were independently adjudicated by two study physicians. In case of disagreement, consensus was sought with a specialist. Follow-up data were complete until 1 January 2012.[9] Information on study model covariates can be found in Supplementary Table 1 (available as Supplementary Data at IJE online).

Genotyping

Genotyping was conducted in self-reported White participants in all three cohorts using the Illumina Infinium HumanHap550K Beadchip in RS-I and RS-II and the Illumina Infinitum HumanHap 610 Quad chip in RS-III at the Genetic Laboratory of the Erasmus MC, Department of Internal Medicine, Rotterdam, The Netherlands. Participants were excluded if they had excess autosomal heterozygosity, mismatch between called and phenotyping sex, or recognized as being outlier with identical-by-state clustering analysis. Before imputation, SNPs with minor allele frequency (MAF) < 0.01, call rate < 95% and departure from Hardy-Weinberg equilibrium cut-off P-value 1 x 10^{-6} were excluded. SNPs were imputed based on the 1000 Genomes cosmopolitan phase 1 version 3 reference.

Construction of the GRS

We searched PubMed using key words 'genome-wide association study', 'GWAS', 'gamma-glutamyltransferase' and 'GGT' and identified one of the largest genome-wide association studies conducted on 61 089 individuals of European descent. In this study, we selected 26 SNPs that passed the genome-wide significance threshold (P-value < 5 x

10^{-8}) for serum GGT levels.[4] Supplementary Table 2 (available as Supplementary Data at IJE online) provides an overview of the SNPs included in the genetic score for GGT and weights assigned to each SNP. The effect allele (coded 0–2) was the GGT raising allele. We then calculated the GRS by multiplying the number of risk alleles at each locus by corresponding reported beta-coefficient and summed the products.

Statistical analysis

For the observational association between GGT and prediabetes and type 2 diabetes, we estimated hazard ratios (HRs) in four adjusted Cox proportional hazard models. Model 1 was adjusted for age, sex and study cohort. Model 2 was further adjusted for body mass index (BMI) and alanine aminotransferase (ALT). In model 3, potential risk factors and confounders of type 2 diabetes were added to model 2 including total and high-density lipoprotein (HDL) cholesterol, triglycerides, CRP (C-reactive protein), waist circumference, current smoking status, systolic blood pressure, antihypertensive medication use, lipid-lowering medication use, prevalent cardiovascular disease (CVD), fasting insulin and fasting glucose levels. CRP, ALT, insulin and HOMA-IR were natural log-transformed. As GGT was not normally distributed, we log-transformed prior to analysis, enabling us to express associations as 'per standard deviation (SD) of log-transformed GGT levels' in both observational and MR analyses.

Associations of individual SNPs and GGT genetic score with GGT levels were assessed with linear regression analysis among participants in the Rotterdam Study. SNPs were modelled per GGT-increasing allele (additive model) and, together with MR results, estimates reflect per-SD change in GGT levels. HRs were computed using Cox proportional hazard models adjusted for study cohort. We further investigated the association of genetic score of GGT with glycaemic traits (fasting glucose, fasting insulin, HOMA-IR). The genetic analysis was repeated in strata of alcohol intake (drinkers vs non-drinkers) and we investigated whether there was a trend between alcohol categories and the GRS. In a sensitivity analysis, participants with prevalent CVD were excluded. To increase the sample size, we examined the association of GRS with a combination of both prevalent and incident type 2 diabetes cases. Taking into consideration any pleiotropic effect of the SNPs included in the genetic score, we reanalysed the genetic estimates excluding SNPs that have previously been reported to be associated with cardiometabolic traits (rs1260326, rs10513686, rs4074793, rs17145750, rs7310409, rs516246).[4]

MR using data from MAGIC and DIAGRAM consortia

To maximize the statistical power, we examined the association of GGT-related genetic variants with diabetes and glycaemic traits using data from the largest GWAS meta-analyses. For diabetes, we used data from the DIAGRAM consortium, which meta-analysed genetic variants in 34 840 case subjects with diabetes and 114 981 control subjects from

37 studies.[10] For fasting glucose (n = 58 074), fasting insulin (n = 133 000) and HOMA-insulin resistance (n = 37 073), we used data from the Meta-Analyses of Glucose and Insulin-related Traits Consortium (MAGIC), which is a collaborative effort that combined data from 55 studies to identify genetic determinants that affect glycaemic traits. Participants were of European ancestry and genotyped with the Metabochip.[11] We selected 26 GGT-related SNPs and extracted effect estimates for diabetes (odds ratios) and for glycaemic traits (beta estimates) together with accompanying standard errors from the published large GWAS data.[3] We applied inverse-variance weighted (IVW) regression and performed MR Egger-regression to calculate causal estimates making use of these summary data.[12] IVW was applied by carrying out a meta-analysis of estimates using both fixed-effects (reported in Supplementary Table 5, available as Supplementary Data at IJE online) and random-effects models (reported in the main text and in Table 1) to obtain pooled estimates of the effect of GGT on diabetes or gly-caemic traits, as previously described.[13] Heterogeneity is quantified in the random-effects model with the parameter I^2, which indicates the percentage of variance in the estimate that is attributable to the variability in the effect size between instruments, as opposed to the variability that is due to measurement error. We also calculated MR Egger estimates—a

Table 1. Inverse-variance-weighted (IVW) estimates and MR-Egger estimates for the effect of GGT on diabetes and glycaemic traits in the large-scale GWAS, DIAGRAM and MAGIC, respectively

	IVW			MR- Egger	
	Effect size (95% CI)	P-value	I^2 (95% CI)	Effect size (95% CI)	P-value
Type 2 diabetes	0.107 (0.275, 0.086)	0.10	49 (20-67)	0.149 (0.187, 0.484)	0.36
				Intercept estimate	
				0.019 (0.045, 0.007)	0.14
Glucose (mmol/L)	0.034 (0.081, 0.024)	0.07	74 (61-82)	0.055 (0.043, 0.153)	0.26
				Intercept estimate	
				0.006 (0.013, 0.001)	0.11
Insulin (pmol/L)	0.015 (0.044, 0.015)	0.16	26 (0-54)	0.019 (0.045, 0.083)	0.54
				Intercept estimate	
				0.002 (0.007, 0.002)	0.29
HOMA-IR	0.020 (0.053, 0.017)	0.11	34 (0-59)	0.015 (0.056, 0.085)	0.66
				Intercept estimate	
				0.003 (0.008, 0.003)	0.33

Abbreviations:

CI, confidence interval; GGT, gamma-glutamyltransferase; GWA, genome wide association study; HOMA-IR, homeostatic model assessment- insulin resistance; I^2, heterogeneity parameter; IVW, inverse-variance weighted method, MR- Egger, Mendelian Randomization Egger method; N, number of cases.

Effect size for diabetes analysis is the effect of genetically determined GGT (per SD log GGT) per odds increase in risk of diabetes. Effect size for glycemic traits analysis is the effect of genetically determined GGT (per SD log GGT) per increase in any of the glycemic traits.

recently proposed method for MR analysis, which is robust to invalid instruments. It is used to test for directional pleiotropy and provides an estimate of the causal effect adjusted for its presence.

For MR analyses of diabetes, we estimated power using the online tool mRnd (http://cnsgenomics.com/shiny/ mRnd/). We used the genetic sample size and case/control ratios together with the proportion of variance of GGT explained by the GRS. We calculated power to detect an effect using type 1 error (a) of 0.05.

Proportional hazard assumptions were inspected visually using log-minus-log plots, with no deviations detected for both the observational and genetic analysis. Statistical analyses were done using SPSS version 20 (IBM, Armonk, NY, USA), Stata release 13 software (StataCorp LP, College Station, TX, USA) and R version 2.15.1 (R Foundation for Statistical Computing, Vienna, Austria). Missing values for all covariates were imputed using expectation maximization in SPSS.

Note: Supplementary Material/Appendeix can be found in the website of the published journal or can be provided on request.

RESULTS

Baseline characteristics of the population used for analysis from the Rotterdam Study are shown in Table 2. Mean age (SD) of participants was 65.6 (9.8), 58% of the population were women and mean serum GGT level was 24 U/L. During a mean follow-up time of 5.7 years, 1125 individuals progressed to prediabetes {incidence rate: 27.1 [95% confidence interval (CI)] per 1000 person-years}. For type 2 diabetes, the mean follow-up was 6.9 years and 811 individuals developed diabetes [incidence rate: 13.6 (95% CI) per 1000 person-years].

The age- and sex-adjusted HRs for prediabetes per 1-SD change in natural logarithm of GGT was 1.26 (95% CI: 1.19–1.34), as shown in Table 3. Further adjustments for BMI and ALT yielded a HR of 1.16 (95% CI: 1.09–1.25). Controlling for conventional risk factors in model 3 diminished the magnitude of the association (HR : 1.10; 95% CI: 1.02–1.19), and adjustment for fasting glucose and fasting insulin did not materially change the estimate. For type 2 diabetes, the age- and sex-adjusted HR was 1.39 (95% CI: 1.30–1.49).

The estimate was attenuated after adjustment for BMI and ALT (HR : 1.27; 95% CI: 1.07–1.2). The association further attenuated after adjustment for confounders and for fasting glucose and fasting insulin (HR : 1.19; 95% CI: 1.1–1.3). Figure 2 depicts a graphical representation of the distribution of GGT levels with incremental increase in HRs for prediabetes and diabetes over the range of GGT in the Rotterdam Study.

Table 2. Baseline characteristics of participants.

Characteristics	Total
Women (n, %)	3,753 (58)
Age (years)	65.6 (9.8)
Waist circumference (cm)	93 (11.8)
Body mass index (kg/m^2)	26.9 (3.9)
Total Cholesterol (mmol/L)	5.7 (0.9)
Triglycerides (mmol/L)	1.4 (0.7)
High density lipoprotein cholesterol (mmol/L)	1.4 (0.4)
Lipid lowering medication (n, %)	907 (13.9)
Systolic blood pressure (mmHg)	139.4 (20.8)
Antihypertensive medication use (n, %)	1,885 (29)
Alcohol (g/day)	10 (13.3)
Non-drinkers (n, %)	1,648 (23.6)
Prevalent coronary heart disease (n, %)	478 (7)
Current smoking (n, %)	1,042 (16)
Alanine aminotransferase (U/L)	21 (16 - 27)
Gamma-glutaryltransferase (U/L)	24 (17 - 36)
C-reactive protein (mg/L)	1.6 (0.6 - 3.5)
Fasting insulin (pmol/L)	73 (51 - 106)
Fasting glucose (mmol/L)	5.4 (0.7)

Values are mean ± standard deviation or median (interquartile range) for characteristics with skewed distributions.

Table 3. Association of natural log transformed GGT levels (per SD) with incident prediabetes and incidence type 2 diabetes.

	Incident prediabetes (n= 1,125/7,263)		Incident type 2 diabetes (n= 811/8,628)	
	HR (95% CI)	***P*-value**	**HR (95% CI)**	***P*-value**
Model 1	1.26 (1.19 - 1.34)	1.0×10^{-14}	1.39 (1.30 - 1.49)	2.0×10^{-16}
Model 2	1.16 (1.09 - 1.25)	1.3×10^{-5}	1.23 (1.14 - 1.33)	1.7×10^{-7}
Model 3	1.10 (1.02 - 1.19)	0.008	1.19 (1.10 - 1.30)	2.6×10^{-5}
Model 4	1.08 (1.01 - 1.17)	0.02	1.11 (1.02 - 1.21)	0.01

Abbreviations: CI confidence interval; HR, Hazard Ratio; SD, standard deviation.
Model 1: age, sex, and Rotterdam Study cohort.
Model 2: Model 1 and BMI, ALT.
Model 3: Model 2 and additionally adjusted for triglycerides, C- reactive protein, body mass index, waist circumference, cholesterol, HDL, smoking, alcohol, systolic blood pressure, indication for hypertension, lipid lowering medication and prevalence CVD.
Model 4: Model 3 and additionally adjusted for fasting insulin and fasting glucose.

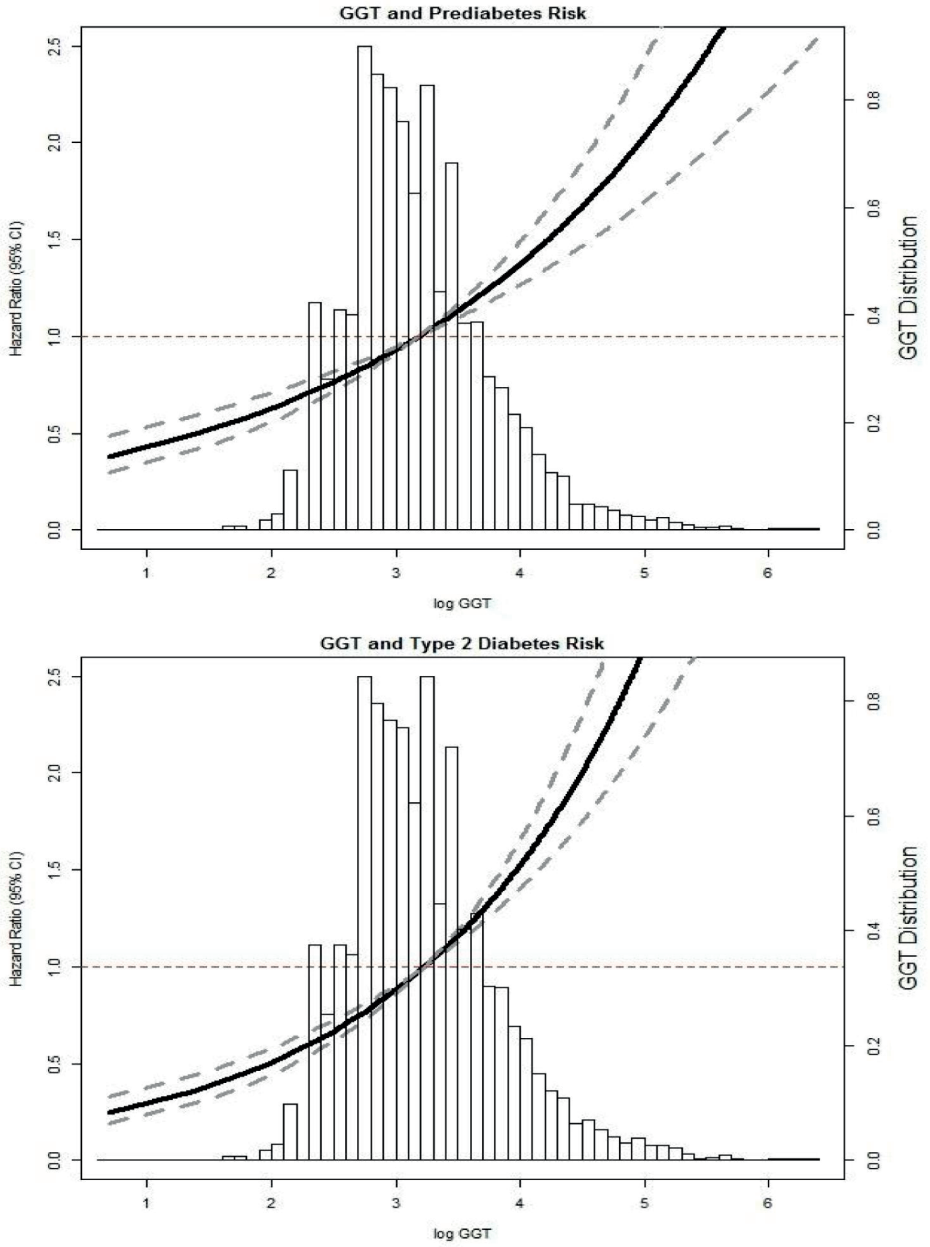

Figure 2. Graphical representation of the distribution of GGT levels with incremental increase in hazard ratios for prediabetes and diabetes over the range of GGT in the Rotterdam Study.

GGT-related genetic variants and risk of prediabetes and type 2 diabetes

Nearly all GGT SNPs were associated with GGT levels in the Rotterdam Study (Supplementary Table 2, available as Supplementary Data at IJE online). However, none of them

was associated with prediabetes and type 2 diabetes (Supplementary Figure 1a and b, available as Supplementary Data at IJE online). The GRS composed of 26 SNPs was normally distributed among the study participants and associated with log-transformed GGT levels (beta per SD log GGT : 0.41, 95% CI: 0.35–0.47, P-value = 2 10^{-16}), explaining 3.5% of the variation in serum GGT (F-statistic : 91.9). Supplementary Figure 2 (available as Supplementary Data at IJE online) shows increasing mean levels of GGT in quartiles of the GRS. The GRS did not associate with the risk of prediabetes (HR per SD in log GGT : 0.90; 95% CI: 0.67–1.22) and type 2 diabetes (HR per SD in log GGT : 0.86; 95% CI: 0.60–1.23) (Table 4). Furthermore, there was no evidence for an effect of GGT genetic score on glycaemic traits in the Rotterdam Study (Table 4). Stratification by drinking status did not affect the genetic association of GGT with the risk of diabetes. Excluding prevalent CVD cases did not affect the estimates (Supplementary Table 3, available as Supplementary Data at IJE online). Combining prevalent and incident cases of type 2 diabetes did not change the results (Supplemental Table 3, available as Supplementary Data at IJE online). Excluding SNPs that had a pleiotropic effect did not affect the as-

Table 4. Association of GGT related genetic risk score and rs2017869 genotype with glycemic traits, incidence prediabetes and type 2 diabetes in the Rotterdam Study.

	N	Effect estimates* (95% CI)	*P*-value
Genetic risk score			
Glucose (mmol/L)	7,383	-0.06 (-0.14, 0.01)	0.1
Insulin (mmol/L)	7,383	-0.006 (-0.06, 0.05)	0.8
HOMA-IR (log units)	7,383	-0.01 (-0.08, 0.04)	0.5
Prediabetes	976/ 6,236	0.90 (0.67 , 1.22)	0.5
Type 2 diabetes	679/ 7,383	0.86 (0.60 , 1.23)	0.4
rs2017869			
Glucose (mmol/L)	7,383	0.0001 (-0.01, 0.01)	0.9
Insulin (mmol/L)	7,383	-0.001 (-0.01, 0.01)	0.8
HOMA-IR (log units)	7,383	-0.001(-0.01, 0.01)	0.8
Prediabetes	976/ 6,236	1.01 (0.95 , 1.08)	0.6
Type 2 diabetes	679/ 7,383	1.04 (0.96 , 1.12)	0.3

Abbreviations: CI, confidence interval; GGT, gamma-glutamyltransferase; HOMA-IR, homeostatic model assessment- insulin resistance; Model adjuststed only for study cohort effect *Effect estimates represent betas for continuous traits and hazard ratios for binary outcomes. Results are reported per SD change in GGT genetic risk score in the Rotterdam Study

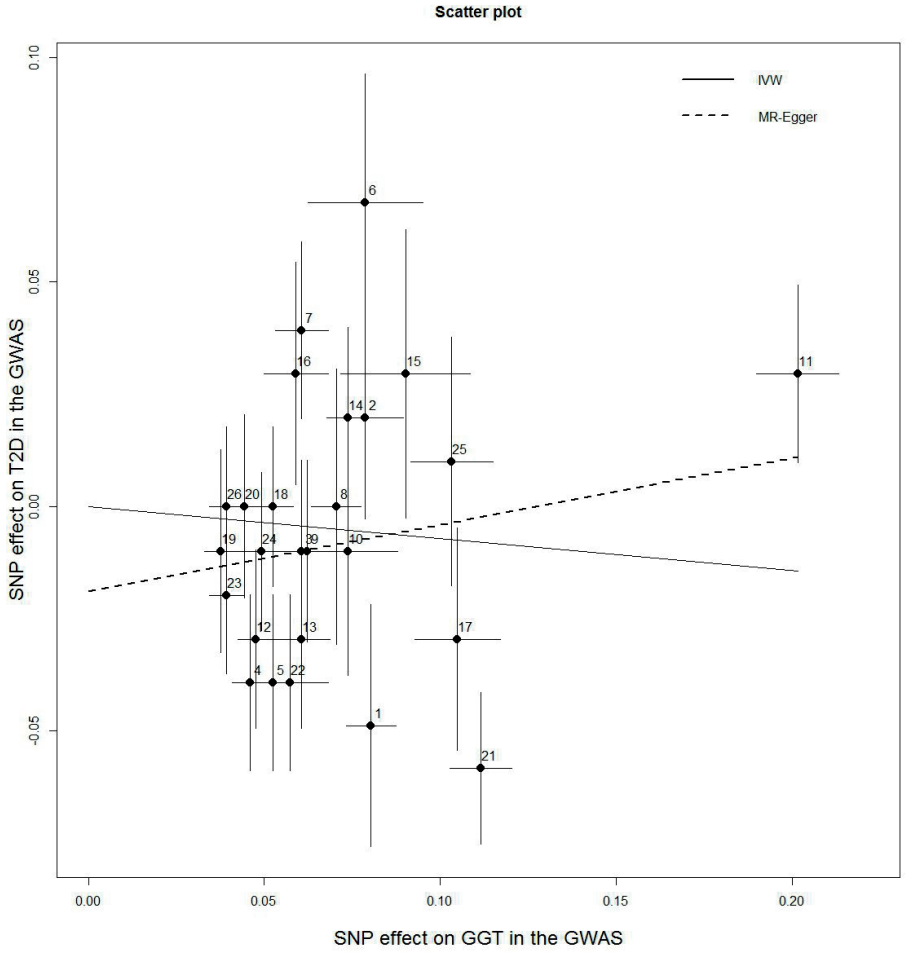

Figure 3. Scatter plot for type 2 diabetes. IVW estimates and MR-Egger estimates of GGT on type 2 diabetes risk utilizing 26 genetic variants as instru-mental variables in GWAS summary data. The included SNPs are shown by Arabic numbering #1, rs10513686 (SLC2A2); #2, rs1076540 (MICAL3); #3, rs10908458 (DPM3, EFNA1, PKLR); #4, rs12145922 (CCBL2, PKN2); #5, rs1260326 (C2orf16, GCKR); #6, rs12968116 (ATP8B1); #7, rs13030978 (MYO1B, STAT4); #8, rs1335645 (CEPT1, DENND2D); #9, rs1497406 (RSG1, EPHA2); #10, rs17145750 (MLXIPL); #11, rs2073398 (GGT1, GGTLC2); #12, rs2140773 (EFHD1, LOC100129166); #13, rs2739330 (DDT, DDTL, GSTT1, GSTT2B,MIF); #14, rs339969 (RORA); #15, rs4074793 (ITGA1); #16, rs4503880 (NEDD4L); #17, rs4547811 (ZNF827); #18, rs4581712 (DYNLRB2); #19, rs516246 (FUT2); #20, rs6888304 (CDH6); #21, rs7310409 (HNF1A, C12orf27); #22, rs754466 (DLG5); #23, rs8038465 (CD276); #24, rs9296736 (MLIP); #25, rs944002 (C14orf73); #26, rs9913711 (FLJ37644, SOX9).

sociation between GGT GRS, prediabetes and type 2 diabetes (Supplementary Table 4, available as Supplementary Data at IJE online). Previously reported genetic variant (rs2017869) in the GGT1 gene was negatively associated with GGT levels (beta per SD in log GGT : –0.06; 95% CI: –0.07 to –0.05; P-value < 2 10^{-16}) and it explained 2.6% of the variance in the GGT levels (F-statistic: 68.82). In a separate genetic analysis of rs2017869,

we found no effect of the genetic variant neither with prediabetes nor with diabetes risk in the Rotterdam Study (Table 4).

Taking advantage of the large sample size, we then evaluated causal estimates in publically available GWAS. With the IVW, we detected no evidence for a causal relation between GGT and diabetes (log OR per SD of log GGT ¼ 0.107, 95% CI: 0.275 to 0.086, P-value ¼ 0.10) and the same null result was observed with MR-Egger estimate (log OR per SD of log GGT ¼ 0.149, 95% CI: (0.187 to 0.484, P-value ¼ 0.36) (Table 1). Scatter plot for the genetic associations of SNPs and type 2 diabetes is depicted in Figure 3. Likewise, no causal association of GGT was detected for any other of glycaemic traits employing IVW or MR-Egger estimates. Visual plots of the genetic association of GGT with glycaemic traits are shown in Supplementary Figure 3a–c (available as Supplementary Data at IJE online).

Making use of the online power calculator, we estimated to have >80% power to detect an HR > 1.11 per SD of log GGT for diabetes.

DISCUSSION

In this population-based cohort study, we found a 10% higher risk of prediabetes and a 19% higher risk of diabetes per SD increase in serum GGT levels. The MR analysis using GGT-related genetic variant as instrumental variables did not support this association to be causal. Altogether, our results suggest that the association could be mainly due to reverse causation or possible confounding.

Although a large and broadly consistent body of evidence has established serum GGT levels as strongly linked to the development of type 2 diabetes, its causal role is uncertain. It is suggested that GGT links to type 2 diabetes through hepatic lipid accumulation and non-alcoholic fatty liver disease (NAFLD), both implicated in impaired hepatic insulin resistance, major features of pathophysiology of type 2 diabetes.[14] In addition, GGT is involved in the catabolism of glutathione and is associated with increased oxidative stress, which is involved in the development of insulin resistance and diabetes.[15,16] This evidence is confirmed from animal models that relate dysregulated glutathione metabolism with impaired insulin action in adipocytes.[17] However, GGT could also be associated with type 2 diabetes and glycaemic traits based on reverse causality. We observed in our study that further adjustment for potential confounders or mediators, including fasting glucose and insulin as well, considerably diluted the association. In agreement with this observation, a recent study from Scott et al. found that a genetically determined insulin resistance was associated with increasing GGT levels,[18] suggesting a causal role of insulin resistance in GGT. We were able to replicate these findings in the Rotterdam Study employing a reverse MR study for GGT. Insulin-related GRS utilizing nine genetic variants[11] was associated with higher GGT levels (effect estimate: 0.001; 95% CI: 0.0001–0.002). Taken altogether, our findings may therefore more favourably indicate reverse causality.

The direction of estimates in the observational analysis is in line with a previous meta-analysis of 24 cohort studies[1] that reported a pooled relative risk of top vs bottom tertiles of GGT levels with type 2 diabetes incidence of 1.34 (1.27–1.42). However, the substantial heterogeneity (I^2 > 70%) observed between studies, together with the younger mean age of participants (50 years old vs 65.6 years old in our study) might explain the difference in magnitude with our study. The effect estimates in our analysis were substantially and steeply attenuated by adjustment for conventional risk factors and confounders across models. This suggests that potential residual and unmeasured confounders remain a concern and might explain the association.

Our genetic analysis suggests that GGT is not causally affecting glycaemic traits, prediabetes or risk of type 2 diabetes. Nevertheless, there is evidence to suggest that the GGT1 locus, which is the main protein-coding gene for GGT, may account for serum GGT levels variation[18,19] and therefore variants within this locus have been used as instrumental variables for MR studies. Conen and colleagues[2] used an MR approach and found evidence for a causal effect of a GGT1 variant on fasting insulin levels in a sample size of 4000 participants. The instrumental variable (rs2017869) was explaining only 1.6% of the variance of GGT levels. These results were not replicated in the Rotterdam Study. Similarly, we could not replicate the causal analysis when using the multi-locus genetic instrument (explaining 3.5% of the variance in GGT levels). Although the Rotterdam Study sample size could not help to yield high-precision estimates from the genetic analysis, we were unable to confirm an association in the GWAS data where we had >80% power to detect an HR of 1.11 of GGT levels on type 2 diabetes risk.[20] MR is a suitable alternative to explore evidence for causality when certain assumptions are met. First, there should be a strong association between the GRS and the risk factor of interest. All SNPs used in our study were associated with GGT levels in a large meta-analyses of GWAS.[4] Second, the effect of genetic instrument on the outcome must be mediated exclusively by the exposure and there should be no direct effects (e.g. a causal pathway between the genetic variants and outcome that does not involve the exposure and that can be introduced by horizontal pleiotropy or population stratification). Third, GRS affects the outcome only through the risk factor of interest. This assumption should be considered using information on the underlying biology, as it is difficult to validate whether the instrument satisfies the no-pleiotropy assumption. In our analysis, GCKR, SLC2A2 and HNF1A have been reported to be associated with glucose[21] and type 2 diabetes.[22] Other genetic variants have been reported to be associated with serum CRP,[23] low-density lipoprotein cholesterol and coronary artery disease.[24] When we excluded the pleiotropic variants from the GRS in a sensitivity analysis, the causal estimates did not change. Thus, it is unlikely that our results are affected by potential pleiotropy. This is in line with our results from MR-Egger-regression estimates utilizing GWAS summary-level data.

The major strength of this study is the large sample size for measurements of both GGT and glycaemic indices, prediabetes and diabetes in the Rotterdam Study, and the additional sample size secured through utilization of the GWAS data. By also examining associations with incident prediabetes, we provided insight into the early development of metabolic dysregulations that could lead to diabetes. Additionally, we used data from a well-characterized prospective population-based cohort study, which allowed us to have a comprehensive assessment of this association using both observational and genetic data. Nevertheless, several issues may compromise our approach in assessing causality. First, it could be argued that, as the biological function for some of the GGT SNPs is yet to be established, there could be alternative biological pathways explaining their association with GGT. Using multiple SNPs to index GGT, we were able to minimize the risk of pleiotropic effects, as the effects of alternative pathways reflected by individual SNPs would be expected to be strongly diluted when combined in a multi-marker score. Second, using SNPs from GWAS, where the lead SNPs with the smallest P-values are typically selected and other significant SNPs are not reported, could lead to overestimation of the SNP-trait effect reflected in the MR analysis. This might be due to chance correlation between these SNPs and potential confounders. However, the genetic variants selected as instruments were strongly associated with GGT levels in both the GWA study and in the current study.

Additionally, we only used GGT measured in serum, but there have been reported differences in plasma levels and different types of GGT fractions.[25,26] Furthermore, the results may not be valid for all ethnic groups, since our population consisted of Caucasian individuals.

In conclusion, we cannot verify any causal effect of GGT on prediabetes and diabetes risk. The observed association between GGT levels and the risk of prediabetes and type 2 diabetes is probably due to reverse causation or residual confounding. This implies that interventions to lower GGT levels are unlikely to result in decreased risk of diabetes.

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Chapter 3.4

Fatty Liver Index and Risk of Diabetes, Cardiovascular Disease and Mortality: The Rotterdam Study

Jana Nano[1,2,3], Tamy Pulido[1], Arjola Bano[1], Adela Brahimaj[1], Loes J.M. Alferink[4], Bledar Kraja[5], Sarwa Darwish Murad[1,4], Abbas Dehghan[1,5], Oscar H. Franco[1,6], Taulant Muka[1,6]

[1] Department of Epidemiology, Erasmus University Medical Center, Rotterdam, the Netherlands
[2] Institute of Epidemiology, Helmholtz Zentrum Munich, German Research Center for Environmental Health, Germany
[3] German Diabetes Center (DZD), Germany
[4] Department of Gastroenterology and Hepatology, Erasmus University Medical Center, Rotterdam, the Netherlands
[5] Department of Gastrohepatology, University Hospital Center Mother Teresa, Tirana, Albania
[5] Department of Biostatistics and Epidemiology, MRC-PHE Centre for Environment and Health, School of Public Health, Imperial College London
[6] Institute of Social and Preventive Medicine (ISPM), University of Bern, Bern, Switzerland
* This authors contribute equally

(Submitted)

ABSTRACT

Background

Fatty liver prevalence is alarmingly rising worldwide with its effects extending beyond the liver.

Objective

We prospectively assessed 1) the association between the fatty liver index (FLI), a proxy measure for fatty liver calculated through an algorithm including body mass index, waist circumference, triglycerides and gamma-glytamyltransferase, with the risk of type 2 diabetes mellitus (T2D), atherosclerotic cardiovascular disease (ASCV), and all-cause mortality, and 2) investigated whether addition of FLI would improve cardiometabolic prediction models.

Method and Results

A total of 7490 participants from the population-based Rotterdam Study, age ≥ 45 years and free of T2D and ASCV at baseline were eligible for analysis. Multivariable hazard ratios (HRs) and 95% confidence intervals (95%CI) were calculated using Cox regression models. After a median follow-up of 7.49 years, 685 subjects developed T2D, 801 subjects developed ASCV and 2308 subjects had died. Higher FLI score was associated with increased risk of T2D (HR (95% CI) = 1.4 (1.19, 1.65), per SD increase) independent of age, sex, socioeconomic status, lifestyle factors and several cardio-metabolic risk factors. Although diabetes risk prediction models improved significantly when FLI was added, the latter did not perform better or beyond of the classical diabetes risk factor, HOMA-IR. FLI was not associated with the risk of ASCV and all-cause mortality after adjusting for lifestyle, socioeconomic and cardio-metabolic factors (HR 1.06 (0.91, 1.23) for ASCV risk, per SD increase; and HR 1.06 (0.96, 1.16) for mortality).

Conclusion

Fatty liver index was associated with incident T2D in middle aged and elderly subjects independent of lifestyle and cardiovascular risk factors. However, FLI has limited utility in predicting either T2D or ASCV in a general population setting.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is defined as the presence of 5% of liver fat, in the absence of competing liver disease etiologies. The prevalence is increasing world-wide, particularly among the elderly ranging from 10% to 30% (1). Emerging data show that fatty liver is associated with an increased risk of type 2 diabetes (T2D) and atherosclerotic cardiovascular disease (ASCV) (2, 3), whereas for all-cause mortality, the reports are less consistent (4). This is not surprising as NAFLD is closely correlated with established cardio-metabolic risk factors nested by the metabolic syndrome including visceral adiposity, dyslipidemia, insulin resistance and hypertension (4-6). Additionally, other factors such as inflammation have been implicated in the pathophysiology of NAFLD, but the role of inflammatory mediators warrants further research (7). Whether these associations are simply due to a shared underlying etiology or because the presence of NAFLD confers an additional risk above these factors, remains a point of discussion. This information has important clinical implications that might influence the decision to perform primary prevention strategies or screening, given the increasing number of patients with NAFLD. Moreover, evidence is still lacking whether adding NAFLD as a predictive factor in risk models or score systems would improve their ability to accurately predict the occurrence of cardiometabolic diseases such as T2D or ASCV.

The gold standard for the diagnosis of NAFLD is a liver biopsy but the procedure can lead to serious complications, such as hemorrhage or infections. In epidemiological and clinical studies, non-invasive techniques such as magnetic resonance, computed tomography, and ultrasound, which can be either costly or are not universally available are often used. To overcome this limitation, the fatty liver index (FLI) has been developed as a surrogate marker of NAFLD based on routine risk factors that can easily be assessed, including body mass index, waist circumference, triglycerides and gamma-glytamyltrasnferase. The FLI is not only applied clinically, but has also been validated in the general population in a large prospective cohort, The Rotterdam Study,(8, 9). The use of this marker is easy to measure and would potentially help to identify individuals of increased cardiometabolic risk, and drive prevention strategies.

Therefore, we aimed to investigate whether 1) FLI predicts the risk of T2D incidence, ASCV incidence, and all-cause mortality independent of traditional cardiovascular risk factors and further, whether 2) FLI improves prediction models or scores of these diseases in middle-aged and elderly individuals. Secondly, we hypothesized that FLI, a proxy for fatty liver, exert its effect on cardiometabolic disease through inflammation. Therefore, we investigated the association of a diverse set of inflammatory markers with FLI.

METHODS

Study population

The Rotterdam Study is a prospective cohort study which started in 1990 in the Ommoord district, in the city of Rotterdam, The Netherlands. In brief, all inhabitants of the Ommoord district aged 45 years or older were invited to participate (n = 10,215). At baseline (1990-1993), 7,983 participants, aged 55 years or older, were included (RS-I). In 2000, an additional 3011 participants were enrolled (RS-II). The third cohort was formed in 2006 and included 3932 participants aged 45 years or older (RS-III). There were no eligibility criteria to enter the Rotterdam Study cohorts except the minimum age and residential area based on postal codes. Participants have been re-examined every 3–4 years, and have been followed up for a variety of diseases. A more detailed description of the Rotterdam Study can be found elsewhere (10). 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.

For the current study, we used data from the third visit of the first cohort (1997–99) and the first centre visit for both the second cohort (2000–01) and the third cohort (2006–08) as baseline. We excluded 314 participants with no informed consent and 1818 individuals without fasting samples or any of the fatty liver index components such as triglycerides, gammaglutamyl transferase or other information on waist circumference and body mass index. Next, we excluded participants with prevalent T2D and ASCV. The final dataset for analysis comprised 7490 participants free of cardiometabolic disease (Figure S1).

Fatty Liver Index

We utilized FLI as a surrogate marker for liver steatosis calculated based on an algorithm including body mass index (BMI), waist circumference (WC), triglycerides (TGs) and Gamma-glutamyltransferase **(**GGT) based on the following formula: FLI = $(e^{0.953*\log(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log(\text{ggt}) + 0.053*\text{waist circumference} - 15.745})/(1 + e^{0.953*\log(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log(\text{ggt}) + 0.053*\text{waist circumference} - 15.745})*100$ (8). Height and weight were measured with the participants standing without shoes and heavy outer garments. BMI was calculated as weight divided by height squared (kg/m^2). WC was measured at the level midway between the lower rib margin and the iliac crest with participants in standing position without heavy outer garments and with emptied pockets, breathing out gently. Serum TG and GGT levels were determined by an automated enzymatic procedure in a fasting blood sample (Roche Diagnostic GmbH, Mannheim, DE). FLI was recently validated in the Rotterdam Study (9).

Assessment of type 2 diabetes, atherosclerotic cardiovascular disease and all-cause mortality

The main outcome measured under study were incident T2D, ASCV and all-cause mortality. At baseline and during follow-up, cases of T2D were ascertained by use of general practitioners' records (including laboratory glucose measurements), hospital discharge letters, and serum glucose measurements from Rotterdam Study visits, which take place roughly every

4 years. According to the WHO guidelines, type 2 diabetes was defined as a fasting blood glucose >7.0 mmol/L, or the use of blood-glucose-lowering medication (11). Information regarding the use of blood-glucose lowering medication was derived from both structured home interviews and linkage to pharmacy records. At baseline, more than 95% of the Rotterdam Study population was covered by the pharmacies in the study area. ASCV events were defined as fatal and nonfatal myocardial infarction (MI), other coronary heart disease (CHD) mortality or stroke as previously described (12, 13). Hard CHD was defined as MI (fatal and nonfatal) and fatal CHD. Data on T2D and ASCV were collected through an automated follow-up system until January 1st 2012. All potential T2D and ASCV were independently adjudicated by two study physicians. In case of disagreement, consensus was sought with a specialist. Data on total mortality were collected using an automated follow-up system until 5th June 2017. ASCV mortality was defined as death due to CHD, cerebrovascular disease or other atherosclerotic diseases.

Other covariates

Baseline information on medical history and medication use was obtained from questionnaires in combination with medical records. During the baseline home interview, participants provided information on smoking habits. Smoking habits were categorized as current, former and never smoking. Education was defined as low (primary education), intermediate (secondary general or vocational education), or high (higher vocational education or university). At home interview, participants self-reported if they were using statins or anti-hypertensive medications. Anthropometrics were measured in the research centre by trained staff. Blood pressure was measured in the sitting position on the right arm and calculated as the mean of two measurements using a random-zero sphygmomanometer. Fasting insulin and glucose levels, lipid levels and C-reactive protein were measured using a COBAS 8000 Modular Analyzer (Roche Diagnostics). Data on fasting glucose and fasting insulin levels were used to calculate the degree of insulin resistance according to homeostasis model assessment for insulin resistance (HOMA-IR) which is calculated by dividing the product of fasting levels of glucose and insulin by a constant (14). To assess overall dietary quality, the Dutch Healthy Diet index (DHD-index) was used as described in detail previously (15). Alcohol intake was assessed in grams of ethanol per

day from food frequency questionnaires. Participants were asked for the average daily consumption of alcohol. Excess alcohol consumption was defined as more than 14gr/day intake.

Fatty liver Index and inflammatory biomarkers

Given the strong inflammatory component underlying the etiology of both T2D and ASCV, we hypothesized that steatosis would exert its effects via inflammation-induced insulin resistance or immune response. therefore, fifty inflammatory markers were quantified in a subset of the first cohort (RSI-3, 669 individuals) using multiplex immunoassay on a custom designed human multi-analyte ELISArray KIT profile. Markers with more than 60% completeness of measurements were selected for analysis (25 from 50) as described previously (16).

Statistical Analysis

Continuous variables were reported as mean ± SD unless otherwise indicated and categorical variables were presented as percentages. The association of FLI with T2D incidence, ASCV incidence and all-cause mortality was assessed by using multivariable Cox proportional hazard regression models. Hazard ratios (HR) and 95% confidence intervals (95% CI) were reported. We present age, sex and BMI-adjusted HRs (Model 1) and performed further adjustment for possible confounders including smoking status (current, former/never), alcohol intake (continuous), physical activity (continuous), education level (low, intermediate, high). A third model was built adjusting further for hypertension treatment, systolic blood pressure, statin use, HOMA-IR, CRP, HDL and total cholesterol (Model 3). FLI was examined as continuous (per SD increase) variable and in categories of FLI corresponding to probabilities of having fatty liver (FLI <30; FLI = 30-60; FL >=60). We also tested separate models with the individual components of FLI included in model 2. In mediation analyses, we calculated the percentage of excess risk mediated $[(\text{hazard ratios } [HR]_{\text{con adj}} - HR_{\text{con + med adj}})/ (HR_{\text{con adj}} - 1)] \times 100\%$, where $HR_{\text{con adj}}$ is the confounder-adjusted HR and $HR_{\text{con + med adj}}$ is the confounder and mediator-adjusted HR. Because of the skewed distribution of HOMA-IR, triglycerides, CRP and gamma-glutamyl transferase, natural log-transformed values were used in the analysis. We constructed multivariate Cox regression models for pooled sexes because formal tests of interaction (sex×FLI) were not statistically significant for any outcome. 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 (time-dependent interaction terms were not significant at the 5% level).

Moreover, we compared the effect of FLI on 10-year risk prediction of T2D and ASCV events by studying the discrimination. Discrimination is the ability of a predictive model

to assign a higher risk to individuals who will develop an event in 10 years compared with those who will not. We quantified discrimination for both models by calculating the c-statistic difference between the base model and the models that additionally included FLI. For type 2 diabetes, there is not yet a unique established risk prediction model. We used KORA basic model (17) (including age, sex, parental history of diabetes, smoking, systolic blood pressure and BMI) since a recent systematic review showed good discrimination and validation of the score in a Dutch population based study when compared to existing prediction models (18). Additionally, we also used Wilson's risk score, including age, sex, parental history of diabetes and BMI (19). To assess the change in predictive power of FLI, we compared the 10-year ASCV risk prediction score (including age, sex, total cholesterol, HDL, smoking, systolic blood pressure, treatment for hypertension and type 2 diabetes) based on the recent ACC/AHA (20) guidelines. The difference in c-statistic between the base model and the model with FLI was performed under 10 iteration of perturbation-resampling (21).

We performed several sets of sensitivity analyses to explore the robustness of our findings: 1) we additionally adjusted Model 2 for coffee intake and the Dutch health diet index score (the original model did not include these variables due to >41% of missing information); 2) fatty liver index was recently validated in the Rotterdam Study, and was found to correlate closely with the presence of liver steatosis on ultrasonography (AUROC 0.81, sensitivity 64%, specificity 83%) (8, 9). We investigated the association of FLI with all-cause mortality, ASCV and T2D according to the cut-offs suggested (FLI<30, FLI from 30 to 60 and FLI>=60); 3) to further elucidate the nature of the relation between FLI with cardiovascular risk, we investigated FLI with separate components of ASCV such as ASCV mortality, hard CHD and stroke events; 4) previous literature suggest that the association between FLI and cardio-metabolic outcomes might be partly explained by obesity and in particular, visceral obesity (22). Therefore, instead of BMI, we rerun the analysis adjusting for waist circumference; 5) we provide effect estimates stratified by sex (men vs women) and age. The latter was grouped based on the cutoff of 65 years, which is closer to the mean and median age of our population; 6) to rule out any potential reverse causation, we investigated the association between FLI and mortality, ASCV and T2D incidence excluding events that occurred during the first 2 years of follow-up.

For the association between FLI and inflammatory markers, we investigated individually a set of 25 markers of low-grade chronic inflammation. After normalizing the markers using z-scaling after natural logarithmic transformation, we investigated the association of the latter with FLI using linear regression models adjusted for age, sex, BMI (model 1) and further C-Reactive Protein and HOMA-IR (model 2). To avoid false positive findings, we applied a Bonferroni corrected p-value of 0.002 (0.05/25 markers).

Approximately 5% of the participants lacked data on one or more of the covariates, except for coffee intake, Dutch health diet index score and alcohol intake which had

41.1%, 22%, 16.8% missing data, respectively. Missing data for these covariates were imputed (n=5 imputations) by using 'mice' package in R. This analyses was performed using R software version 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria) and SPSS statistical software (SPSS, version 21.0; SPSS Inc, Chicago, Illinois).

RESULTS

We included 7490 participants with a maximum follow-up time of 19.9 years and a median of 7.0, 7.49 and 5.69 years for T2D, ASCV and all-cause mortality, respectively. A total number of 685 subjects (incidence rate, 14.3 per 1000 person-years) developed T2D, 801 subjects developed ASCV (incidence rate, 15.9 per 1000 person-years) whereas 2308 deaths (incidence rate, 78.2 per 1000 person-years) occurred during the follow-up time. Anthropometrical, lifestyle, clinical and biochemical characteristics of all study participants are presented in **Table 1**.

Association between fatty liver index and incident type 2 diabetes

Higher FLI score was associated with a higher risk of type 2 diabetes both when investigating it continuously (hazard ratio, 95% confidence interval [HR 95% CI]: 1.96 (1.71, 2.25) and among three fatty liver cut-offs (**Table 2**)). Estimates remained statistically significant after adjusting for lifestyle, socio-economic factors (HR continues: 1.98 (1.72, 2.28)) and cardiovascular factors (HR continues: 1.4 (1.19, 1.65)). The same trend was observed for both FLI= 30-69 and FL≥ 60 when compared with the FLI< 30 group for model 1 and model 2. The group with FL >=60 was associated with a hazard ratio of 1.46 for type 2 diabetes incidence when compared to the group with FLI<30. HOMA-IR conferred the highest percentage of excessive risk mediating the association between FLI and type 2 diabetes incidence, followed by similar contributions of BMI and WC.

Association between fatty liver index and incident atherosclerotic cardiovascular disease

There was a positive association of FLI score with the risk of incident ASCV events both when assessed continuously (HR, 1.31 (1.15, 1.5) or when we compared FL >=60 with the reference category (HR, 1.48 (1.14, 1.91); 1.39 (1.07, 1.8), respectively). However, addition of several cardio-metabolic risk factors explained the observed association in model 3. Again, BMI was mediating the highest percentage of excess risk to ASCV incidence (**Table 3**).

Association between fatty liver index and all-cause mortality

Increasing FLI score were associated with higher hazard ratios of all-cause mortality (1.11 (1.03, 1.21) per SD increase in FLI in model 1. The association attenuated after con-

Table 1. Baseline characteristics of study participants

N	7490
Gender (Women)	4488 (59.9)
Age (years)	63.6 (9.56)
Waist circumference (cm)	92.48 (11.76)
Body Mass Index (kg/m2)	26.97 (4.07)
Gamma-glutamyltransferase (U/L)	23.00 [17.00, 34.00]
Glucose (mmol/L)	5.45 (0.57)
Insulin (pmol/L)	69.00 [49.00, 97.00]
HOMA-IR (units)	2.38 [1.66, 3.44]
LDL cholesterol (mmol/L)	3.72 (0.90)
Total cholesterol (mmol/L)	5.81 (0.99)
HDL cholesterol (mmol/L)	1.44 (0.41)
Triglycerides (mmol/L)	1.47 (0.78)
C-reactive protein (mg/L)	1.75 [0.70, 3.86]
Systolic blood pressure (mm Hg)	135.99 (20.90)
Diastolic blood pressure (mm Hg)	78.38 (11.49))
Hypertension	2879 (23.8)
Use of statin	655 (8.7)
Fatty Liver Index	42.57 [21.81, 66.62]
Coffee intake (grams/day)	500.00 [375.00, 625.00]
Dutch Health Diet Index	6.75 (1.91)
Alcohol intake(grams/day)	4.29 [0.54, 12.86]
Physical activity (MET-hours/week)	67.20 [36.9, 100.2]
Family history of diabetes	673 (9.0)
Highest level of education (%)	
Primary	866 (11.6)
Lower/intermediate	3043 (40.6)
Intermediate / higher	2175 (29.0)
Higher	1406 (18.8)
Smoking history	
Never	2405 (32.3)
Ever	1930 (25.9)
Current	3118 (41.8)
Follow up time for type 2 diabetes	7.00 [4.00, 11.00]
Follow-up time for atherosclerotic events	7.49 [4.46, 11.75]
Follow up time for mortality	5.69 [4.15, 9.54]

Data are n (%), mean(SD), or median[IQR, interquartile range; for characteristics with skewed distributions]
HOMA-IR: homeostatic model assessment –insulin resistance;

Table 2. Association of Fatty Liver Index (FLI) and type 2 diabetes incidence, atherosclerotic cardiovascular disease incidence and all-cause mortality in the Rotterdam Study.

		Model 1 (HR, 95% CI)	Model 2 (HR, 95% CI)	Model 3 (HR, 95% CI)
Type 2 Diabetes	n= 7484, number of events= 684			
FLI continuous		**1.96 (1.71, 2.25)**	**1.98 (1.72, 2.28)**	**1.4 (1.19, 1.65)**
FLI <30	2667	Reference		
FLI = 30-60	2478	**1.7 (1.33, 2.16)**	**1.68 (1.32, 2.13)**	1.23 (0.96, 1.57)
FL >=60	2345	**2.7 (2.03, 3.59)**	**2.65 (1.99, 3.54)**	**1.46 (1.07, 2.00)**
Atherosclerotic Cardiovascular disease	n= 7490, number of events= 801			
FLI continuous		**1.31 (1.15, 1.5)**	**1.26 (1.1, 1.44)**	1.06 (0.91, 1.23)
FLI <30	2667	Reference		
FLI = 30-60	2478	1.17 (0.96, 1.42)	1.15 (0.94, 1.4)	0.98 (0.8, 1.2)
FL >=60	2345	**1.48 (1.14, 1.91)**	**1.39 (1.07, 1.8)**	1.05 (0.79, 1.38)
All-cause mortality	n= 4809, number of events= 2291			
FLI continuous		**1.11 (1.03, 1.21)**	1.06 (0.97, 1.15)	1.06 (0.96, 1.16)
FLI <30	2667	Reference		
FLI = 30-60	2478	1.01 (0.9, 1.13)	0.98 (0.88, 1.1)	0.97 (0.86, 1.09)
FL >=60	2345	1.11 (0.95, 1.3)	1.04 (0.89, 1.22)	1.04 (0.88, 1.23)

Model 1: age, sex, cohort, body mass index
Model 2: Model 1 + smoking, alcohol intake, physical activity, education
Model 3: Model 2 + treatment for hypertension, systolic blood pressure, statins use, cholesterol levels, HDL, C-reactive protein, HOMA -insulin resistance
Abbreviations: BMI, body mass index; CRP, C-Reactive Protein; HOMA-IR, Homeostasis Model Assessment for Insulin Resistance; FLI, fatty liver index; HR, hazard ratio; CI, confidence interval; WC, waist circumference;

trolling for other lifestyle and socio-economic factors (model 2) and cardio-metabolic risk factors (model 3). No association was observed among the groups with different cut-offs of FLI. Among other components of FLI, BMI conferred the largest excess risk in the association (**Table 3**).

Association between fatty liver index and markers of low-grade chronic inflammation

After adjusting for Bonferroni correction, we identified 11 out of 25 inflammatory markers significantly associated with FLI adjusting for age, sex and BMI. Adding HOMA-IR and CRP to the model, attenuated six of these associations whereas IL13, Complement 3,

Table 3. Association of fatty liver index with type 2 diabetes incidence, atherosclerotic cardiovascular disease incidence and all-cause mortality in the Rotterdam Study by adding in the model components of the index

	Type 2 Diabetes		Atherosclerotic Cardiovascular Disease		All-cause mortality	
	HR, 95% CI	Percentage of excess risk mediated	HR, 95% CI	Percentage of excess risk mediated	HR, 95% CI	Percentage of excess risk mediated
Model 1	1.83 (1.69, 1.99)		1.11 (1.03, 1.2)		0.98 (0.94, 1.03)	
Model 1 + BMI	1.98 (1.72, 2.28)	8.2	1.26 (1.11, 1.45)	13.52	1.06 (0.98, 1.15)	7.82
Model 1 + WC	1.98 (1.71, 2.29)	8.19	1.17 (1.02, 1.35)	5.34	0.99 (0.91, 1.07)	0.57
Model 1 + GGT	1.76 (1.6, 1.93)	3.99	1.09 (1, 1.19)	2.45	0.95 (0.9, 1)	3.6
Model 1 + Triglycerides	1.69 (1.54, 1.87)	7.47	1.06 (0.97, 1.16)	4.75	0.99 (0.94, 1.05)	0.76
Model 1 + CRP	1.77 (1.62, 1.93)	3.19	1.1 (1.02, 1.19)	1.05	0.96 (0.91, 1)	2.7
Model 1 + HOMA-IR	1.43 (1.3, 1.58)	21.69	1.17 (1.07, 1.29)	5.45	0.99 (0.94, 1.05)	0.83
Model 1 + other cardiovascular mediators*	1.62 (1.48, 1.78)	11.48	0.99 (0.90, 1.08)	11.11	0.96 (0.91, 1.01)	2.3

Model 1: age, sex, cohort, smoking, alcohol intake, physical activity, education
*cholesterol, HDL, systolic blood pressure, hypertension treatment, statin use
Abbreviations: BMI, body mass index; CRP, C-Reactive Protein; HOMA-IR, Homeostasis Model Assessment for Insulin Resistance ; HR, hazard ratio; CI, confidence interval; WC, waist circumference;

Interleukin 1 Receptor Antagonist (IL1ra), Human CC chemokine-4 (HCC4) and Macrophage migration inhibitory factor (MIF) remained significant with P values ranging from 0.001 to 9.86×10^{-16}.

Risk prediction models

For 10-year T2D risk for the KORA basic model, the c-statistic was 0.663 (0.638, 0.688) and further improved with addition of FLI to 0.689 (0.662, 0.717) with a significant difference of 0.026 (0.013, 0.040) (**Table S6**). Similarly, the c-statistic of the Wilson's base model for 10-year risk of T2D was lower than previous (0.649 (0.630, 0.668)) and adding FLI improved the model with a significant difference of 0.033 (0.020, 0.046). However, in both predicted models of 10-year diabetes risk, HOMA-IR revealed higher c-statistic values with significant predictive changes of 0.043 (0.028, 0.058) and 0.048 (0.033, 0.064), respectively. FLI showed no further improvement of the model beyond HOMA-IR when

Table 4. Association between inflammatory markers and fatty liver index in a subset of participants of the Rotterdam Study.

Marker	Model 1			Model 2		
	Beta	SE	P value	Beta	SE	P value
IL13, pg/mL	**-0.18**	**0.018**	**2.55E-21**	-0.15	0.02	**9.86E-16**
Complement 3, mg/mL	**0.11**	**0.019**	**2.45E-09**	0.06	0.02	**0.001**
IL1ra, pg/mL	**0.1**	**0.018**	**1.43E-08**	0.06	0.02	**0.0005**
HCC4, ng/mL	**0.1**	**0.018**	**1.26E-07**	0.06	0.02	**0.0003**
FAS, ng/mL	**0.08**	**0.018**	**4.69E-06**	0.05	0.02	0.0034
MIF, ng/mL	**0.08**	**0.017**	**1.51E-05**	0.06	0.02	**0.0006**
EN-RAGE, ng/mL	**0.08**	**0.018**	**2.73E-05**	0.03	0.02	0.06
CD40, ng/mL	**0.08**	**0.02**	**0.000125**	0.03	0.02	0.14
MIP1 beta, pg/mL	**0.07**	**0.018**	**0.000131**	0.04	0.02	0.01
MDC, pg/mL	**0.06**	**0.018**	**0.001**	0.04	0.02	0.02
TNFRII, ng/mL	**0.06**	**0.019**	**0.001**	0.02	0.02	0.22
IL8, pg/mL	0.05	0.018	0.004	0.03	0.02	0.13
IL18, pg/mL	0.05	0.018	0.006	0.02	0.02	0.34
TRAILR3, ng/mL	0.04	0.018	0.028	0.001	0.02	0.94
CFH, ug/mL	0.04	0.018	0.036	0.03	0.02	0.1
MCP1, pg/mL	0.04	0.018	0.049	0.04	0.02	0.02
IL17, pg/mL	0.03	0.018	0.111	0.04	0.02	0.03
CD40 ligand, ng/mL	0.03	0.019	0.128	0.05	0.02	0.01
IL16, pg/mL	0.03	0.018	0.135	0.01	0.02	0.52
PARC, ng/mL	-0.02	0.018	0.205	-0.02	0.02	0.16
RANTES, ng/mL	0.02	0.018	0.397	0.01	0.02	0.73
Eotaxin, pg/mL	-0.01	0.019	0.49	-0.003	0.02	0.88
sRAGE, ng/mL	0.003	0.019	0.85	0.01	0.02	0.41
Resistin, ng/mL	0.002	0.018	0.925	-0.01	0.02	0.45
MIP1 alpha, pg/mL	-0.0004	0.018	0.984	-0.02	0.02	0.2

Model 1 adjusted for age, sex, BMI, cohort

Model 2 adjusted for age, sex, cohort, BMI, C-Reactive Protein, HOMA-IR

Bonferroni adjusted P value (0.05/25=0.002)

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;

the latter was added at the baseline model (**Table S6**). The c-statistic of the traditional ASCV risk score model for 10-year risk was 0.723 (0.71, 0.735). When we added FLI to the model, the c-statistic did not show any improvement (0.723 (0.71, 0.736)).

Sensitivity analysis

Adding coffee intake and dietary quality to model 2 did not affect the associations with all three outcomes. (**Table S1**); neither did the substitution of waist circumference instead of BMI in the models (**Table S2**). Sensitivity analysis excluding excessive alcoholic intake and those individuals within 2 years of follow-up did not change the results (**Table S3**). Similar to the ASCV events, the association between FLI and hard CHD events and stroke events were explained by lifestyle, socioeconomic and cardiovascular risk factors **(Table S4**). In a sex and age stratified analysis for diabetes incidence, higher hazard ratios were reported for women (rather than men) and among individuals < 65 years (vs ≥ 65 years) of age **(Table S5)**. There were no significant sex or age differences for ASCV or all-cause mortality**.**

DISCUSSION

In the present study, the FLI was associated with higher risk of type 2 diabetes independent of potential confounding such as lifestyle, socio-economic and cardio-metabolic risk factors. However, FLI did not improve diabetes prediction models better than HOMA-IR. For cardiovascular incidence and all-cause mortality, the association was explained by these factors and addition of FLI did not improve the prediction. FLI was associated with a set of proinflammatory markers; among them, IL13 prevailed strongly.

Until now, only three studies have evaluated the association between FLI and incident diabetes in a German, French and Korean populations (22-24). In line with our results, a recent meta-analysis investigating fatty liver (measure by ultrasound) reported two fold increase in diabetes risk (3). In our study, FLI was associated with diabetes risk even after adjusting for BMI and waist circumference, two important components of the index. This finding reinforce the consensus that liver fat content is associated with insulin resistance stronger than total and visceral fat mass (25, 26). Moreover, we found higher hazard ratios among women and <65 years old individuals in our sensitivity analysis. The prevalence of fatty liver is known to increase with age and is highest in males between 40 and 65 years, after which the prevalence declines (27). Post-menopausal women exhibit worst cardio-metabolic profile than men and therefore might have a progressive form of fatty liver disease that could explain such results (28, 29). Lower hazard ratios for ≥ 65 year old individuals might reflect decreased fatty liver changes in advanced stages such as nonalcoholic steatohepatitis or fibrosis (30).

Although we noticed a significant drop in risk estimates when adjusting for potential confounders, in particular HOMA-IR, the association between FLI and type 2 diabetes risk remained strong implying that other factor that we are not capturing might be involved in the relationship. Beside traditional cardio-metabolic risk factors, other potentially contributing factors that might influence fatty liver development include increased levels of circulating pro-inflammatory markers that are likely to be synthesized in the liver, in particular in the presence of progression to other stages of fatty liver (31, 32). We investigated the association of FLI with a diverse set of pro-inflammatory biomarkers and found that more than half of them were significantly associated with FLI. However, the effect estimates attenuated after further adjustment for CRP and HOMA-IR levels, but remained significant for IL13, Complement 3, Il1ra, HCC4 and MIF after stringent Bonferroni correction. Most of them have been previously reported as biomarkers related to cardio-metabolic diseases (33-37) and IL13, one of the markers with the strongest association, was recently reported as a novel marker for risk of prediabetes, diabetes and initiation of insulin therapy (16). IL13 is a cytokine mainly produced by T-helper lymphocytes and has been previously concluded that IL13 mitigates proinflammatory response in mice and regulates glucose homeostasis via the IL-13ra1–STAT3 signaling pathway in the liver, and that this pathway might provide a target for glycemic control in type 2 diabetes (38).

There is an emerging evidence for the positive association between fatty liver and ASCV risk reported in many studies (39), but the quality of studies remains poor. Contrary to the lack of association in our study for this outcome, a recent comprehensive meta-analysis of 16 observational studies found that fatty liver conferred an odds ratio of 1.64 (95% CI 1.26–2.1) for fatal and non-fatal incident ASCV events (40). Nevertheless, the heterogeneity of the studies was remarkably high (I^2 = 86%) with diverse definition of exposure (NAFLD measured with different techniques) or outcome (fatal vs non-fatal ASCV). Some of these studies have suggested that the increase in ASCV risk may be limited to subgroups of patients with NAFLD such as those with type 2 diabetes, increased GGT levels or advanced stages of fibrosis (41, 42). In our study, we excluded patients having pre-existing chronic diseases such as T2D and CVD, and thus provided data on a homogenous population. Although increased levels of cardio-metabolic factors such as hypertension and carotid atherosclerotic plaques have been recently reported in association with FLI from cross-sectional studies, to our knowledge, there is no report on the relation between FLI and ASCV risk (43, 44). Overall, although available evidence in the literature demonstrates an association between NALFD and ASCV risk, further high quality prospective design studies should investigate the role of FLI on ASCV incidence and most importantly, whether current ASCV risk prediction models can apply to patients with NAFLD. This is of paramount importance in clinical practice where this information would help make decisions to start primary prevention strategies.

Our results of no association between FLI, as a surrogate measure for fatty liver, and all-cause mortality are in line with a recent meta-analysis of seven studies (among which five were cohort studies) (2). Consistently, the relation between FLI and all-cause mortality were explained by adjusting for HOMA-IR in the Cremona Study, (45). On the other hand, LURIC study reported an increased risk of all-cause mortality (46). However, these study included patients who were referred to coronary angiography who might have been already at higher risk of mortality.

In our study, the addition of FLI as a predictor of diabetes risk improved their ability to accurately predict future events. However, FLI was not performing better than HOMA-IR alone. Therefore, in clinical practice, the use of FLI, which contains four clinical measurements such as BMI, WC, GGT and triglycerides, does not provide more accurate information than HOMA-IR itself, calculated based on fasting glucose and insulin levels. The same results were observed for cardiovascular disease. Altogether, the clinical utility of FLI remains limited.

The strengths of our study include its prospective design, the long follow-up up to nearly 20 years with extensive data on mortality and morbidity, the comprehensive adjustment for a broad range of possible confounders and the diversity of the available inflammatory biomarkers. Moreover, this is the first population-based study to investigate the association of FLI with risk of incident ASCV. Some limitations warrant mention. Ultrasonography or biopsy data for the assessment of hepatic steatosis could have helped compare our results and shed light whether the accuracy of the prediction models for ASCV and T2D risk would have been improved. We used only one measurement of FLI, which can change during time and potentially lead to attenuation of the results. However, major components of the index such as BMI and WC are not prone to large variations among the elderly. Although FLI has a sensitivity of 81% as compared to ultrasound measurement of NAFLD, restricting the study population with FLI >=60 yielded similar results for our outcomes. Our population is ≥ 45 years and Caucasians. Therefore, generalization of the results to a younger age category and other ethnicities should be with caution.

Conclusion

We conclude that FLI is associated with increased risk of type 2 diabetes independent of cardio-metabolic risk factors in a healthy population. Other markers of inflammation might further mediate such relation. Our data imply that FLI improve prediction of diabetes events but outperforms compared to HOMA-IR which limits its use in clinical setting.

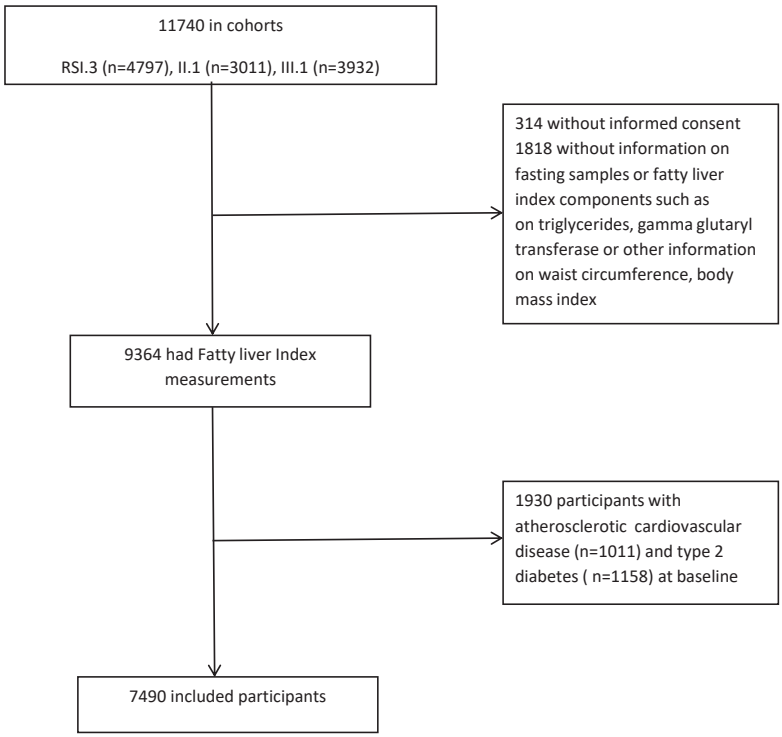

Figure S1. Flow chart for the selection of study participants

Table S1. Association of Fatty Liver Index (FLI) and type 2 diabetes incidence, atherosclerotic cardiovascular disease incidence and all-cause mortality in the Rotterdam Study further adjusting for coffee intake and dietary quality in model 2.

	Model 2	Model 2 +coffee intake and diet quality
	HR (95% CI)	HR (95% CI)
Type 2 Diabetes	**1.98 (1.72, 2.28)**	**2.00 (1.74, 2.3)**
Atherosclerotic Cardiovascular Disease	**1.26 (1.1, 1.44)**	**1.17 (1.02, 1.35)**
All-cause mortality	1.06 (0.97, 1.15)	1.06 (0.97, 1.15)

Abbreviations: HR, hazard ratio; CI, confidence interval;

Table S2. Association of Fatty Liver Index (FLI) and type 2 diabetes incidence, atherosclerotic cardiovascular disease incidence and all-cause mortality in the Rotterdam Study adjusting for waist circumference.

	Model 2: adjusted for waist circumference, age, sex, cohort, smoking, alcohol intake, physical activity, education	Model 3: model 2 further adjusted for treatment for hypertension, systolic blood pressure, statins use, cholesterol levels, HDL, C-reactive protein, HOMA -insulin resistance
	HR (95% CI)	HR (95% CI)
Type 2 Diabetes	**1.97 (1.71, 2.29)**	**1.38 (1.16, 1.64)**
Atherosclerotic Cardiovascular Disease	**1.17 (1.02, 1.34)**	0.98 (0.84, 1.14)

All-cause mortality	0.98 (0.91, 1.07)	0.97 (0.89, 1.07)

Abbreviations: HR, hazard ratio; CI, confidence interval;

Table S3. Association of Fatty Liver Index and type 2 diabetes incidence, atherosclerotic cardiovascular disease incidence and all-cause mortality excluding participants with excessive alcohol intake and excluding participants within the first two years of follow-up (model adjusted for age, sex, cohort, body mass index, smoking, alcohol intake, physical activity, education , treatment for hypertension, systolic blood pressure, statins use, cholesterol levels, HDL, C-reactive protein, HOMA -insulin resistance)

		Excluding alcohol >14 gram/day		Excluding 2 years of follow up
		HR (95% CI)		HR (95% CI)
Type 2 Diabetes	n= 5640, number of events= 538	**1.35 (1.12, 1.63)**	n= 7216, number of events= 591	**1.35 (1.12, 1.63)**
Atherosclerotic Cardiovascular Disease	n= 5646, number of events= 601	1.12 (0.93, 1.35)	n= 7230, number of events= 662	1.07 (0.90, 1.28)
All-cause mortality	n= 3652, number of events= 1807	1.00 (0.90, 1.11)	n= 4676, number of events= 2158	1.06 (0.97, 1.17)

Abbreviations: HR, hazard ratio; CI, confidence interval;

Table S4. Association of FLI with CVD mortality, hard CHD and stroke events in the Rotterdam Study

n= 7490, number of events= 355	
CVD mortality	HR (95% CI)
Model 1	**1.35 (1.11, 1.65)**
Model 2	**1.31 (1.07, 1.61)**
Model 3	1.18 (0.93, 1.5)
n= 7473, number of events= 414	
Hard CHD	HR (95% CI)
Model 1	**1.44 (1.2, 1.73)**
Model 2	**1.38 (1.15, 1.66)**
Model 3	1.06 (0.85, 1.3)
n= 7490, number of events= 454	
Stroke	HR (95% CI)
Model 1	**1.21 (1.01, 1.44)**
Model 2	1.16 (0.97, 1.39)
Model 3	1.05 (0.86, 1.28)

Model 1: age, sex, cohort, body mass index
Model 2: Model 1 + smoking, alcohol intake, physical activity, education
Model 3: Model 2 + treatment for hypertension, systolic blood pressure, statins use, cholesterol levels, HDL, C-reactive protein, HOMA -insulin resistance
Abbreviations: ASCV, atherosclerotic cardiovascular disease; BMI, body mass index; CHD, coronary heart diseases; CRP, C-Reactive Protein; HOMA-IR, Homeostasis Model Assessment for Insulin Resistance; FLI, fatty liver index; HR, hazard ratio; CI, confidence interval; WC, waist circumference.

Table S5. Association of Fatty Liver Index and type 2 diabetes incidence, atherosclerotic cardiovascular disease incidence and all-cause mortality stratified by gender and age (model adjusted for age, sex, cohort, body mass index, smoking, alcohol intake, physical activity, education , treatment for hypertension, systolic blood pressure, statins use, cholesterol levels, HDL, C-reactive protein, HOMA -insulin resistance)

		Incident Type 2 Diabetes		Incident Atherosclerotic Cardiovascular Disease events		Mortality
Strata	Events/Total number	HR (95% CI)	Events/Total number	HR (95% CI)	Events/Total number	HR (95% CI)
Gender						
Women	n= 4460, number of events= 401	**1.46 (1.18, 1.80)**	n= 4465, number of events= 422	1.07 (0.86, 1.33)	n= 2888, number of events= 1307	1.01 (0.89, 1.14)
Men	n= 2987, number of events= 279	**1.35 (1.02, 1.77)**	n= 2988, number of events= 370	1.12 (0.88, 1.42)	n= 1921, number of events= 984	1.13 (0.97, 1.32)
P for interaction		0.4		0.1		0.7
Age						
< 65 years	n= 4410, number of events= 283	**1.59 (1.23, 2.06)**	n= 4413, number of events= 169	1.03 (0.73, 1.44)	n= 1906, number of events= 420	1.03 (0.83, 1.27)
≥ 65 years	n= 3037, number of events= 397	**1.33 (1.07, 1.67)**	n= 3040, number of events= 623	1.11 (0.93, 1.32)	n= 2903, number of events= 1871	1.04 (0.94, 1.16)
P for interaction		0.05		0.6		0.2

Abbreviations: BMI, body mass index; CRP, C-Reactive Protein; HOMA-IR, Homeostasis Model Assessment for Insulin Resistance; FLI, fatty liver index; HR, hazard ratio; CI, confidence interval.

Table S6. Evaluation of c-statistic in diabetes risk model prediction.

Variable	c-statistics (95%CI)	c-statistics changes (95% CI) from base model
Base model 1: age, sex, family history of diabetes, hypertension, BMI, smoking history	0.663 (0.638, 0.688)	
+ FLI	0.689 (0.662, 0.717)	**0.026 (0.013, 0.040)**
+ HOMA-IR	0.706 (0.682, 0.730)	**0.043 (0.028, 0.058)**
+ HOMA-IR*, FLI	0.711 (0.686, 0.736)	*0.005 (-0.002, 0.013)
Base model 2: age, sex, family history of diabetes, BMI	0.649 (0.630, 0.668)	
+ FLI	0.682 (0.656, 0.708)	**0.033 (0.020, 0.046)**
+ HOMA-IR	0.697 (0.677, 0.718)	**0.048 (0.033, 0.064)**
+ HOMA-IR*, FLI	0.705 (0.681 0.728)	*0.007 (-0.001 0.015)

Abbreviations: BMI, body mass index; HOMA-IR, Homeostasis Model Assessment for Insulin Resistance; FLI, fatty liver index; HR, hazard ratio; CI, confidence interval; *Base model + HOMA-IR, compared with the addition of FLI in the model

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CHAPTER 4

Epigenetics of Type 2 Diabetes and Its Risk Factors

Chapter 4.1

The role of global and regional DNA methylation and histone modifications in glycemic traits and type 2 diabetes: a systematic review.

Taulant Muka, MD[1,2*], Jana Nano, MD[1*], Trudy Voortman, PhD[1,2], Kim V.E. Braun, RD, MSc[1], Symen Ligthart, MD [1], Saverio Stranges, MD, PhD[3], Wichor M. Bramer, MSc[4], John Troup, MD, PhD[5], Rajiv Chowdhury, MD, PhD[6], Abbas Dehghan, MD, PhD[1], Oscar H. Franco, MD, PhD[1]

[1] Department of Epidemiology, Erasmus MC, University Medical Center, Rotterdam, the Netherlands.
[2] Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, Mass, USA.
[3] Department of Population Health, Luxembourg Institute of Health, Luxemburg.
[4] Medical Library, Erasmus MC, University Medical Center, Rotterdam, the Netherlands.
[5] Research and Development, Metagenics, Inc.
[6] Department of Public Health & Primary Care, Cardiovascular Epidemiology Unit, University of Cambridge, Cambridge, CB1 8RN, United Kingdom.
* Authors contributed equally

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ABSTRACT

Background

New evidence suggests the potential involvement of epigenetic mechanisms in type 2 diabetes (T2D) as a crucial interface between the effects of genetic predisposition and environmental influences.

Aim

To systematically review studies investigating the association between epigenetic marks (DNA methylation and histone modifications) with T2D and glycemic traits (glucose and insulin levels, insulin resistance measured by HOMA-IR).

Method

Six bibliographic databases (Embase.com, Medline (Ovid), Web-of-Science, PubMed, Cochrane Central and Google Scholar) were screened until 28th August 2015. We included randomized controlled trials, cohort, case-control and cross-sectional studies in humans that examined the association between epigenetic marks (global, candidate or genome-wide methylation of DNA and histone modifications) with T2D, glucose and insulin levels and insulin metabolism.

Results

Of the initially identified 3,879 references, 53 articles, based on 47 unique studies met our inclusion criteria. Overall, data were available on 10,823 participants, with a total of 3,358 T2D cases. There was no consistent evidence for an association between global DNA-methylation with T2D, glucose, insulin and insulin resistance. The studies reported epigenetic regulation of several candidate genes for diabetes susceptibility in blood cells, muscle, adipose tissue and placenta to be related with T2D without any general overlap between them. Histone modifications in relation to T2D were reported only in 3 observational studies.

Conclusions and relevance

Current evidence supports an association between epigenetic marks and T2D. However, overall evidence is limited, highlighting the need for further larger-scale and prospective investigations to establish whether epigenetic marks may influence the risk of developing T2D.

INTRODUCTION

Type 2 diabetes mellitus (T2D) and its complications constitute a major and growing health problem worldwide, necessitating the delineation of underlying pathogenic mechanisms [1, 2]. A better understanding of factors contributing to development of T2D is essential to improve prevention and treatment strategies [3]. Physical activity, obesity, diet, and aging, account for the most important non-genetic (environmental) risk factors [4, 5]. Ample evidence emphasizes the contribution of genetics in the pathophysiology of diabetes as well. Candidate gene and genome-wide association studies have identified approximately 153 single-nucleotide polymorphism (SNP) across human genome that explain only a minor fraction of the inter-individual variation in the susceptibility for T2D [6]. However, the causative polymorphisms are still unknown challenging the translation of genetic information into clinical practice [7].

T2D is the result of a combination of genetic and environmental factors playing a crucial role in etiological processes [8]. Even though both genetic and environmental can be assessed, the informative value in predicting disease or unraveling underlying biological mechanism remains restricted. The role of epigenetic determinants are increasingly being recognized as a potential important link between environmental exposure and disease risk and thus may be a benchmark to capture both their influences [9]. In contrast to genetic modifications, which lead to a change in the base sequence of DNA, epigenetic changes are relatively susceptible to modifications by the environment as well as dysregulation over time. Epigenetics refers to information transmitted during mitosis or meiosis that is very important in gene function. DNA methylation, histone modification, and non-coding RNA are three major types of epigenetic marks [10]. DNA methylation refers to the addition of a methyl group to cytosine at CpG dinucleotides that further influences the function of DNA: activating or repressing the transcriptional activity of a gene. Posttranslational histone modifications are another type of epigenetic mark that affect gene expression mainly by altering chromatin structure. Noncoding RNAs have recently emerged as important regulators of gene expression [11]. The genome-wide distribution of these marks and regulators refers as "epigenome" [12].

Following the advent of epigenome investigations, many array-based methods are now opening new windows to further identify epigenetics marks on genome scale related to complex diseases. Currently, Illumina Infinium Methylation450 bead Chip is one of the most widely used platforms praised for its cost-effectiveness, high number of sites and overall good accuracy [13]. Although providing easily reproducible data across studies, deep sequencing technologies are another emerging field that despite drawback of high costs, offer a wider exploration of the genome [14]. Methods investigating site or regions differentially methylated as opposed to genome-wide approaches are continually being used to overcome issues associated with multiple testing corrections. Such example is the bisulfite pyrosequencing, which is based on sequencing-by-syn-

thesis methodology and is relatively cost- and time- effective, mostly suitable for DNA methylation analysis of single gene loci [15]. Despite the developments in technology to characterize DNA methylation, the interplay of genes and environment in complex disease such as diabetes is complicated and still remains poorly understood.

The aim of this paper is to systematically review the current body of evidence on the role of epigenetic marks and T2D and glycemic traits including glucose levels, insulin levels and insulin resistance index (HOMA) specifically focusing on human studies. Studies investigating the association between major types of epigenetics signatures including global DNA methylation, site-specific or genome-wide DNA methylation and histone modification with type 2 diabetes and glycemic traits are summarized and further discussed. Noncoding RNAs were not addressed in this review. A critical appraisal of current limitations in the field is also presented.

METHODS

Studies identified by search strategy

Six bibliographic databases (Embase.com, Medline (Ovid), Web-of-Science, PubMed, Cochrane Central and Google Scholar) were searched to identify relevant studies published from conception until 28th August 2015 (described in detail in the method section in the Online Supplemental Material). A total of 3,879 potentially relevant references were retrieved from electronic searches (Figure 1). Based on the title and abstracts, full texts of 65 articles were selected for detailed evaluation. In the full-text assessment, 53 of these articles, based on 47 unique studies met our eligibility criteria and were therefore included in this review.

Note: Supplementary Material/Appendix can be found in the website of the published journal or can be provided on request.

RESULTS

Characteristics of the included studies

Characteristic details of the included studies are summarised in **Supplementary Table 1**. The majority of studies were performed among Chinese, Swedish and American populations and mostly assessed epigenetic signatures in blood. Overall, 10,823 unique participants were included in the analysis, with a total of 3,358 T2D cases. All available studies, except two that were longitudinal studies, had a cross-sectional or case control design and were judged to be of low or medium quality (*methods used to assess the quality of the included studies are described in details in the method section in the Online Supplemental Material*). Given the heterogeneous nature of the study characteristics, exposures evaluated and outcomes, we did not perform a quantitative summarization

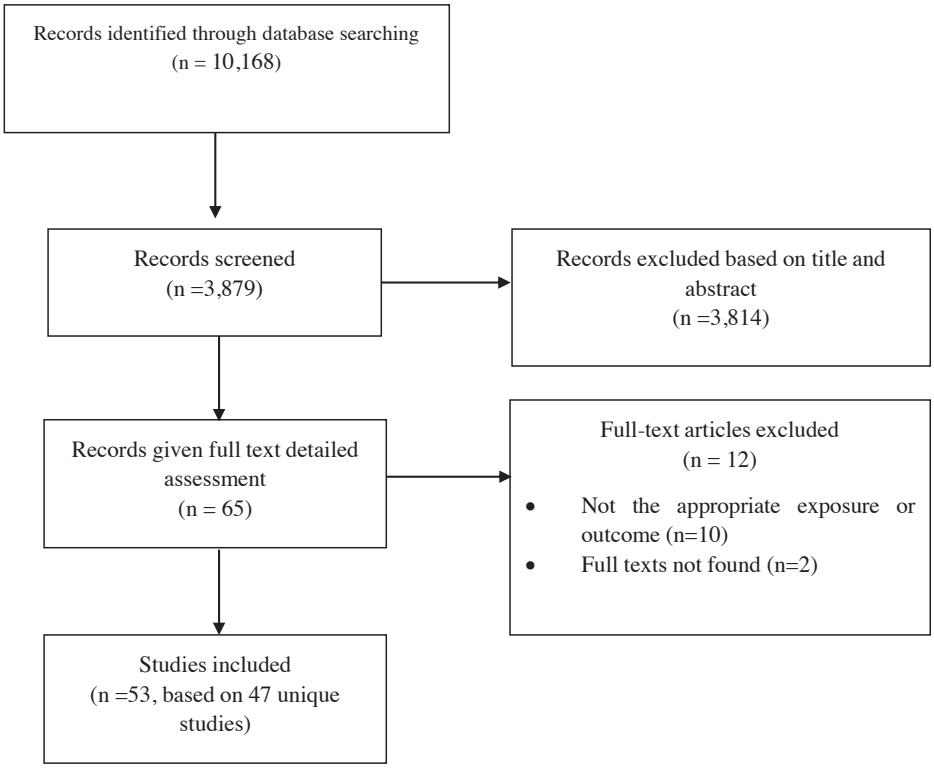

Figure 1. Flowchart of studies included in the systematic review.

of the studies included in this review. Instead, a narrative synthesis and construction of descriptive summary tables was done. A schematic representation of the effect of epigenetic alterations and summary of the results are depicted in **Figure 2.**

Global DNA Methylation, Type 2 Diabetes and Glycemic Traits

Global methylation refers to the overall level of methylcytosine in the genome, expressed as percentage of total cytosine. A large portion of methylation sites within the genome are found in repeat sequences and transposable elements, such as Alu and long-interspersed nuclear element (LINE-1) and correlate with total genomic methylation content [16, 17]. Such elements have served as a useful proxy for global DNA methylation as they are commonly heavily methylated in normal tissue and are spread ubiquitously throughout the genome [18, 19]. Other methods (e.g., Luminometric Methylation Assay, LUMA and the [^{3}H]-methyl acceptance

based method) that assess global genomic DNA methylation are primarily based on the digestion of genomic DNA by restriction enzymes such as HpaII and MspI [20].

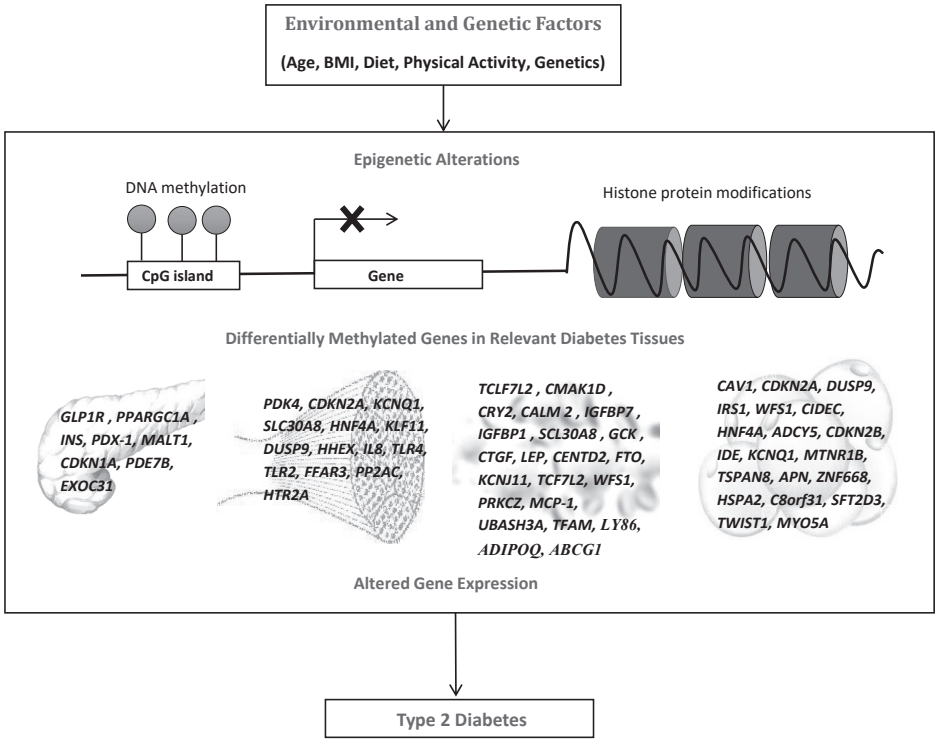

Figure 2. Schematic model illustrating how altered epigenetic marks from the influence of environmental and genetic factors may contribute to risk of type 2 diabetes. Altered DNA methylation and expression patterns are specific to tissue.

Type 2 diabetes

Eight studies [21-28] examined the association between global DNA methylation and T2D (Table 1). Five studies assessed DNA methylation in blood cells whereas the other investigations were performed in skeletal muscle, adipose tissue and pancreatic islets. Six studies reported no association between global DNA-methylation and T2D, whereas one study showed lower levels of global DNA-methylation in T2D cases compared to healthy controls [21] (Table 4). One study reported cell-specific global DNA methylation patterns: in T2D patients, compared to controls, there was an increased degree of global DNA-methylation in B-cells and natural killer cells, but no difference was found overall in peripheral blood (PB) mononuclear cells, lymphocyte cells and monocytes [25].

Glycemic Traits

Six studies examined the association between global DNA-methylation and plasma glucose (5 studies assessed fasting plasma glucose levels [21, 25, 28-30] and 1 study plasma glucose 2 hours after the oral glucose tolerance test [24]) (**Table 1**). Studies did not report consistent associations between global DNA methylation and glucose levels.

Table 1. Global DNA methylation in type 2 diabetes and glycemic traits(glucose levels, insulin levels and HOMA-IR.

Tissue type	Population	Result, reference	Outcome (nr of cases)
LINE-1 methylation			
WB	M and F, n=56	No association [1]	T2D (24)
Skeletal muscle and SAT	M and F, n=24	No association [2]	T2D (12)
PI	Male, n=16	No association [3]	T2D (5)
Lymphocytes	F, n=173	Positive association [4]	Fasting glucose levels
Skeletal muscle and subcutaneous adipose tissue	M and F, n=24	Positive association [2]	2-h plasma glucose levels
PB	M and F, n=228	Positive association [5]	Fasting glucose levels
Lymphocytes	F, n=173	No association [4]	Fasting insulin levels
PB	M and F, n=228	No association [5]	Insulin secretion
PBMC	F, n=470	No association [6]†	HOMA-IR
5mdC			
PB	M and F, n=104	No association [7]	T2D (66)
PBMCs	M, n=44	No association [8]*	T2D (12)
Lymphocyte B and Natural Killer cells	M, n=44	No association [8]*	Fasting glucose levels
Lymphocyte B	M, n=44	Positive association [8]	Fasting insulin levels
Natural Killer cells		No association [8]*	
Mathylcytosine/cytosine ratio			
PBL	M and F, n=738	Hypomethylated [9]	T2D (286)
PBL	M and F, n=738	Negative association [9]	Fasting glucose levels
HpaII/MspI ratio			
PB	M and F, n=44	No association [10]	T2D (26)
Abdominal Adipose tissue (subcutaneous and visceral fat)	M and F, n=51	No association [11] **	T2D (13)
LUMA			
Abdominal Adipose tissue (subcutaneous and visceral fat) and leucocyte	M and F, n=51+509‡	No association [11]	Fasting glucose levels
Abdominal Adipose tissue (subcutaneous and visceral fat) and leucocyte	M and F, n=51+509‡	No association [11]	Fasting insulin levels
Alu			
PBL	M, n=168	Positive association [12]	HOMA-IR

5mdc, 5 methyl deoxy cytidine; F, female; LINE-1, long interspersed nuclear element 1; LUMA, Luminometric-based assay; M, male; PB, Peripheral blood; PBL, peripheral blood leucocytes; PBMCs, Peripheral blood mononuclear cells; PI, pancreatic islets; SAT, subcutaneous adipose tissue; T2D, type 2 diabetes; WB, whole blood

*No difference in global DNA methylation in PBMCs (comprising both lymphocytes and monocytes), or in the lymphocyte or monocyte populations assessed separately was found between T2D cases and non-T2D controls. However, in T2D patients, compared to controls, there was an increased methylation levels in B (p=0.022) and natural killer cells (p=0.004) but not in T helper (p>0.05) and T cytotoxic cells (p>0.05).

**Assessed by LUMA method

†Significant interaction with circulating folate concentrations were observed and a lower degree of methylation and lower plasma folate concentrations were associated with higher HOMA-IR: OR=1.78, 95%CIs: 1.02-3.13, P=0.041)

‡The study included two study populations, one investigating global methylation in the adipose tissue (n=51) and in the other assessing global methylation in leucocyte (n=509).

Table 2. DNA methylation and type 2 diabetes: candidate gene and epigenome-wide approaches.

Author, Publication year	No. of cases	Tissue type	Population	Methylation sites/method	Main findings
Ling C. et al 2008 [13]	10	PI	M and F, n=19	*PPARGC1A* gene, 4 target sites/ EZ DNA Methylation Kit	*PPARGC1A* promoter gene showed a two fold increase in DNA methylation levels in diabetic islets compared with non-diabetic islets *(P <0.04)*
Olsson A. et al. 2011[14]	9	PI	M and F, n=64	*NDUFA5* (6 CpG sites), *COX11* (12 CpG sites), *ATP6V1H* (16 CpG sites) genes / EZ DNA Methylation Kit, Sequenom EpiTyper MassARRAY	None of the CpG sites in the three genes showed differences in DNA methylation
Yang B.T. et al. 2011[15]	9	PI	M and F, n=57	Insulin promoter gene, 25 CpG sites/ Sequenom EpiTyper MassARRAY	Four CpG sites showed increased DNA methylation in islets from patients with T2D compared to controls (p=0.03 for each of the CpGs).
Kulkarni S. et al 2012 [16]	33	Skeletal muscle	M and F, n=112	*PDK4* gene/ bisulphite sequencing	Methylation levels of the PDK4 promotor were lower in T2D patients.
Liu Z.H. et al 2012 [17]	32	PBMCs	M and F, n=47	MCP-1 gene/EpiTect Bisulfite Kit	Methylation of the MCP-1 gene was reduced in T2D patients.
Ribel-Madsen R. et al 2012 [2]	12	Skeletal muscle and SAT	M and F, n=24	49 susceptibility genes for mono- or polygenetic T2D, 136 CpG/ Bisulphite sequencing	25 CpG sites located in 17 genes were differentially methylated between T2D twins and non-T2D twins (*CDKN2A, DUSP9, HNF4A, HHEX, KCJNQ1, KLF11, PPARGC1A* and *SLC30A8* in muscle and *ACY5, CAV1, CIDEC, CDKN21, CDKN2B, DUSP9, HNF4A, IDE, IRS1, KCNQ1, MTNR1B, TSPAN8* and *WFS1* in subcutaneous adipose tissue; Two CpG sites in adipose tissue (*CDKN2A* and *HNF4A*) remained significant after adjustment for multiple testing- P < 0.02) • Hypomethylated genes in muscle: *CDKN2A*, *KCNQ1* and *SLC30A8* • Hypomethylated genes in subcutanues adipose tissue: *CAV1*, *CDKN2A, DUSP9, IRS1* and *WFS1*. • Hypermethylated genes in muscle: *HNF4A, KLF11, DUSP9, HHEX and PPARGC1A*. Hypomethylated genes in subcutanues adipose tissue: *CIDEC, HNF4A, ADCY5, CDKN2B, IDE, KCNQ1, MTNR1B* and *TSPAN8*

Table 2. (continued)

Author, Publication year	No. of cases	Tissue type	Population	Methylation sites/method	Main findings
Yang B.T. et al. 2012 [18]	9	PI	M and F, n=64	*PDX-1* gene / 12 CpG and 14 CpG sites for the promoter and enhance region respectively/ Pyrosequencing and Sequenom EpiTyper MassARRAY	There was no difference in DNA methylation of CpG sites located in the proximal promoter region close to the PDX-1 transcription start between T2D and non-T2 subjects (1.9 vs. 2.0, P =0.57). 10 of the analyzed CpG sites in the distal promoter region and the enhancer region of *PDX-1* showed increased DNA methylation in T2D compared to non-T2D subjects (the absolute increase in the degree of DNA methylation in T2D islets ranged between 6.2 to 18.0% for the analyzed regions, representing fold changes between 1.15 and 1.47).
Yang M. et al. 2012 [19]	178	PB		Leptin gene/ methylation-specific polymerase chain reaction	The methylation rates of leptin gene in T2D group were higher compared to normoglycemic subjects (P < 0.01)
Zou L. et al. 2012 [20]	152	PBL	M and F, n=272	*PRKCZ*, 9CpG sites/ bisulfite sequencing	7 out of 9 CpG sites were hypermethylated in T2D cases compared to non-T2D subjects (P < 0.01 for 6 CpGs and P < 0.05 for one CpG).
Canivelli S. et al. 2013 [21]	93	PB	M and F, n=186	*GIPR* gene, 13 CpG sites/ Sequenom EpiTyper MassARRAY	9 out of 13 CpG sites showed a significant trend toward hypomethylation in T2D patents as compared to controls (P < 0.004). Mean *GIPR* promoter methylation was lower in T2D patients as compared to controls.
Gillberg L. et al. 2013 [22]	25	Skeletal Muscle	F, n=124	*PPARGC1A* gene / bisulfite pyrosequencing	There was no difference in DNA methylation between T2D-cases and normal glucose subjects.
Gu H.F. et al. 2013 [23]	100	PB	M and F, n=340	*IGFBP7* gene, 3 CpGs/bisulphite sequencing	DNA methylation levels were significantly increased in newly diagnosed T2D patients compared with non-T2D patients at all the three CPG sites. Combined data from all three CpG sites showed that genomic DNA methylation levels of the *IGFBP7* gene in T2D were increased.
Gu T. et al. 2013 [24]	164	PB	M, n=402	*IGFBP1* gene, 6 CpGs/bisulphite pyrosequencing	DNA methylation of the 6 CpG sites were significantly higher in T2D patients compared with those in non-T2D subjects (P < 0.001)
Hall E. et al 2013 [25]	10	PI	M and F, n=65	*GLP1R* gene,12 CpG sites / Sequenom EpiTyper MassARRAY	One CpG site showed a small increase in DNA methylation in islets from donors with T2D compared to non-diabetic donors (P = 0.02, but did not persist after correction for multiple testing).

Table 2. (continued)

Author, Publication year	No. of cases	Tissue type	Population	Methylation sites/method	Main findings
Ma J. et al 2013 [26]	48	PB	M and F, n=96	*IRS-1* gene, 3 CpG sites/ bisulphite pyrosequencing technology	No difference in methylation.
Canivelli S. et al. 2014 [27]	93	PB	M and F, n=186	*TCLF7L2* gene, 22 CpG sites/ Sequenom EpiTyper MassARRAY	14 out of 13 CpG showed significant differences in DNA methylation values between T2D patents as compared to controls ($P < 0.05$). After adjustment for multiple testing, only 13 CpGs remained significant.
Cheng J. et al. 2014 [28]	48	PB	M and F, n=96	*CMAK1D* (9 CpGs), *CRY2* (5 CpGs) and *CALM 2* (4 CpGs) genes, bisulphite sequencing	The promoters of the three genes in the PB exhibited low methylation levels for T2D compared to controls ($P < 005$).
Remely M. et al. 2014 [29]	24	WB	M and F, n=56	*TLR4, TLR2,* 4 and 7 CpG sites respectively/ bisulfite pyrosequencing	The mean methylation of 4 the CpGs (and of each CpG) in the first exon of *TLR4* gene in the obese group were lower compared to T2D subjects whereas no difference in methylation was observed between T2D cases and lean controls ($P =0.95$). Lower methylation levels of seven CpGs (and of each single CpG) in the promoter region of *TLR2* were observed in T2D subjects compared to lean subjects but no difference in methylation with obese controls was shown to obese.
Remely M. et al. 2014 [1]	24	WB	M and F, n=56	*FFAR3*/ bisulfite pyrosequencing	There was significant lower methylation in T2D subjects compared to lean mass subjects($P = 0.003$)
Simar D. et al. 2014 [8]	12	B-cells	M, n=44	*GAPDH, TFAM, TRIM3, UBASH3A* genes/bisulphite sequencing	In the region tested, only few cytosine residues were found differentially methylated, notably cytosine -235 relative to the transcription start site of the *UBASH3A* gene, which was significantly hypomethylated in T2D compared to controls ($P < 0.05$).
Tang L. et al 2014 [30]	96	PB	M and F, n=96	*GCK* gene/EpiTech Bisulfite Kits	Significant elevation of methylation levels of *GCK* CpG4 were observed in T2D patients than in the healthy controls ($P=0.004$). This association was specific to males ($P=0.002$)
Tang L. et al 2014 [31]	96	PB	M and F, n=96	*BCL11A* gene/EpiTech Bisulfite kit	No difference in methylation was observed.
Tros F. et al 2014 [32]	48	WB	M and F, n=95	*Pp2ac*/ bisulfite pyrosequencing	The gene was significantly demethylated in T2D patients compared to controls.

Table 2. (continued)

Author, Publication year	No. of cases	Tissue type	Population	Methylation sites/method	Main findings
Zhang H. et al. 2014 [7]	66	PB	M and F, n=104	*CTGF*/ bisulfite pyrosequencing	The *CTGF* promoters in individuals in the non-T2D (82.9%) group showed significantly higher methylation levels than those in patients in the diabetes with (31.6%) and without nephropathy (43.2%) ($P < 0.05$). *CTGF* promoter methylation level was 72.2 ± 19.3 in the non-T2D group, which was significantly higher than in the T2D without nephropathy (49.2 ± 8.0%, P=0.045) and with nephropathy (22.0 ± 12.9%, $P < 0.001$).
Jun Z. et al. 2015 [33]	26	Adipose tissue	M and F, n=124	*APN* promoter/ Denaturing high performance liquid chromatography	Methylation positive rate was the lowest in control, middle in obesity and highest in T2D group and the differences were statistically significant ($P < 0.05$).
Seman N.A. et al. 2015 [34]	516	PB	M and F, n=992	*SLC30A8* gene promoter, 6 CpG sites/ Bisulfite treatment and pyrosequencing	DNA methylation levels at five CpG sites of the gene (except CpG2) in T2D patients were found to be higher than those in NGT subjects, respectively (CpG1 83.9% vs. 81.9%, P = 0.031; CpG3 82.1% vs. 84.8%, P = 0.003; CpG4 69.6% vs. 66.3%, P = 0.001; CpG5 86.2% vs. 83.7%, P = 0.004; and CpG6 79.8% vs. 78.1%, P = 0.001). Combining all six CpG sites together, total mean values of *SLC30A8* DNA methylation levels were significantly increased in T2D patients compared with NGT subjects (82.9%, 95% CI = 79.2% to 80.5% vs. 80.1%, 95% CI = 75.4% to 78.6%, P = 0.014).

Table 2. (continued)

Epigenome wide approaches						
Author, Publication year	**Tissue**	**Discovery sample**	**Validation sample**	**Measurement**	**Correction for multiple tests**	**Main finding**
Toperoff G. et al. 2012 [35]	PB	M and F, n= 1169	NA	Affymetrix SNP6 microarray In-deep sequencing: 454 FLX Titanium genome sequencer/ PyroMark Q24 bench-top sequencer	Benjamini and Hochberg procedure /q-values	Pool-based genome-scale screen revealed an excess of differentially methylated site in genomic regions previously associated with T2D from genetic studies. Further ultra deep sequencing was performed at selected tip-ranking regions. 13 out of 93 CpG sites showed cace-control differences. A CpG site in the first intron of the FTO gene showed small but significant (P = 0.000021) hypomethylation of cases relative to controls.
Ribel-Madsen R. et al 2012 [2]	SM and SAT	M and F, n=24	NA	HumanMethylation 27 BeadChip	Permutation correction for multiple testing (P adj=0.02)	One CpG site in muscle (*IL8*) and 7 sites in SAT (*ZNF668, HSPA2, C8orf31, CD320, SFT2D3, TWIST1, MYO5A*) were displaying differentially methylated sites.
Volkmar M.et al 2012 [36]	PI	M, n=16	NA	HumanMethylation 27 BeadChip	15% group-wise difference of methylation levels was set as a cutoff additional to P Mann–Whitney <0.01	276 CpG loci affiliated to promotors of 254 genes displaying differential DNA methylation in diabetic islets
Dayeh T. et al. 2014 [37]	PI	M and F, n=49	NA	HumanMethylation 450 K BeadChip	FDR < 5%	1,649 CpG sites with differential DNA methylation (q<0.05 and difference in DNA methylation ≥5%) in pancreatic islets from 34 non-diabetic versus 15 T2D human donors.
Yuan W. et al. 2014 [38]	WB	M and F, n= 27 twin pairs	M and F, n=263	MeDIP-seq	FDR <5%	The strongest signal is in the promoter of the MALT1 (mucosa-associated lymphoid tissue lymphoma translocation protein 1) gene.
Kulkarni H. et al 2015 [39]	WB	M and F, n= 850	NA	HumanMethylation 450 BeadChip	FDR <5%	51 CpG sites were significantly associated with T2D.

Epigenome wide approaches						
Author, Publication year	**Tissue**	**Discovery sample**	**Validation sample**	**Measurement**	**Correction for multiple tests**	**Main finding**
Chambers J. et al 2015 [40]	WB	M and F, n= 1074 incident cases	M and F, n=377 incident cases	HumanMethylation 450 K BeadChip	$P < 5 \times 10^{-7}$	Methylation markers at five loci (*ABCG1, PHOPHO1, SOCS3, SREBF1,TXNIP*) were associated with incident T2D.

F, female; FDR, false discovery rate; M, male; P, p-value; PB, Peripheral blood; PBL, peripheral blood leucocytes; PBMCs, Peripheral blood mononuclear cells; T2D, type 2 diabetes; PI, pancreatic islets; WB, whole blood; SAT, subcutaneous adipose tissue; * longitudinal study

Three studies showed increased degree of methylation in LINE-1 elements assessed in blood, skeletal muscle and adipose tissue with increasing levels of plasma glucose [24, 29, 30]. Three studies used other methods to assess global DNA methylation [25, 28] reporting either no association [21] or inverse relationship (**Table 1**).

Insulin metabolism and global DNA methylation was investigated in six studies. Three studies examined fasting plasma insulin levels [25, 28, 29], one study insulin secretion [30] and two studies insulin resistance [31, 32] (**Table 1**). Three studies assessed global DNA-methylation in LINE-1 elements in blood cells and showed no association with insulin (**Table 1**). Similarly, there was no association with global methylation assessed in adipose abdominal tissue by LUMA method and fasting insulin [28]. However, one study reported an interaction of global DNA-methylation with circulating folate concentrations in relation to insulin resistance and showed that a lower degree of methylation and lower plasma folate concentrations were associated with higher insulin resistance (odds ratio = 1.78; 95% Confidence Intervals: 1.02- 3.13) [31]. One study assessed global DNA methylation as a percentage of 2′-deoxycytidine plus 5mdC in genomic DNA and showed a positive association between insulin levels and global DNA-methylation assessed in lymphocyte B cells, but no association in natural killer cells [25]. Another study assessed global DNA-methylation in Alu elements and reported a positive association with insulin resistance [32].

Gene Specific DNA Methylation, Type 2 Diabetes and Glycemic Traits

Investigating gene-specific DNA methylation is one of the most widely studied approaches in epigenetics because it allows a relatively simple analysis. However, how these epigenetic marks regulate gene expression is not deeply entangled. The association of DNA methylation with gene expression depends on where within the gene sequence the methylation occurs. DNA methylation in the promoter region of the gene down-regulates its expression whereas higher methylation in the gene-body promotes the expression of the gene [33]. Most of the studies included in our review have used a hypotheses-driven and few studies used a candidate gene approach.

Candidate Gene Studies

Type 2 Diabetes

There were 25 unique studies that examined methylation sites in or near known candidate genes for diabetes susceptibility (Table 2). Overall, the candidate gene studies showed that T2D cases compared to controls, have higher degree of methylation of IGFNP7, IGFBP1, TLR2, SLC3OA8, GCK, PRCKZ, CTGF and leptin gene in PB cells; PPARGC1A, PDX-1, insulin promoter gene and GLP1R gene in pancreatic islets and APN gene in the adipose tissue. Lower methylation levels were observed for GIPR, CMAK1D, CRY2, CALM2, MCP1, TLR4, FFAR3, PP2AC and CTGF gene in the PB cells; UBASH3A gene in B-cells and PDK4

gene in the skeletal muscle. Five studies found no difference or clear pattern in methylation of the following genes: TCLF7L2 and IRS-1 in the PB; GADPH, TFAM and TRIM3 in B-cells; GLP1R in pancreatic islets and PPARGC1A in the skeletal muscle. One study [24] that examined 49 (135 CpG sites) known susceptibility genes for mono- or polygenetic diabetes found 25 CpG sites located in 17 genes to be differentially methylated between diabetes twins and non-diabetes twins (muscle tissue: hypermethylated in T2D - HNF4A, KLF11, DUSP9, HHEX and PPARGC1A; hypomethylated - CDKN2A, KCNQ1 and SLC30A8); in subcutaneous adipose tissue: hypermethylated: CIDEC, HNF4A, ADCY5, CDKN2B, IDE, KCNQ1, MTNR1B and TSPAN8; hypomethylated: CAV1, CDKN2A, DUSP9, IRS1 and WFS1. However, only two CpG sites in adipose tissue (CDKN2A and HNF4A) remained significant after adjustment for multiple testing [24].

Glycemic traits

There were eight investigations that examined methylation sites in or near known candidate genes in relation to fasting plasma glucose and glucose 2 hours after the oral glucose tolerance (Table 3). Overall, these studies found higher degree of methylation of L286 gene in the PB and lower levels of methylation of GIPR gene and PPARGC1A gene in the PB and skeletal muscle respectively with increasing levels of plasma glucose. Two studies did not show any association between DNA methylation levels of LEP and ADIPOQ [34] in PB or GLUT4 [28] in abdominal adipose tissue and fasting glucose. Three unique studies [35-38] included pregnant women and found negative correlations between glucose concentrations and methylation levels of ADIPOQ, LPL, IGF1R and IGFBP3 in placenta tissue, and a positive association between plasma glucose and ABCA1 gene methylation in placenta, whereas no association was found for INSR and IGF1.

There were 14 studies that examined methylation sites in or near known candidate genes in relation to plasma insulin, insulin expression and insulin resistance (Table 3). These studies found a positive correlation between plasma insulin and methylation at PPARGC1A gene in liver, HTR2A and LY86 genes in blood cells and lower levels of methylation of PPARGC1A gene in skeletal muscle and of insulin promoter gene with increased levels of plasma insulin or mRNA insulin expression. Also, inverse associations were found between insulin resistance and the degree of methylation of TFAM and GIPR3 genes in blood cells and PPARGC1A gene in skeletal muscle. Two studies included pregnant women and reported methylation levels of the maternal side of placenta of ADIPOQ negatively correlated with insulin resistance [37] and methylation frequency of IGFBP3 positively correlated with fasting insulin levels and insulin resistance [38]. No association was found between DNA methylation of IGF1R in placenta tissue and fasting insulin or insulin resistance [38]. Collectively, these studies provide evidence that T2D and glycemic traits are associated with altered epigenetic regulation of a number of metabolically important genes.

Table 3. DNA methylation and glycemic traits (glucose levels, insulin levels and HOMA-IR): candidate and epigenome-wide approaches.

Author, Publication year	Phenotype	Tissue type	Population	Methylation sites/ method	Main findings
Sookioan S. et al.2010 [41]	Fasting Insulin	Liver	M and F, n=39	Promoters of *PPARGC1A* (peroxisome proliferator-activated receptor y activator 1α)	Liver *PPARGC1A* promoter methylated DNA/unmethylated DNA ratio was significantly correlated with plasma fasting insulin (Person correlation r=0.51, P <0.01).
				Promoter *TFAM* (mitochondrial transcription factor A), 3 CpG sites/ Bisulphite sequencing	Liver *TFAM* promoter methylated DNA/unmethylated DNA ration was inversely associated with fasting insulin levels (r=-0.49,, P <0.04).
Gemma C. et al. 2010 [42]	Fasting Insulin HOMA-IR	WB	M and F, n=122	*TFAM* gene promotor/ bisulfite pyrosequencing	The status of the promoter methylation of *TFAM* correlated negatively with fasting insulin (r:-0.26, P <0.004) and HOMA-IR ((r:-0.27, P <0.002)
Yang B.T. et al. 2011[15]	Insulin mRNA expression	PI	M and F, n=57	Insulin promoter gene, 25 CpG sites/ Sequenom EpiTyper MassARRAY	9 out of 25 CpGs in the insulin promoter gene were inversely correlated with insulin mRNA expression (Spearman correlation coefficient varied from -0.70 to -0.32, P <0.01)
Bouchard L. et al. 2012 [43]	2-h plasma glucose	Placenta Tissue	F, n=98	*ADIPOQ* gene/ Sequenom EpiTYPER system	Lower DNA methylation levels on the fetal side of the placenta were associated with higher maternal 2-h post oral glucose tolerance test levels during pregnancy ((r < -0.21, P < 0.05). Lower DNA methylation levels on the maternal side of the placenta were not associated with 2h post-oral glucose tolerance test levels.
	HOMA-IR				Lower DNA methylation levels on the maternal side of the placenta were associated with higher insulin resistance index during pregnancy (r =< -0.27, P < 0.05). No association was observed between DNA methylation at the fetal side and HOMA-IR
Canivelli S. et al. 2013 [21]	Fasting glucose	PB	M and F, n=186	*GIPR* gene, 13 CpG sites/ Sequenom EpiTyper MassARRAY	Lower levels of methylation at *GIPR* promoter gene were associated with higher levels of fasting glucose.
	HOMA-IR				Lower levels of methylation at *GIPR* promoter gene were associated with higher HOMA-IR.
Gillberg L. et al. 2013 [22]	Fasting glucose	Skeletal Muscle	F, n=124	*PPARGC1A* gene, 4CpG sites / bisulfite pyrosequencing.	The average *PPARGC1A* methylation at four CpG sites situated 867-624 bp from the transcription start was borderline negatively correlated with fasting glucose (β=-3.86, 95%CIs=-7.8;0.07), P = 0.05)

Table 3. (continued)

Author, Publication year	Phenotype	Tissue type	Population	Methylation sites/ method	Main findings
	Fasting Insulin (and insulin sensitivity)				The average *PPARGC1A* methylation at four CpG sites situated 867-624 bp from the transcription start was borderline negatively correlated with fasting insulin (β=-0.83, 95%CIs=-1.64;-0.02), P = 0.05). Out of the four CpG sites, one site was associated with whole-body insulin sensitivity (b =0.12; P = 0.03).
	HOMA-IR				The average *PPARGC1A* methylation at four CpG sites situated 867-624 bp from the transcription start was borderline negatively correlated with HOMA-IR (β=-1.65, 95%CIs=-3.02;-0.27), P = 0.02)
Houde A. et al. 2013 [44]	2-h plasma glucose	Placenta tissue and cord blood	F, n=100	*ABCA1* gene/ bisulfite pyrosequencing	DNA methylation levels on the maternal side of the placenta were positively correlated with maternal glucose 2h post oral glucose tolerance test (r=0.25; P =0.02). Cord blood DNA methylation levels were negatively correlated with maternal glucose 2h post oral glucose tolerance test (r =-0.24; P =0.03).
Canivelli S. et al. 2013 [21]	Fasting Insulin (and insulin sensitivity)	Skeletal Muscle	F, n=124	*PPARGC1A* gene, 4CpG sites / bisulfite pyrosequencing.	The average *PPARGC1A* methylation at four CpG sites situated 867-624 bp from the transcription start was borderline negatively correlated with fasting insulin (β =-0.83, 95%CIs=-1.64;-0.02), P =0.05) . Out of the four CpG sites, one site was associated with whole-body insulin sensitivity (β =0.12; P =0.03).
Wang X. et al. 2014 [30]	Fasting Glucose	PB	M and F, n=703	*LY86* gene, 6 CpG sites/ Bisulphie pyerosequencing	DNA methylation level of *LY86* gene was positively associated with fasting glucose (partial r =0.082, P =0.04)
	Fasting Insulin (and insulin sensitivity)				DNA methylation level of *LY86* gene was positively associated with fasting insulin (partial r=0.086, P =0.03)
					DNA methylation level of *LY86* gene was inversely associated with insulin sensitivity (partial r=-0.091, P =0.02)
Houde A. et al. 2014 [45]	2-h plasma glucose	Placenta tissue and cord blood	F, n=126	*LPL* gene/ bisulfite pyrosequencing	Placental DNA methylation levels at *LPL*-CpG1 and CpG1 were negatively correlated with maternal glucose

Table 3. (continued)

Author, Publication year	Phenotype	Tissue type	Population	Methylation sites/ method	Main findings
Desgagne V. et al. 2014 [46]	Impaired glucose tolerance. 2-h plasma glucose and fasting glucose	Placenta tissue	F, n=170	*IGF1R* (35CpGs), *IGFBP3* (6 CpGs), *INSR* (25 CpGs) and *IGF1* (11 CpGs)/ bisulfite pyrosequencing	*IGF1R*-L1 to L5 CpGs and *IGFBP3*-L1 were found significantly hypomethylated in placentas exposed to IGT (initial sample, n=140) and only IGF1R-L4 was replicated in another set (n=30). No difference was found for the other regions studied. *IGF1R*-L4 (7 CpGs) and *IGFBP3*-L1 DNA methylation levels were negatively correlated with maternal glucose 2h post oral glucose tolerance test (*IGF1R*-L4: r=-0.228; P =0.01; *IGFBP3*-L1: r=-0.195; P =0.028) and fasting glucose at the second trimester of pregnancy (*IGF1R*-L4: r=-0.239; P =0.007; *IGFBP3*-L1: r=-0.152; P =0.089).
	Impaired Fasting insulin and HOMA-IR				*IGFBP3*-L1 DNA methylation levels were positively correlated with the third semester fasting insulin (r=0.217, P =0.02) and HOMA-IR (r=0.280; P =0.003). No association was found for *IGF1R*-L4 and for the second trimester insulin or HOMA-IR.
Keller M. et al. 2014 [11]	Fasting glucose	Adipose tissue (SAT and visceral fat)	M and F, n=51	*GLUT4* promoter and the first intron, 22 CpGs/ bisulfite pyrosequencing	Methylation levels did not withstand adjustments in their association with glucose homeostasis.
	Fasting Insulin				No association was found.
Hidalgo B. et al. 2014 [47]	Fasting Insulin and HOMA-IR	PB	M and F, n=837	470,000 CpG sites/ Infinium Human Methylation 450K Bead Chip	Differential (hyper) methylation of CpG site within *ABCG1* was associated with fasting insulin and HOMA-IR
Perez-Cornago et al.2014 [48]	Insulin	WB	M and F, n=41	*HTR2A* gene promotor/ bisulphite sequencing	Higher levels of *HTR2A* gene methylation were associated with higher levels of insulin.

Table 3. (continued)

Author, Publication year	**Phenotype**	**Tissue type**	**Population**	**Methylation sites/ method**	**Main findings**
Garcia-Cardona M.C. et al. 2014 [49]	Fasting Insulin and HOMA-IR	PB	M and F, n=106	*LEP* promoter (located at -51 and -31 nt relative to the transcription start site) and *ADIPOQ* promoter (located at -238 and -74 nt relative to the transcription start site)/ bisulfite pyrosequencing.	Methylation frequency of the *LEP* promoter at both CpGs was negatively associated with HOMA-IR and insulin whereas no association with these traits was observed for *ADIPOQ* methylation.
Xie X. et al. 2015 [50]	Glucose concentrations after a 75g-OGTT	Cord blood and placenta	F, n=58	*PPARGC1A*, 6 CpGs sites/ pyrosequencing	The maternal gestational glucose level was positively correlated with placental DNA methylation, and negatively correlated with cord blood DNA methylation of the *PPARGC1A* promoter in a CpG site-specific manner.
Kulkarni H*. et al 2015 [39]	HOMA-IR	PB	F (63%)	446 356 CpG sites/ HumanMethylation450 BeadChip	24 CpG sites were significantly associated with fasting glucose, but not significant after correction for anti-diabetic medication use.

*Epigenome wide approach

β, beta estimates; PB, Peripheral blood; PBL, peripheral blood leucocytes; PBMCs, Peripheral blood mononuclear cells; PI, pancreatic islets; WB, whole blood.

Table 4. Histone modifications and type 2 diabetes

Lead Author, Publication Date	No. of cases	Tissue type	Population	Methylation sites/ method	Result	Main findings
Miao F. et al. 2007 [51]	6	PBMC	n=12	H2K9me2 in PTEN coding and IL-1A promoter region/ Chip assay	Increased	T2D subjects had significant higher levels of histon H3K9me2 around the IL-1A promoter and PTEN coding regions relative to those in the normal control group (P <0.0125).
Hou C. et al. 2011 [52]	12	PBMC	M and F, n=24	H3 acetylation of Promoter region of TNF-α and Cox-2 mRNA	Increased	H3 acetylation at the TNF-α (1.54 ± 0.43 vs. 0.97 ± 0.39, P =0.0094) and COX-2 (1.20 ± 0.58 vs. 0.64 ± 0.21, P =0.0161) gene promoter region was elevated in PBMCs from T2D patients compare to controls.
Paneni F. et al.2014 [53]	44	PBMC	M and F, n=68	H3K4m1 in the NF-kB promoter region (-480/-240)/Chip assay	Increased	Subjects with T2D showed Set7-dependent monomethylation of lysine 4 of histone 3 on NF-Kb p65 promoter (P <0.0001).

F, female; M, male; PB, Peripheral blood; PBL, peripheral blood leucocytes; PBMCs, Peripheral blood mononuclear cells; T2D, type 2 diabetes; WB, whole blood.
The references for all tables can be found at the of the manuscript.

Epigenome-wide analysis, type 2 diabetes and glycaemic traits

Two studies investigated diabetes-associated differentially methylated sites in pancreatic islets [27, 39], one study in skeletal muscle and subcutaneous adipose tissue [24] and four studies in blood cells [40-43] (Table 2). In the pancreatic islets, up to 853 genes (including reported diabetes loci - TCFL72, FTO and KCNQ1) were found to be differentially methylated in diabetes cases compared to healthy controls [39]. In a study of Ribel-Madsen et al., they reported only one CpG site (IL8) in muscle and 7 CpG sites in adipose tissue (ZNF688, HSPA2, C8orf31, SFT2D3, TWIST1, MYOA5) after correction for multiple testing [24]. Epigenome-wide association analysis in whole blood revealed ~69 CpG were reported including loci such as MALT1, ABCG1, PHOPHO1, SOCS3, SREBF1 and TXNIP.

As for glycemic traits, two studies performed epigenome-wide analysis [42, 44], but only one significant CpG site (ABCG1) was reported for fasting insulin and insulin resistance after correction (Table 3).

Histone Modifications and Type 2 diabetes

Three studies examined the association between histone modifications and T2D (Table 4). Patients were reported to have elevated histone H3 acetylation of tumor necrosis alpha and COX-2 in the PB mononuclear cells, higher levels of histon H3K9me2 around the IL-1A promoter gene and PTEN coding regions and Set7- dependent monomethylation of lysine 4 of histone 3 on promoter gene.

DISCUSSION

The present work is the first to systematically review the current evidence for the role of epigenetic marks in type 2 diabetes and glycemic traits. The findings of this study indicate no consistent associations between global DNA methylation and diabetes. Conclusive evidence in alterations of histones are still lacking. Although a few diabetes differentially methylated sites have been reported (summarized in Figure 2), the majority of studies have used a candidate-gene approach. No overlap was found between the significant genes differentially methylated on epigenome-wide association studies and studies that used a candidate-gene approach.

Global DNA methylation

The lack of consistency in the associations of global DNA methylation with both T2D and glycemic traits is not reported only across different methods of measurement, but also across different tissues. However, the available information is rather limited and heterogeneous to draw any firm conclusions. The global DNA methylation assessed at long-interspersed nuclear element (LINE-1) was inversely associated with CVD, independent of established cardiovascular risk factors. Conversely, a higher degree of global DNA methylation measured at Alu repeats or by the LUMA method was associated with the presence of CVD [45]. Similarly, no consistent association is observed between global DNA methylation and obesity [46]. Changes in global methylation can affect expression, genomic stability, and chromosomal structure [47]. These contradictory results observed with global DNA methylation may be due to distinct functions between LINE-1 and Alu elements. LINE-1 and Alu repeats represent distinct measures of dispersed DNA methylation, and might have different functions [48]. The quantitative assessment of DNA methylation at ALU is about one-third to one-fourth of methylation at LINE-1, which may suggest that epigenetic changes at LINE-1 and ALU are measuring different traits [48]. Also, the differences in the reported results may come from the assay used to assess global DNA methylation. For example, global DNA methylation assessed by LUMA modestly correlates with LINE-1 methylation [49]. Additionally, this could be explained by the small sample size and therefore, the lack of power of studies investigating the association between global DNA methylation and T2D until now.

Histone modifications

This review underscores a gap in the literature concerning T2D and histone modifications. Specific patterns of post-translational modifications to histones act like a molecular "code" recognized and used by non-histone proteins to regulate chromatin functions [50]. These in turn, can be associated with either an active or repressive state of the gene depending on where the modification takes place [51]. Many of the enzymes are involved in the regulation of histones including processes such as acetylation, methyla-

tion, phosphorylation, sumoylation and ubiquitination, which may play important roles in the pathogenesis of diabetes [52]. Various families of proteins have been identified to be active in these processes (such as histone deacetylases (HDACs), K-acetyltransferases (KATs), K-methyltransferases (KMTs), and K-demethylases (KDMs)) [53]. For instance, a genome-wide association study identified chromosomal region 6q21 associated with both type 1 and type 2 diabetes [54, 55]. Notably, one type of HDAC maps in this region. Further evidence pointing the role of histone modification in T2D can be found in studies conducted using animal models [56, 57]. Taken together, many important enzymes/proteins are involved in histone regulation processes. Further studies are needed to elucidate the role of these mechanism in the pathogenesis of type 2 diabetes.

EWAS and candidate gene approach

Overall, epigenome-wide association studies have provided further insights into the DNA methylation changes associated to T2D. Despite the recognized value of EWAS in uncovering potential novel pathways for common disorders, several limitations are present with regards to the current costs that generally limit the sample size, the varying reproducibility related with different platforms or appropriate analysis approaches [58, 59]. Further attempts to standardized approaches would be a great step in improving the reliability of results and thus the possibility of replicating findings across different studies. Unlike genetic information, which does not very across tissues, methylation is highly variable [58]. Candidate gene approaches, on the other hand, have less stringent multiple testing thresholds on the expense of a narrower focus on genes. Most studies examining epigenetic changes in T2D and glycemic traits in our systematic review have taken this approach. However, there was an absence of reproducibility of results in the reviewed EWAS.

Tissue relevance

The ideal tissue to study directly diabetes pathophysiology could be considered to be the pancreatic B-cells. However, collection of this biological material is invasive and not feasible on a large scale. Peripheral blood is the best accessible alternative tissue that reflects multiple metabolic and inflammatory pathways [60, 61]. There is great interest to perform methylation profiling in blood to find methylation disease-related associations since specific methylated regions could be used as potential biomarkers with a great promise of potential clinical utility [62]. Although white blood cell subtype proportions have been previously reported to be associated with T2D [63], methylation changes were not confound by differential cell types as suggested by Yuan et al [40]. However, methylation patterns are thought to be tissue-specific thus we might not extrapolate the methylation pattern found in blood to the methylation pattern in other tissues [64].

Bias of the data and Confounding

A number of studies in our review adjusted for age and gender in their analysis, but others are still lacking of these basic covariates. More than 50% of the studies were classified as low quality where most of the part due to lack of proper adjustments. Controlling for different confounders and mediators is important in epigenetic studies since the association is susceptible by temporal factors and spatial effects including age, gender, demographics, ethnicity, environmental, cellular composition, comorbidities and medication use [65-67]. Only a few studies mention adjustment for lifestyle factors such as smoking, alcohol consumption, BMI which might be more prevalent and affecting diabetes patients and have been associated with methylation changes. Comorbidities may also confound epigenetic analysis. Unlike genetic association studies which are less prone to confounding, it is important to incorporate a set of confounding factors in epigenetic analysis.

Causality and study designs

Although studies have reported associations between epigenetic markers and diabetes, identifying causal processes is very difficult due to the unstable nature of the epigenome. This is commonly acknowledged in epigenetics epidemiology; unraveling the direction of the association between DNA methylation and the phenotype remains challenging. In human studies, advantages of longitudinal designs could be of help. In all the studies included in this review, except two [41, 68], methylation levels and outcome were measured at the same time point, making it impossible to define whether specific DNA methylation marks are a cause or consequence of the disease. Cohort studies give dynamic information of methylation changes during lifetime and help provide stronger evidence towards causation. The gold standard for establishing causation would be to conduct a longitudinal study with repeated measurements of DNA methylation before disease development and after it. Although this would be costly and logistically challenging, such efforts are on the way. Statistical approaches like Mendelian Randomization where genetic variants are used as proxies for DNA methylation and the outcome of interest can offer an opportunity to study the direction of causation from cross-sectional data [69].

Clinical Implications

Epigenetics might help further identify and understand causes of disease, but is also concerned with risk prediction. Indeed, epigenetics has been shown to be associated with age and moreover, it is a promising biomarker of accumulative environmental exposures [70]. Without being directly involved in the etiology of disease, biomarkers such as epigenetic signatures with high sensitivity and specificity for diabetes would improve the diagnosis, resulting in earlier detection of new cases and introducing new

potential therapeutical interventions [41]. Likewise, these epigenetic biomarkers can be used as prognostic tools on diseased individuals.

Perspective and conclusion

Promising results has been reported in the field of diabetes and epigenetics, but still there is long way to go in our understanding of the role of the epigenetics in modifying and regulating human health and disease. Although the field is running on a fast pace, more stringent criteria should be applied to current studies. An increasing tendency to harmonize appropriate methods for DNA methylation detection and reference standards is rapidly evolving and the first steps are made to identify potential DNA methylation biomarkers for type 2 diabetes. Larger sample sizes, replication of results, controlling for a wide range of confounders are paramount. Large-scale longitudinal studies with markers collected over time would undoubtedly contribute to our understanding of the role of epigenetic mechanisms in the evolution of a complex disease. Eventually this may help in predicting an individual's risk and opens possibilities for introducing targeted strategies to prevent and treat type 2 diabetes.

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Chapter 4.2

Epigenetics and inflammatory markers: a systematic review of the current evidence.

Valentina González-Jaramillo MD[1,2], Eliana C. Portilla-Fernandez MSc[1], Marija Glisic MD, MSc[1], Trudy Voortman PhD[1], Mohsen Ghanbari MD, PhD[1, 3], Wichor Bramer MSc[4], Rajiv Chowdhury MD, PhD[5], Tamar Nijsten MD, PhD[6], Abbas Dehghan MD, PhD[1,7], Taulant Muka MD, PhD[1], Oscar H. Franco MD, PhD[1,2], Jana Nano MD, DSc[1,8,9]

[1] Department of Epidemiology, Erasmus MC, Erasmus University Medical Center, Rotterdam, the Netherlands.
[2] Institute of Social and Preventive Medicine (ISPM), University of Bern, Bern, Switzerland.
[3] Department of Genetics, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.
[4] Medical Library, Erasmus MC, Erasmus University Medical Center, Rotterdam, the Netherlands.
[5] Department of Public Health and Primary Care, University of Cambridge, Strangeways Research Laboratory, Cambridge, UK
[6] Department of Dermatology, Erasmus MC, Erasmus University Medical Center, Rotterdam, the Netherlands
[7] Department of Biostatistics and Epidemiology, MRC-PHE Centre for Environment and Health, School of Public Health, Imperial College London
[8] Helmholtz Zentrum München, German Research Center for Environmental Health, Institute of Epidemiology, Neuherberg, Germany
[9] German Center for Diabetes Research (DZD), Munich-Neuherberg, Germany

(Submitted)

ABSTRACT

Objective

Epigenetic mechanisms have been suggested to play a role in the genetic regulation of pathways related to inflammation. Therefore, we aimed to systematically review studies investigating the association between DNA methylation and histone modifications with circulatory inflammation markers in blood.

Approach and Results

Five bibliographic databases were screened until 21 November of 2017. We included studies conducted in humans that examined the association between epigenetic marks (DNA methylation and/or histone modifications) and a comprehensive list of inflammatory markers. Of the 3,759 identified references, 24 articles were included, involving, 17,399 individuals. There was suggestive evidence for global hypomethylation but better-quality studies in the future have to confirm this. Epigenome-wide association studies (EWAS) (n=7) reported most of the identified differentially methylated genes to be hypomethylated in inflammatory processes. Candidate genes studies reported 18 differentially methylated genes related to several circulatory inflammation markers. There was no overlap in the methylated sites investigated in candidate gene studies and EWAS, except *TMEM49,* which was found to be hypomethylated with higher inflammatory markers in both type of studies. The relation between histone modifications and inflammatory markers was assessed by one study only.

Conclusions

This review supports an association between epigenetic marks and inflammation, suggesting hypomethylation of the genome. Important gaps in the quality of studies were reported such as inadequate sample size, lack of adjustment for relevant confounders and failure to replicate the findings. While most of the studies have been focused on C-Reactive Protein, further efforts should investigate other inflammatory markers.

INTRODUCTION

Inflammation is a critical response to pathogens and injuries in the human body. Specifically, chronic low-grade inflammation plays a key role in the pathogenesis of chronic conditions and diseases like obesity, diabetes mellitus, and cardiovascular disease (1-3). A better understanding of factors that contribute to the development of inflammation and its consequences on disease is essential to improve prevention strategies in inflammation-related disorders.

Genome-wide association studies have identified several genetic variants associated with inflammatory markers such as C-reactive protein, the most widely studied marker (4, 5), but the explained variance is relatively small. In addition, non-genetic factors such as smoking and dietary behaviours have been shown to exhibit a strong influence on the inflammatory response (6, 7). Emerging evidence suggests that epigenetic processes, reflecting changes in gene expression that occur without sequence mutations, may offer opportunities to understand the pathophysiology of inflammation processes. The role of epigenetic determinants is increasingly being recognized as a link between (external) environmental factors and disease risk. Moreover, epigenetic modifications are also involved in differentiation of the immune cells, a key component of the inflammatory process. Epigenetics is defined as a group of chemical modifications of the DNA sequence, which could be affected by external factors such as BMI, smoking, inflammation and can be transmitted from one generation of cells to the other (8). The molecular basis of epigenetic mechanisms are complex and comprise DNA methylation, modifications of histones and gene regulation by non-coding RNAs (9). Unlike genetic variation, epigenetic modifications are dynamic and potentially reversible and, therefore, could be modified by lifestyle and other therapeutic approaches.

Until now, a comprehensive and systematic appraisal of the current literature on the role of epigenetic modifications in inflammation measured by levels of inflammatory markers is missing. Therefore, we aimed to identify and synthetize all available evidence conducted in humans and quantify the association of two of the major epigenetic modifications, DNA methylation and histone modifications, with circulation inflammatory markers in blood.

RESULTS

In total, after deduplication, we identified 3,759 potentially relevant citations. Based on the title and abstracts, full texts of 80 articles were selected for detailed evaluation. After full-text assessment, 24 of these unique studies met our eligibility criteria, and were included in this review. The other 56 articles were excluded for reasons shown in **Figure 1**.

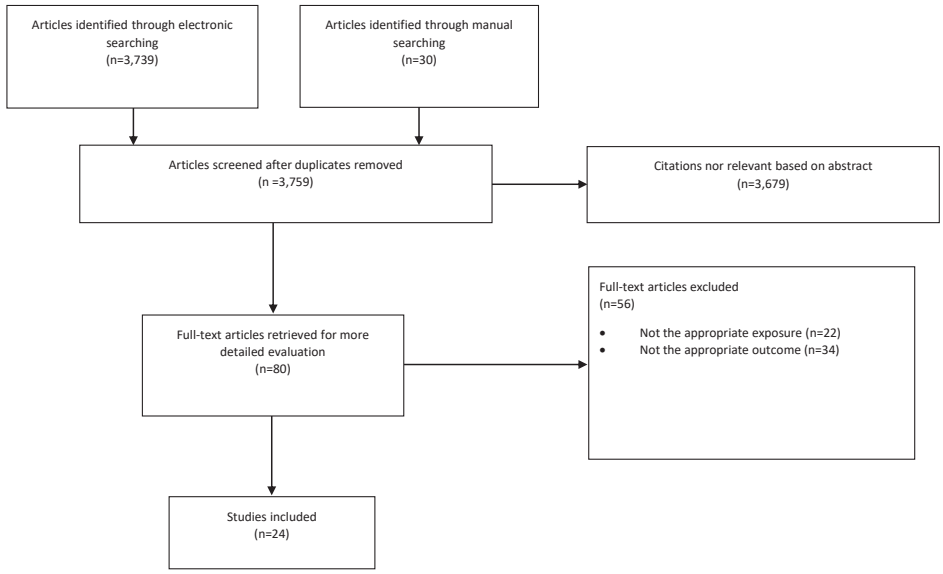

Figure 1. Flowchart of studies included in the systematic review.

Characteristics of the included studies

Detailed characteristics of the included studies are summarized in Tables 1-3. All included studies were of cross-sectional design, except one study of prospective design (10). Overall, 17,399 individuals were participating in these studies. Nine studies included participants from the USA, three studies from China, three studies from Canada and the rest included participants from Brazil, Colombia, India, Ireland, Germany, Greece, Mexico, Spain, and Sweden. One of the studies (11) included participants from different cohorts such as USA, UK, Italy, Germany, and Netherlands. The majority (n=23) of studies assessed epigenetic signatures in blood, whereas other assessed epigenetic marks in tumour specimens (glioblastomas).

Of the 24 studies included, four studies assessed global DNA-methylation only, eleven studies assessed DNA methylation in specific candidate genes, seven studies used genome-wide approaches, while one additional study examined both DNA methylation in specific candidate genes and globally (12) . Only one study assessed histone modification in relation to inflammation markers (13).

The most studied marker was C- Reactive Protein (CRP), which was evaluated in 17 studies. Interleukins like IL-4, IL-6, IL-8, IL-9, IL-10, and IL-18 were evaluated in 11 studies. TNF-α was assessed in three studies, fibrinogen in two and other markers such as VCAM, ICAM, VEFG, COX2, leptin, TNFR2, C-CAM1, alpha interferon and TGF-β were assessed in one single time.

Nine studies were judged at medium risk of bias whereas the rest at high risk of bias.

Global DNA methylation and inflammation markers

Five studies examined the association between global DNA methylation and inflammatory markers in blood samples (Table 1). All of them used blood samples to assess DNA methylation. Four of these studies assessed methylation in long-interspersed nuclear element (LINE-1). A large portion of methylation sites within the genome are found in these repeat sequences and transposable elements, and correlate well with total genomic methylation content. From the four studies, two (12, 14) reported no association between global DNA methylation and CRP levels, while the others showed lower methylation to be related with higher CRP levels (15, 16).

Table 1. Global DNA methylation and inflammatory markers.

Author, Year	Study Design	Outcome	Population Sex/Age/Population/ Country	Tissue type	Adjustment	Association, reference
LINE-1 methylation						
Baccarelli et al., 2010(14)	CS	VCAM-1, ICAM-1 and CRP	M /73.8 ± 6.7/n=593/ USA	WB	Age, BMI, smoking, pack years of smoking, IHD or stroke.	Inverse for VCAM-1, no association for ICAM-1 and CRP.
Perng et al., 2012(15)	CS	CRP	M and F/ 8.8 ± 1.7/ n=568/ Colombia	WBC	Sex, vitamin A, maternal BMI and household socioeconomic stratum.	Higher CRP was related to lower LINE-1 mehtylation.
Zhang et al., 2012(12)	CS	CRP	M and F/ 18-78/n=165/ USA	WBC		No association (β coefficient=-0.02, p=0.81).
Narayan & Dangi, 201.(16)	CS	CRP	M and F/7.9 ± 1.5/ n=600/India	WB	Sex, plasma Vitamin A, socioeconomic status	Global DNA methylation was inversely related to CRP concentrations and the association was stronger in male children.
5mdC						
Murphy et al., 2015(60)	CS	IL-6 (protein and serum levels)	M and F/mean=33.04/ n=47/Ireland	WB		No association. (r = -0.125, p=0.46).

Abbreviations: CS: cross-sectional; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intercellular adhesion molecule 1; CRP: C-Reactive protein; M: men; W: women; WB: whole blood; BMI: body mass index; WBC: white blood cells; IL: interleukin.

One study (14), in addition to CRP levels, also evaluated the association of global DNA methylation at LINE-1 with VCAM-1 and ICAM-1, and reported an inverse association with VCAM-1 but no association with ICAM-1. One study quantified global DNA methylation by measuring the amount of methylated cytokines in the sample (5 mc) relative to global cytidine (5mC + dC) in a positive control and found no association between global DNA methylation and IL-6 serum levels.

Gene specific DNA methylation and inflammatory markers

There were twelve studies that examined methylation sites in, or near, candidate genes in relation to inflammatory markers (Table 2). One study measured DNA methylation in tumour specimens, whereas the other studies used blood samples to assess the DNA methylation. Of the twelve studies, eight did not report any level of adjustment or control for confounders, one of them (17) controlled for age and sex whereas the others (12, 18, 19) controlled for at least these two confounders. Of the twelve studies, three focused solely on CRP as outcome, one solely on interleukins, one solely in leptin and the others assessed a set of inflammatory markers including interleukins, TNF-α and fibrinogen.

In total, eight studies assessed CRP as inflammatory marker. Overall, these studies found higher levels of CRP to be associated with higher degree of methylation of SOCS-1 (20), LY86 (18) and EEF2 (21) with lower degree of methylation of AIM2 (22), IL-6 (23) and IL-6 promoter gene (17). One additional study (12) that examined methylation levels of IL-6 promoter and CRP reported no association. In addition, no association was found between methylation status of F2RL3 in peripheral blood cells and CRP levels.

Five studies evaluated the association of gene specific DNA methylation with IL-6. They found higher degree of methylation of MGMT, RARβ, RASSF1A, and CDH13 in tumour specimens and of SOCS-1 in peripheral blood with higher levels of IL-6, while others found less degree of methylation of USP2, TMEM49, SMAD3, DTNB and IL-6 promoter with higher levels of IL-6. Other interleukins such as IL-8, IL-10 and IL-18 were only evaluated once. No significant correlation was found for IL-8, whereas for IL-10 and IL-18 inverse association was found with DNA methylation in IL-10 promotor and F2RL3, respectively (Table 2).

Two studies evaluated leptin as outcome, showing contradictory results. One (21) reported inverse association between leptin levels and Leptin Receptor methylation, whereas the other reported no association between Leptin promoter and leptin levels (24).

Two studies assessed the association of DNA methylation and TNFα levels. Higher levels of methylation of EEF2 (21) and SOCS-1 (20) were found with higher levels of TNFα.

Also, six studies reported the association between methylation at different genes (MGMT, RARβ, RASSF1A, CDH13, USP2, TMEM49, EEF2, COL18A1, IL4I1, LEPR, PLAGL1,

Table 2. Specific gene methylation and inflammatory markers.

Author	Study design	Outcome	Tissue type	Population Sex/Age/ Population/ Country	Methylation sites/ method	Adjustment	Main findings	Clinical condition associated with the main findings*
Candidate gene approach								
Piperi et al., 2010(61)	CS	IL-6, IL-8, VEGF, COX-2	Tumour specimens	M and W/25-76/n=23/ Greece	*MGMT, RARβ, RASSF1A, CDH13*/MS-PCR		IL-6: positive correlation with the four genes; IL-8 and COX-2: no correlation for any gene; VEGF positive correlation with *MGMT* and *RARβ* no correlation with RASSF1A and *CDH13*.	IPA: Cancer, neurological disease, ophthalmic disease
Uddin et al 2010(23)	CS	IL-6, CRP	PBMC	M and W/ 45.3±16.7 6/ n=100/ USA	*IL-6*/Illumina HumanMethylation27K DNA Analysis BeadChip		Among patients with lifetime depression, there was a significant inverse correlation between methylation of *IL-6* and serum levels of IL-6 and CRP (Pearson r=-0.54, p=0.001 and Pearson r=-0.48, p=0.006, respectively.	*IL-6*: Rheumatic diseases, inflammatory bowel disease, Kaposi sarcoma.
Fu et al., 2011(62)	CS	IL-10 (mRNA)	PBMC	M and W/39 ± 10.8/ n=40/ China	*IL-10 promoter*, 5 CpG sites/ Pyro Q-CpG system		Hypothmethylation of -145C was correlated with higher IL-10 mRNA expression (*r*=-0.746, *P*=0.001), The authors did not report the results for the other CpG sites.	*IL-10*: Susceptibility to HIV type 1, rheumatic conditions, cutaneous leishmaniosis.
Zhang et al., 2012(12)	CS	CRP	PB	M and W/18-78/ n=165/ USA	*IL-6 promoter*, 6 site/ bisulfite treatment	Age, sex, race, dietary folate intake, prudent diet pattern, western diet	No association was found, with a spearman correlation coefficient of 0.11 (p=0.18).	Not applicable

Table 2. (continued)

Author	Study design	Outcome	Tissue type	Population Sex/Age/ Population/ Country	Methylation sites/ method	Adjustment	Main findings	Clinical condition associated with the main findings*
García-Cardona et al., 2014(24)	CS	Leptin	PB	M and W/ 10-16/ n=106/ Mexico	*LEP promoter*/ MS-PCR		No significant correlation was observed between the circulating levels of leptin and the methylation frequencies of the two selected CpG sites of the *LEP* promoter (at − 51 and − 31 nt).	Not applicable
Lai et al., 2014(20)	CS	IL-6, TNF-α and CRP	PB	M and W/ 36-80/ n=46/ China	*SOCS-1* gene, 11 CpG sites/ Bisulfite method		A positive trend between the levels of SOCS-1 methylation and CRP levels was observed (R^2=0.1127, P=0.0278). Patients with serum IL-6 above median showed a significantly higher *SOCS-1* methylation than the patients with serum IL-6 below median (P<0.001). Similar results were observed for TNF-α (P<0.001).	*SOCS-1*: Cancer, hepatic system disease, ophthalmological disease.
Smith et al., 2014(10)	CS and prospective	sTNFR2, IL-6	PBMC	W/56.4 ± 9.4/ n=61/USA	*USP2, TMEM49, SMAD3, DTNB*, 8 CpG sites/ HumanMethylation450 Bead Cheap		At baseline, lower methylation at each of the 8 CpG sites was significantly correlated with increased sTNFR2 and IL-6.	IPA: Gastrointestinal diseases, hepatic system diseases, cancer (like gynaecological cancer), dermatological diseases.

Table 2. (continued)

Author	Study design	Outcome	Tissue type	Population Sex/Age/ Population/ Country	Methylation sites/ method	Adjustment	Main findings	Clinical condition associated with the main findings*
Wang et al., 2014(18)	CS	Fibrinogen and CRP	PB	M and W/16.2 ±1.2/ n=703/ USA	*LY86* gene, 6 CpG sites / HumanMethylation27 BeadChip and ThumanMethylation450 BeadChip from Illumina	Age, sex, race, BMI and batch	They performed a principal component analysis to combine the six CpG sites into one score. The score of these CpG sites was significantly associated with fibrinogen (partial r=0.145, p<0.001) and CRP (partial r=0.114, p=0.005).	*LY86*: Pelvic inflammation, pulmonary interstitial emphysema
Wei et al., 2016(17)	CS	CRP	WBC	M and W/ / n=673/China	*IL-6* promoter/EZ DNA Methylation Kit	Age and sex	Plasma CRP levels were significantly associated with *IL-6* promoter methylation (P = 0.025). One interquartile range increase in plasma CRP was associated with a decrease in *IL-6* methylation by 0.78% (95% CI: -1.47% to -0.1%).	*IL-6*: Rheumatic diseases, inflammatory bowel disease, Kaposi sarcoma.
Arpón et al., 2017(21)	CS	TNF-α , VCAM-1, sICAM-1, CRP, leptin	PB	M and W/63.8±2.74/ n=36/ Spain	*EEF2, COL18A1, IL4I1, LEPR, PLAGL1, IFRD1, MAPKAPK2* and *PPARGC1B*/ Ilumina Infinium HumanMethylation450K BeadChip		Results showed correlations between *LEPR* methylation and concentration of LEP (r=-0.24, p=0.047). Also, between *EEF2* methylation and concentration of TNF-α (r=0.24, p= 0.0408) and CRP (r=0.24, p=0.0457).	IPA: Inflammatory response, cardiovascular disease, reproductive system disease.

Table 2. (continued)

Author	Study design	Outcome	Tissue type	Population Sex/Age/ Population/ Country	Methylation sites/ method	Adjustment	Main findings	Clinical condition associated with the main findings*
Jhun. et al., 2017(19)	CS	CRP, IL-6, IL-18, fibrinogen	PBL	M and W/66 7.5/n=822/ USA	Cg0363183 in *F2RL3*/ Illumina Infinium HumanMethylation27 BeadChips and the Illumina BeadXpress reader.	Age, sex, four principal component, five cell proportions, plate and random intercepts for family.	DNA methylation level of Cg03636183 in F2RL3 was significantly associated with log (IL-18) levels (-0.11, 95% CI (-0.19, -0.04)).	Unknown.
Miller et al., 2017(22)	CS	CRP	WBC	M and W/32.08±8.36/ n=286/ USA	*AIM2*,cg10636246/ Ilumina Infinium HumanMethylation450K		Log CRP levels were negatively correlated with cg10636246 ($r = -0.264$, $p < 0.001$).	*AIM2*: Skin disease, melanoma.

*We used Ingenuity Pathway Analysis (IPA) for studies that found significant association between multiple inflammatory markers and/or methylation in multiple genes (10) (21, 61). For the other studies, the connection between findings and disease was assessed through literature review and gene cards (https://www.genecards.org/).

Abbreviations: CS: cross-sectional; IL: interleukin; VEGF: vascular endothelial growth factor; Cox-2: cyclooxygenase; M: men; W: women; MS-PCR: methylation-specific PCR; IPA: Ingenuity pathway analysis. PBMC: peripheral blood mononuclear cells; PB: peripheral blood; TNF- α: tumour necrosis factor-alpha; sTNFR2: soluble tumour necrosis factor receptor 2; BMI: body mass index; WBC: white blood cells; VCAM-1: vascular cell adhesion molecule 1; sICAM-1: soluble intercellular adhesion molecule 1; PBL: peripheral blood leucocytes.

IFRD1, MAPKAPK2, PPARGC1B, SMAD3, DTNB, LY86 and F2RL3) with levels of several inflammatory markers other than CRP and interleukins (VEGF, VCAM1, C-CAM1, COX-2, sTNFR2 and fibrinogen) (Supplement Table 1).

Epigenome-wide analysis and inflammatory markers

Seven studies investigated differentially methylated regions in the genome in a hypothesis-free approach. Six of them adjusted at least for age and sex and four of them, additionally, for BMI, smoking or other confounders. All of the studies used blood samples to assess DNA methylation. Five studies assessed CRP, two studies evaluated TNF and interleukins such as IL-1β, IL-6, IL-8 and IL-10 (Table 3). One study assessed 121 inflammatory biomarkers related with inflammation, cancer, and cardiovascular disease (25). Three out of seven studies used replication to validate their findings: two of them (11, 26) used external validations and one (27) internal validation. The identified genes were enriched by pathways such as atherosclerosis, IL-6, IL-9, IL-8, growth hormone, and JAK/STAT, signalling pathways. Among the genes reported to be differentially methylated, SOCS3 and BCL3 were found to be significantly hypomethylated in two studies (11, 26). BCL3 was no longer significant in the replication cohort, whereas SOCS3 remained significant after replication.

Histone modifications and inflammatory markers

There was only one study that examined the association between histone modifications and inflammatory markers (13). The authors assessed levels of acetylated histone H4 in the peripheral blood mononuclear cells of CODP patients, and reported higher acetylation levels in patients with higher IL-8 levels and in patients with lower IL-4 levels.

DISCUSSION

This is the first attempt to summarize current literature on the role of epigenetic marks in chronic inflammation. There is suggestive evidence for hypomethylation of overall genome in inflammatory processes, but better-quality studies have to confirm these results. Histone modification and inflammatory markers are scarcely investigated. Given the complexity and variability of proteins involved in the inflammation network, most of the studies focused on exploring CRP levels with few studies on IL-6 and fewer investigations on IL-8, IL-10, IL-18, VEGF, Cox-2, TNF-α, sTNFR2, leptin and fibrinogen levels. The largest epigenome wide association study up to date found AIM2 and SOCS3 to be top genes related to CRP levels in whole blood.

Table 3. Genome-wide and histone acetylation approaches and inflammatory markers.

Author	Study design	Outcome	Tissue type	Population Sex/Age/ Population/ Country	Methylation sites/ method	Adjustment	Main findings
Epigenome-Wide Association Study							
Guénard et al., 2013(27)	CS	CRP	WB	M and W/12.25±5.77/ n=50/Canada	Infinium HumanMethylation450K BeadChip	Age and sex	From 17 genes involved in the IL-8 signalling pathway, significant correlations between gene methylation and plasma CRP levels was found for 16 genes. Of these, 9 showed inverse correlation and 7 positive. Out of those 16 genes, 13 remained significant after adjustments.
Sun et al., 2013(36)	CS	CPR	PBL	M and F/ 66.27±7.58 n=966/ USA	Infinum HumanMethylation27K BeadCheap	Age, sex, BMI, smoking	207 out of 257 CRP-associated DNAm sites, showed an inverse correlation of greater methylation with lower level of CRP. Twenty-four out of the top 30 CpGs remained significant in both replication subsets with *KLK10, LMO2* and *TM4SF4* as top genes ($p=5.85x10^{-12}$, $p=1.69x10^{-11}$ and $p=2.05x10^{-10}$, respectively).
Ligthart et al., 2016(11)	CS	CRP	WB	M and W/mean age between 60 and 87/n=8863/ Consortia	Illumina Infinium HumanMethylation 27K and 450K BeadChip.	Age, sex, white blood cell proportion, technical covariates, smoking, BMI.	Of the 218 CpG sites (125 CpGs positively associated and 93 negatively associated) significantly associated with CRP, 58 CpG sites, in 47 genes, were still significantly associated in the replication cohort (n=4111). The top CpG site were located in *AIM, RPS6KA2 and PHOSPHO1* ($P = 2.53x10^{-27}$, $2.06x10^{-26}$ and $4.87x10^{-25}$, respectively). .

Table 3. (continued)

Author	Study design	Outcome	Tissue type	Population Sex/Age/ Population/ Country	Methylation sites/ method	Adjustment	Main findings
Marzi et al., 2016(26)	CS	CRP	PB	M and W/60.9±8.89/ n=1741/ Germany	Illumina HumanMethylation450K BeadChip	Age, sex, BMI, smoking, white blood cells composition.	Four CpG sites located at *AQP3, BCL3, SOCS3*, and intergenic at chromosome 19p13.2 were significantly hypomethylated at high CRP concentrations. Those four sites were replicated in three subcohorts. CpG at *AQP3* remained significant in two of the subcohorts and the one at *SOCS3* remained significant in one of the subcohorts.
Ahsan et al., 2017(25)	CS	121 biomarkers related with inflammation, cancer, and cardiovascular disease.	PBL	M and W/ 14-97/ n=698/Sweden	Illumina HumanMethylation450K BeadChip	Age, sex, batch and plate effects, year of sampling and cell fractions.	For 36% (44/121) of the studied biomarkers, the abundance level was associated with DNA methylation, but for 52% these biomarkers (23/44), the associations were explained by genetic variants. For a subset of biomarkers, the association with DNA methylation was confounded by environmental factors (e.g., smoking), but for the majority of the associations, no such relationship could be found.
Verschoor et al., 2017 ("The relation between…")(63)	CS	TNF, IL-6, IL-8, IL-10	WBL	M and W/48-78/ n=14/Canada	Illumina Infinium HumanMethylation 450 K BeadChip	Age and sex	Serum IL-10 levels exhibited the most substantial association to DNA methylation patterns, followed by TNF, IL-6 and IL-8.

Table 3. (continued)

Author	Study design	Outcome	Tissue type	Population Sex/Age/ Population/ Country	Methylation sites/ method	Adjustment	Main findings
Verschoor et al., 2017 ("DNA methylation…") (64)	CS	TNF, IL-6, IL-1β, IL-10 and CRP	PBMC	M and W/82-98/ n=23/Canada	Illumina Infinium HumanMethylation 450 K BeadChip		Authors performed linear regression between each factor assessed and the scores of top 10 principal components (PCs) of the DNA methylation dataset. Only IL-6 and IL-10 were found to be associated, both of which with PC7 (ln IL-6, p = 0.002; ln IL-10, p= 0.03). Ln CRP was positively associated with DNAmAge using Hannum's approach (β = 0.21, p = 0.007), which relates to approximately 5-years age acceleration per 1-unit change in ln CRP (β= 0.20, p =0.008).
Histone acetylation							
da Silva et al., 2017(13)	CS	IL-4, IL-6, IL-9, INF- γ and TGF-β	PBMC	M and W/ 68.5±6.49/n=10/ Brazil	Global Histone H4 Acetylation Assay Kit		At 24th session, the basal values of global histone H4 acetylation levels were correlated with basal IL-4 and IL-8 levels (r =−0.65, p= 0.04 and r=0.85, p=0.01, respectively).

Abbreviations: CS: cross-sectional; WB: whole blood; M: men; W: women; IL: interleukin; PBL: peripheral blood leucocytes; BMI: body mass index; PB: peripheral blood; TNF: tumour necrosis factor; WBC: white blood cells; PBMC: peripheral blood mononuclear cells; INF-γ: interferon-gamma TGF-β: transforming growth factor-beta.

Global DNA methylation

There were either no or an inverse association of inflammatory markers such as CRP, VCAM-1 and ICAM-1 in whole blood. Because we identified only a small number of studies, we cannot make any firm inferences on the overall hypomethylation of the genome due to inflammation. Moreover, populations were hardly comparable as two of the studies were conducted in children while the others in adults. As global DNA hypomethylation has become the hallmark of most human cancers, stroke and heart disease (28-31), the need to measure this epigenetic signature has become more essential. Global methylation would enable the ability to associate for example, LINE-1 or 5-mdC levels with correlative factors such as patient history or clinical outcome. The observed hypomethylation could lead to activation of dormant repeat elements and the subsequent aberrant expression of associated genes or may contribute to genomic instability and increased mutation rates. More intense efforts in studies investigating global DNA methylation through different methodologies such as Alu repeats and LUMA can hold future prospects for guiding risk stratification in individuals with high levels of inflammatory markers at an increased risk of chronic diseases.

EWAS vs candidate gene approaches

Ligthart et al. identified and validated 58 CpG sites located in 45 unique loci in whole blood in 12,974 individuals of European and African descent (11). The top signal near AIM2 gene was found to be inversely associated with gene expression levels and with lower CRP levels. AIM2 is a key regulator of human innate immune response implicated in defence mechanism against bacterial and viral pathogens (32, 33). Several of these hits including cg18181703 (SOCS3), cg06126421 (TUBB), and cg05575921 (AHRR) were associated with future incidence of coronary heart disease and smoking (11), whereas two other CpGs were recently identified in an EWAS of type 2 diabetes (34). The gene SOCS3, suppressor of cytokine signalling 3, plays a pivotal role in the innate immune system as a regulator of cytokine signalling along the JAK/STAT pathway and was previously reported to be important in atherosclerosis processes (35). Moreover, another epigenome-wide association study in 1,741 individuals of European descent reported SOCS3, among others, as significantly associated with systemic CRP levels, not only in peripheral blood tissue, but also in human liver tissue (26).

Given the reported association of CRP levels and these cardiometabolic clinical outcomes, it seems that inflammation-related epigenetic features may explain part of the observed associations reported in epidemiology. However, the results should be interpreted with caution, as the association of CRP and DNA methylation were not adjusted for these factors. Most of the replicated CpG sites reported in the study of Lighart et al. were associated with different cardiometabolic phenotypes (body mass index, fasting glucose, fasting insulin, triglycerides, total cholesterol, HDL-cholesterol), highlighting

the evidence of a pleiotropic network of epigenetics across various phenotypes. This information is promising as it holds new insights into shared epigenetic mechanisms and provide opportunities to link inflammation processes with clinical outcomes. Moreover, a general inverse association between hypomethylation and higher levels of CRP was observed by two large cohorts: KORA and GENOA study (26, 36). The latter reported a similar trend of hypomethylation among individuals of older age and suggested that these pattern of modifications of DNA methylation on CpG islands between aging and inflammatory markers may indicate shared molecular mechanisms underlying chronic diseases through epigenetic changes (36).

Differentially methylated genes associated with CRP levels and other inflammatory markers did not directly overlap with the genes identified from previously reported genome wide association studies influencing CRP levels and other biomarkers. The non-overlap between GWAS and EWAS identified genes shows that clinical phenotypes are being influenced by different molecular mechanism, all of them important to explain phenotypical variation. Most of the identified genes are involved in common inflammation pathways related to cancer, rheumatic diseases and gastrointestinal pathologies (20, 23). Nevertheless, candidate gene approaches have less stringent criteria to assign significance on the expense of a narrower focus on genes. This might explain the absence of reproducibility of results in the reviewed epigenome wide association studies, except for TMEM49, which was found to be inversely associated with sTNFR2 and IL-6 levels in the candidate gene approach study of Smith et al, and shared the same direction of association with CRP levels, in the EWAS study of Lighart et al.

Histone modification

This review demonstrated that evidence involving inflammation and histone modification mechanisms are inexistent. Histone modifications are another epigenetic mark that play a pivotal role in the epigenetic regulation of transcription and other functions in cells. In addition, histone modifications have been linked to other inflammatory-related disorders, such as dyslipidaemia, obesity, diabetes, cancer and cardiovascular disease (37-39). Future studies on histone modifications and inflammation markers might shed light into their functional role in chronic diseases and might provide novel target therapies for inflammatory conditions.

Bias, confounding, and tissue specificity

There is quite ample evidence showing differential DNA methylation differing by ethnicity (40). Therefore, it is recommended that studies investigating epigenetics of genes related to inflammation should replicate their findings in diverse populations. The largest to date epigenome wide association study investigating DNA methylation and CRP levels used as discovery set a large European population (n = 8,863) and sought

trans-ethnic replication in African Americans (n = 4,111) (11). As in genetic studies, the importance of replication of the significant findings in epigenetic association studies is a paramount in order to prevent false-positive results (41, 42).

Unlike genetic association studies that are less prone to confounding, epigenetic signatures throughout the genome, are highly labile due to temporal or spatial factors affecting DNA such as age, gender, demographics, lifestyle, comorbidities, and medication used. It has been shown that methylation investigations harbour new information in explaining the variation of complex traits such as inflammation characterized by a strong influence of environment (4, 11, 43). CRP, one of the most studied inflammatory markers, and others, are affected by both genetic and environmental factors. Therefore, controlling for confounders in epigenetic studies is crucial. In our review, the majority of our studies (62.5%) were classified as low quality largely explained by the lack of proper adjustment in the statistical models. Whereas epigenome wide studies controlled for life-style factors such as smoking, alcohol consumption and BMI, candidate-gene approach studies were heavily suffering from incomplete adjustments.

Most of the inflammatory markers and especially the ones of the acute phase are predominantly synthesized in liver cells, hepatocytes, and regulated via transcription factors such as STAT3, C/EBP family members and NF-kappa B by the pro-inflammatory cytokines IL-6 and IL-1beta (44, 45). Nevertheless, extra-hepatic expression to a lesser degree has been reported for adipose tissue and blood cells (45). DNA methylation profiles have been commonly studied in whole blood due to the easy access to the biological samples. Environmental exposure signatures such as smoking, alcohol and other condition involving the circulatory system and the immune response are well reflected in whole blood. This tissue is primarily composed of leukocytes, a key component of the human immune system and therefore, highly relevant to systematic inflammation. However, since peripheral blood constitutes a heterogeneous admixture of different cell populations, it is possible that the results reflect inflammation-related DNA methylation changes that influence a single cell type component of blood cells. Adjusting for measured or estimated blood cell proportions or future studies conducted in cell specific tissues would help to rule out presence of any residual confounding caused by white blood cell distribution.

Causality and study designs

In the last years, the GWAS have resulted in the identification of many genetic variants that are associated with clinical traits and diseases but together, these variants explain only a small fraction of the variability. It has been suggested that epigenetics might hold promise to uncover the rest of the missing heritability. Moreover, it has been commonly hypothesized whether epigenetic signatures might be a cause for disease, rather than consequence. With the current evidence, it is unclear if epigenetic variation is causal to

these inflammatory markers. In a recent study of Ahsan et al, the authors investigated the genetic and epigenetic influence in a large set of disease related inflammatory markers (25). Combining results of GWAS/EWAS in around 1,069 individuals and employing a complex bidirectional model to asses causality between genetic variation-DNA methylation-inflammation markers, concluded that DNA methylation has a limited direct effect on inflammatory markers and it reflects the underlying pattern of genetic variants, environmental exposures or secondary effect of the pathogenesis of disease. In line with recent evidence, DNA methylation seems to be a consequence of clinical traits rather than a cause, for example, BMI (46).

All of the included studies in this systematic review were of cross-sectional design, except for one (40), meaning that both epigenetic signatures and outcomes were measured at the same time. This design challenges further inferences concerning causal directionality of associations, a typical vulnerability of epigenetic studies. In longitudinal cohort designs, repeated measurements for both inflammatory markers and dynamic methylation changes could improve our knowledge of directionality of events. While performing direct experiments with randomization of individuals into exposed and not exposed to a specific inflammatory marker would be of preference, these requires a large amount of resources. Therefore, statistical approaches like Mendelian Randomization, in which genetic variants are used as proxies for DNA methylation and the outcome of interest, offer new opportunity to investigate the directionality of evidence from cross-sectional data (47). The identification directionality and molecular pathways underlying the relation between epigenetic signatures and inflammatory markers represent promising targets for future functional studies.

Epigenetic screening

In the last years, many advances in technologies related to measurements of epigenetic signatures have been developed to respond to the fast-growing pace of the field (48). These techniques allow to investigate DNA methylation either on candidate genes or on the whole-genome level. However, as the number of genes of interest increases along with the number of tissues of relevance, investigating the role of DNA methylation in different clinical traits could be very costly and time consuming. Progressing to more cost-effective solutions, high-throughput technologies have open new opportunities for epigenome wide investigations in large-scale screening such as in population-based cohort studies. Furthermore, gene-specific assays such as bisulfite conversion provide a quick and efficient result for epigenetic investigations requiring relatively low DNA input with minimum DNA loss (49, 50). Cloning, the gold standard method for gene-specific DNA methylation studies, followed by Sanger sequencing is another technological option (51). Although the time for the procedure itself has been significantly reduced, the sequencing step might introduce several sources of errors (48, 52). Another technique,

pyrosequencing, represents a high throughput quantitative method used for bisulfite sequencing (53, 54). This technique, which can be used for both DNA methylation and genetic variation (single nucleotide polymorphism) analysis, takes less time than cloning providing accurate reads within each run. Yet, optimal DNA quality is important to avoid misreads of pyrosequencing (48). Mass spectrometry assay, on the other side, is a tool that can be used for the discovery and quantification of DNA methylation sites based on difference in fragments weights that have been cleaved depending on the methylation status (52). This technology is highly sensitive and has the ability to sequence reads up to 600bp, which is considerably longer than other methods. Quantitative Polymerase Chain Reaction (qPCR) arrays are another alternative of methylation quantification techniques operating on fluorophore-labelled probes that emit fluorescence when bound to a complementary DNA sequence. This method might not be ideal for regions with multiple CpG sites because many probes need to be created, resulting rather costly. However, if a region is characterized by a few CpGs, qPCR method might provide a simple and relatively inexpensive way to conclude a high-powered study (48).

Other chip techniques for epigenetic studies, in particular for histone modifications, include chromatin immunoprecipitation (ChIP), methylated DNA immunoprecipitation (MeDIP) platforms, as well as methyl-binding protein immunoprecipitation platforms. A major limitation to these techniques in epigenome-wide analysis is the quality of the antibody, which plays an important role in the proper enrichment of DNA. In general, the immunoprecipitation techniques require the availability of large sample volumes and only measure relative enrichment of epigenetic markers.

Concerning large-scale epigenetic analysis, the most widely used platforms, as shown from our review, are from Illumina. Illumina Methylation profiling is based on bisulfite converted DNA genotyping (55). For example, The Illumina Infinium HumanMethylation27 (27,000 CpG site) and Human-Methylation450 Bead (450,000 CpG sites) arrays provide genome-wide coverage, featuring methylation status at CpG islands, CpG shores, nonCpG sites, promoter regions, 5′ UTR, 3′ UTR, as well as gene bodies. More recent platforms such as Infinium MethylationEPIC BeadChip Kit, have increased the number of interrogated sites to more than 850,000 CpGs across the genome at single-nucleotide resolution for only of 250ng DNA as input quantity (56). Moreover, TruSeq Methyl Capture EPIC Library Prep Kit, is another option that combines whole-genome bisulfite sequencing with methylation arrays that can support both screening and biomarker discovery studies targeting over 3.3 million CpGs (57). These technologies rapidly produce a large amount of data at relatively low costs and are mostly preferred in population studies. On the other hand, epigenome-wide sequencing is another technology that is holding high hopes for future discoveries in the field of epigenetics. Currently, its widespread use is hampered by the high costs and computation burden of the analysis.

Clinical implications

Understanding the epigenomic regulation of loci related to inflammatory markers might hold the possibility to discover attractive targets to control inflammatory processes and consequently, improve therapeutical interventions for chronic diseases that share in their aetiology, inflammatory-related pathophysiology. The identified epigenetic patterns may be used not only in functional studies to provide further insights into molecular mechanisms of inflammatory processes but also in biomarker studies using whole blood to improve the prediction of inflammation related clinical disorders or events.

Conclusions

Current evidence suggests a potential role of epigenetics on the level of inflammatory markers in blood. Studies reporting on the association of inflammation with global DNA methylation show a hypomethylation trend. However, this evidence is not conclusive. Further studies are recommended to explore this relation. Moreover, studies on the role of histone modifications in inflammation markers are scarce. While most of the studies have been focused on CRP, reporting replicated genes across cohorts such as SOCS3, further efforts should focus on other biomarkers of the inflammatory cascade such as interleukins. Most importantly, given the systemic nature of inflammation, validation of the methylation sites among different tissues is paramount. The identified and reported genes so far involve epigenetics of inflammation with cardiometabolic factors, but also cancer and rheumatic diseases highlighting the potential of these regions as translational targets in the future. Given that we observed a lack of high quality investigations included in this review, we recommend future studies to improve some of the most urging factors such as design of studies (choosing for example, repeated measurements of epigenetic marks or prospective designs of conducted studies that would allow to draw insights on one of the most important drawbacks of epigenetic data, assessing the directionality of effects), increase the sample size (to provide adequate power) and perform proper adjustment of analysis to account for the role of environment on both epigenetics and inflammation. Lastly, the identified genes need to be validated in functional (in vitro and in vivo) studies in order to draw valuable and conclusive insights into the epigenetic mechanisms of inflammatory markers.

METHODS

Literature search

This review was conducted and reported using a predefined protocol and in accordance with the PRISMA (58) and MOOSE (59) guidelines (Supplement Material 1 and 2). We sought studies published before 21 November of 2017 (date last searched) in five electronic databases: Embase.com, Medline (Ovid), Web-of-Science, Cochrane Central and

Google Scholar. We did the search with the help of an experienced medical information specialist. In databases where a thesaurus was available (Embase and Medline), articles were searched by thesaurus terms, title and/or abstract; in other databases only by title and/or abstract. The search combined terms related to the exposure (e.g. epigenetic, methylation, demethylation, hypomethylation, hypermethylation, DNA methylation) and outcome (e.g. inflammation, C-reactive protein, cytokine). We did not apply any language restriction, but we restricted the search to studies on humans alive. The full search strategies of all databases are provided in Supplement Material 3. The study identification also included manual search, based on the screening of the citations of the relevant studies.

Information about study selection and inclusion criteria, data extraction process, and risk of bias assessment is described in Supplement Material 4.

Supplement Table 1. Genes methylation in candidate gene approach

Inflammatory marker	Tissue	Hypomethylated genes	Hypermethylated genes	Null association	Differentially methyalted
IL-6	Tumor specimens		*MGMT, RARβ, RASSF1A, CDH13*		
	PB		*SOCS-1*		
	PBMC	*IL-6, USP2, TMEM49, SMAD3, DTNB*			
CPR	PBMC	*IL-6*			
	PB		*SOCS-1, LY86, EEF2*	*IL-6* promoter	
	WBC	*IL-6* promoter, *AIM2*			
IL-8	Tumor specimens			*MGMT, RARβ, RASSF1A, CDH13*	
VEGF	Tumor specimens		*MGMT, RARβ*	*RASSF1A, CDH13*	
Cox-2	Tumor specimens			*MGMT, RARβ, RASSF1A, CDH13*	
IL-10	PBMC				*IL-10* promoter
Serum leptin	PB	*LEPR*		*LEP* promoter	
TNF-α	PB		*SOCS-1, EEF2*		
sTNFR2	PBMC	*USP2, TMEM49, SMAD3, DTNB*			
IL-18	PBL	*F2RL3*			
Fibrinogen	PBL		*LY86*		

PB: Peripheral blood; PBMC: Peripheral blood mononuclear cell; PBL: Peripheral blood leukocyt

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Chapter 4.3

Epigenome-wide association study identifies methylation sites associated with liver enzymes and hepatic steatosis

Jana Nano[1], Mohsen Ghanbari[1,2], Wenshi Wang[3], Paul S. de Vries[4], Klodian Dhana[5], Taulant Muka[1], André G. Uitterlinden[1,6], Joyce B.J. van Meurs[6], Albert Hofman[1,7], BIOS consortium, Oscar H. Franco[1], Qiuwei Pan[3*], Sarwa Darwish Murad[3*], Abbas Dehghan[1]

[1] Department of Epidemiology, Erasmus University Medical Center, Rotterdam, the Netherlands
[2] Department of Genetics, School of medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[3] Department of Gastroenterology and Hepatology, Erasmus University Medical Center, Rotterdam, the Netherlands
[4] Human Genetics Center, University of Texas Health Science Center at Houston, Houston, TX, USA
[5] Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA
[6] Department of Internal Medicine, Erasmus University Medical Center, Rotterdam, the Netherlands
[7] Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA.
* These authors contributed equally in this work.

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ABSTRACT

Background & aims

Epigenetic mechanisms might be involved in the regulation of liver enzyme level. We aimed to identify CpG sites at which DNA methylation levels are associated with blood levels of liver enzymes and hepatic steatosis.

Methods

We conducted an epigenome-wide association study in whole blood for liver enzymes levels including gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT), and aspartate aminotransferase (AST), among a discovery set of 731 participants of the Rotterdam Study and sought replication in a non-overlapping sample of 719 individuals. Significant DNA methylation changes were further analysed to evaluate their relation with hepatic steatosis. Expression levels of the top identified gene were measured in 9 human liver cell lines and compared with expression profiles of its potential targets associated with lipid traits. The candidate gene was subsequently knocked down in human hepatoma cells using lentiviral vectors expressing small hairpin RNAs.

Results

Eight probes annotated to *SLC7A11, SLC1A5, SLC43A1, PHGDH, PSORS1C1, SREBF1, ANKS3* were associated with GGT and one probe annotated to *SLC7A11* was associated with ALT after Bonferroni correction (1.0×10^{-7}). No probe was identified for AST levels. Four probes for GGT levels including cg06690548 (*SLC7A11),* cg11376147 (*SLC43A1)*, cg22304262 (*SLC1A5)* and cg14476101 (*PHGDH)*, and one for ALT cg06690548 (*SLC7A11)* were replicated. DNA methylation at *SLC7A11* was associated with reduced risk of hepatic steatosis in participants (odds ratio, 0.69; 95% CI= (0.55 - 0.93; *P*-value: 2.7×10^{-3}). In functional experiments, *SLC7A11* was highly expressed in human liver cells; its expression is positively correlated with expression of a panel of lipid-associated genes, indicating a role of *SLC7A11* in lipid metabolism.

Conclusions

Our results provide new insights into epigenetic mechanisms associated with markers of liver function and hepatic steatosis, laying the groundwork for future diagnostic and therapeutic applications.

INTRODUCTION

High concentrations of liver enzymes including gamma-glutamyl transferase (GGT), alanine aminotransferase, and aspartate aminotransferase (ALT, AST) are used as markers of liver injury (1). There is strong evidence of prospective associations between liver enzymes and hepatic steatosis (2), cancer (3), type 2 diabetes (4), cardiovascular disease (5) and all-cause mortality (6). Moreover, abnormal liver function is a common reason for terminating new clinical therapeutic agents, representing a major challenge for the global pharmaceutical industry (7).

Liver enzymes can be influenced by drug therapy or lifestyle factors such as diet, physical activity, alcohol consumption and smoking (8). Several studies have also revealed a genetic impact on liver enzyme levels (9). Genome-wide association studies identified a total of 42 genetic loci associated with liver enzymes, explaining up to 2.3% of the variation (10, 11). The role of epigenetic determinants are increasingly being recognized as an important link between environmental exposures, genetic determinants and disease risk (12). DNA methylation, the major type of genetic mark, refers to the addition of a methyl group to cytosine at Cytosine-Guanine dinucleotides (CpG) that further influences the function of DNA: activating or repressing expression levels of genes (13). Unlike genetic variation, DNA methylation is dynamically remodeled over time and can be affected by environment; methylation could therefore, influence liver enzymes, and vice versa. So far, no previous study has been investigating differential methylation in relation to liver function.

Hence, we performed the first epigenome-wide association studies (EWAS) of DNA methylation in whole blood for liver enzymes GGT, ALT, and AST in 731 participants of the Rotterdam Study, a population-based cohort study. Further, we investigated whether the identified methylation probes are associated with expression levels of nearby genes and whether genetic variants are determining methylation. Subsequently, the relationship between observed DNA methylation changes and hepatic steatosis was explored. Finally, we integrated these results and performed experimental studies to provide evidence for the function of the identified gene in relation to hepatic steatosis.

METHODS

Study population

This study was performed among participants of the prospective population-based Rotterdam Study. In 1989, all 10,275 residents aged 55 years or older in Ommoord, a suburb of Rotterdam, were invited to participate in the study. In 2000, the Rotterdam Study was extended by including 3,011 participants that moved to Ommoord or people who turned 55 (RS-II). The third cohort was formed in 2006 and included 3,932 participants 45 years and older (RS-III). Participants have been re-examined every 3-4 years and have

been followed up for a variety of diseases. The Rotterdam Study has been approved by the medical ethics committee according to the Population Screening Act: Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. All participants in the present analysis provided written informed consent to participate and to obtain information from their treating physicians. A more detailed description of the Rotterdam Study can be found elsewhere (14).

Discovery and replication panel

For the discovery panel, we used data from RSIII-1 compromising 3,932 participants examined between February 2006 and December 2008. EWAS measurements were performed on a random subset of 731 subjects. We sought replication of the identified CpG sites in a set of 719 participants from the third visit of RS-II (468 individuals) and the second visit of RS-III (251 individuals), after excluding 48 participants with missing values on any of the liver enzymes. The individuals in the replication study were not included in the discovery study.

Liver enzyme measurements and hepatic steatosis

Blood samples were collected by venipuncture, and immediately frozen (−20°C). Serum GGT, ALT and AST levels, were determined within two weeks using a Merck Diagnostica kit on an Elan Autoanalyzer (Merck). Non-fasting samples were considered acceptable as fasting status does not greatly affect serum liver enzyme levels (15). Abdominal ultrasonography was performed by a certified and experienced technician (Pavel Taimr) on Hitachi HI VISION 900. Images were stored digitally and re-evaluated by a single hepatologist with more than ten years of experience in ultrasonography. The diagnosis of steatosis was determined by the ultrasound technician according to the protocol by Hamaguchi et al (16). Study covariates information is found in the supplementary material.

DNA methylation data

DNA was extracted from whole peripheral blood (stored in EDTA tubes) by standardized salting-out methods. Genome-wide DNA methylation levels were measured using the Illumina Human Methylation 450K array (17). In short, samples (500ng of DNA per sample) were first bisulfite-treated using the Zymo EZ-96 DNA-methylation kit (Zymo Research, Irvine, CA, USA). Next, samples were hybridized to the arrays according to the manufacturers' protocol. The methylation proportion of a CpG site was reported as a beta-value ranging between 0 (no methylation) and 1 (full methylation). The data preprocessing was additionally performed in both datasets using an R programming pipeline based on the pipeline developed by Tost & Toulemat (18), which includes additional parameters and options to preprocess and normalize methylation data directly from idat files. 11,648

probes at X and Y chromosomes were excluded to avoid gender bias. These filtering criteria left us with 731 samples and 463,456 probes in the first dataset, and 767 samples and 419937 probes in the second dataset. The raw beta values were then background-corrected and normalized using the DASEN option of the WateRmelon R-package (19).

Liver cell lines

Human hepatoma cell lines including Hep3B, Huh6, Huh7, PLC/PRF/5, SNU182, SNU398, and SNU449 were cultured in Dulbecco's modified Eagle's medium (Invitrogen-Gibco, Breda, the Netherlands) complemented with 10% (v/v) fetal calf serum (Hyclone, Lonan, UT), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Invitrogen-Gibco). The hepatoblastoma cell line HepG2 was cultured on fibronectin/collagen/albumin-coated plates (AthenaES) in Williams E medium (Invitrogen-Gibco, Breda, the Netherlands) complemented with 10% (v/v) fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. The human liver progenitor cell line HepaRG was cultured in William's E medium supplemented with 10% (v/v) fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin (Sigma-Aldrich, St. Louis, MO), and 50 µM hydrocortisone hemisuccinate (Sigma-Aldrich, St. Louis, MO). Identity of all cell lines was confirmed by STR genotyping.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was isolated with a Machery-NucleoSpin RNA II kit (Bioke, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was synthesized from total RNA using a cDNA Synthesis Kit (TAKARA BIO INC). The cDNA of all target genes was amplified for 50 cycles and quantified with a SYBRGreen-based real-time PCR (Applied Biosystems) according to the manufacturer's instructions. GAPDH was considered as a reference gene to normalize gene expression. Relative gene expression was normalized to GAPDH using the formula $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{control}$). All the primer sequences are included in Supplementary Table 1.

Gene knockdown by lentiviral vectors

Lentiviral pLKO knockdown vectors (Sigma–Aldrich) targeting SLC7A11 or control were obtained from the Erasmus Biomics Center and produced in HEK293T cells. After a pilot study, the shRNA vectors exerting optimal gene knockdown were selected (shRNA sequence: CCGGCCCTGGAGTTATGCAGCTAATCT CGAGATTAGCTGCATAACTC-CAGGGTTTTTG; target sequence: CCCTGGAGTTATGCAGCTAAT). Stable gene knockdown cells were generated after lentiviral vector transduction and puromycin (2.5 µg/ml; Sigma) selection.

Statistical analyses

GGT, ALT and AST levels were log-transformed using a natural-log to obtain normal distribution. The characteristics of the discovery and replication population were compared using linear regression models adjusted for age, sex and cohort (when appropriate) for continuous variables and logistic regression models for categorical variables. In the discovery stage, we modeled associations between Dasen normalized beta-values of the CpG sites as the dependent variable and log-transformed measure of GGT, ALT, and AST as the independent variable. We used linear mixed effect models adjusting for age, sex, white blood cell proportions and technical covariates (array number and position on array). Technical covariates were modeled as random effects. Estimated leukocyte proportions (B-cells, CD4+ T-cells, CD8+ T-cells, granulocytes, monocytes and NK-cells) were calculated as described by Houseman and implemented in the minfi package in R (20, 21). We corrected for multiple testing using a robust Bonferroni corrected P-value of $1.0 \times 10\text{-}7$ as the threshold for significance (0.05 / 463,456 probes). The probes identified in the discovery analysis were tested for replication in the independent samples from the Rotterdam study. We used identical models with the addition of cohort (RS-II or RS-III) as a variable in the model to adjust for a potential cohort effect. A Bonferroni corrected P-value of 0.05 divided by the number of significant findings in the discovery study was used as a threshold of significant replication. In addition, we performed a fixed effects meta-analysis using the inverse-variance weighted method implemented in METAL combining the discovery and replication samples (22). The first meta analyzed model was adjusted for age, sex, technical covariates and cell counts, whereas the second model was further adjusted for BMI, smoking history and alcohol consumption.

Integration of EWAS with existing genetic variation and expression data

Since DNA methylation may have an effect on gene expression, we tested the association between DNA methylation and mRNA expression levels of genes from the replicated CpG sites. We examined the association of significant CpG sites using data from five Dutch biobanks in 3,841 whole blood samples (http://www.genenetwork.nl/biosqtlbrowser/), integrating information on genetic variants influencing methylation levels of close or far-away genes (cis- and trans- methylation quantitative loci (meQTL)). Additionally, we explored if DNA methylation levels were affecting expression of near-by genes (cis- expression quantitative methylation (eQTM)). We subsequently tested the identified genetic variants for further associations with corresponding liver enzyme levels in the Rotterdam Study. Investigation of genetic confounding was carried out to identify whether the observed associations between liver enzymes and methylation levels were due to genetic variants being associated with both liver enzyme levels and DNA methylation. Enrichment analysis was performed to test whether the findings were over-represented for liver enzymes-related genes (Supplementary material). Furthermore, to investigate

whether any of the identified methylation changes are a cause or a consequence of liver enzymes, we performed bi-directional Mendelian Randomization (MR) approach using the Rotterdam Study data as well as a recently published epigenome-wide association study. In Mendelian Randomization, causality is inferred from associations between genetic variants that mimic the influence of a modifiable exposure and affect the outcome of interest. Similar to a clinical trial, two randomly assigned groups with different levels of exposure (carriers of genetic variants) are compared to each other. Because gene variants do not change over time and are assigned randomly during gamete formation, they are not prone to reverse causation and are free from confounding. More details about the MR analysis can be found in supplementary material.

Association of DNA methylation with prevalent hepatic steatosis

To assess the association of the observed liver enzyme- related CpGs with the presence of hepatic steatosis in the replication dataset, generalized linear mixed effects models were fitted using the R package lme4. Three models were analyzed. The first model was adjusted for age, sex, smoking history and whole blood cells proportions. In the second model, we additionally included body mass index, alcohol consumption, coffee intake, hypertension medication, lipid levels (HDL and triglycerides), glucose levels, and in the third model liver enzymes were further included. We used Bonferroni corrected p-value of 0.05 divided by the number of significant CpGs as a threshold for significance for the clinical outcome association. The same was done for fatty liver index in the discovery set.

All analysis was performed using the statistical package R, version 3.0.2. Figure 1 depicts an overall overview of the study flow.

Note: Supplementary Material/Appendix can be found in the website of the published journal or can be provided on request.

RESULTS

Characteristics of all 1,450 participants under study are summarized in Table 1. Discovery and replication data sets did differ significantly with regards to mean age of participants. Although AST and ALT mean levels were different between the two samples, the cohort effect was not significant in a regression model with liver enzymes as outcome and age, sex, cohort as covariates. In the discovery sample, the mean age of participants was 59.6 years; 54% were female. No differences were observed for BMI, smoking history, alcohol consumption or other cardiometabolic risk factors. All participants were from European ancestry.

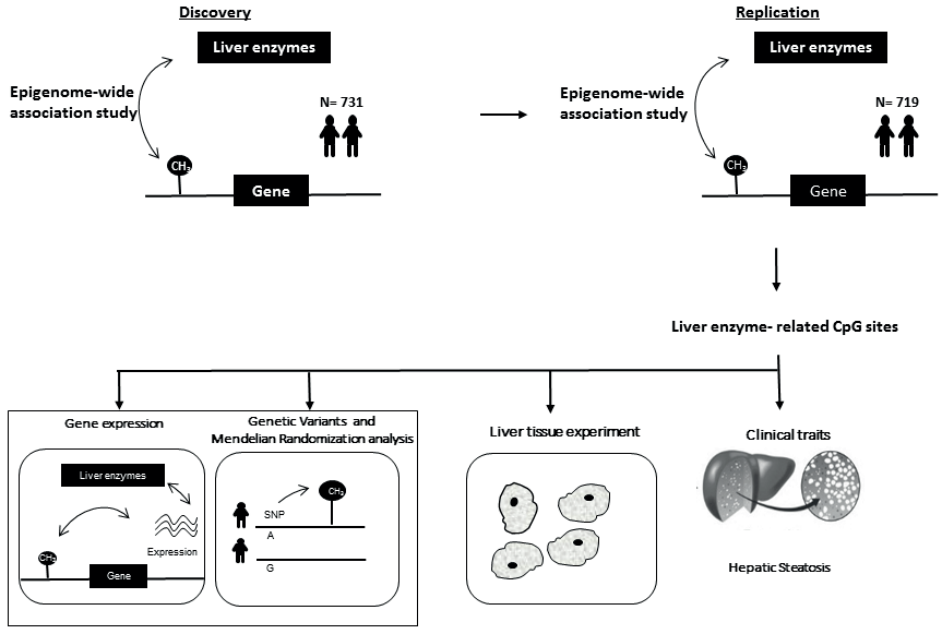

Figure 1. Illustration of overall study flow.

Discovery

Eight CpG sites in 7 unique loci were associated with GGT levels (P-values varying from 1.1 × 10-8 to 7.6 × 10-14). Table 2 shows the DNA methylation probes significantly associated with liver enzymes. The most significant CpG site in SLC7A11 with GGT levels, cg06690548 (P-value = 1.5 × 10-14), was also the only epigenome-wide significant hit for ALT (P-value = 1.8 × 10-8). None of the CpG sites investigated was associated with AST levels after correction for multiple testing. Manhattan plots for the association of GGT and ALT with markers of methylation are shown in Figure 2 A and Figure 2 B.

Replication

We replicated findings from our discovery set in a replication data set of 719 independent participants of the second and third cohort of the Rotterdam Study using a Bonferroni threshold of < 6.2 × 10-3. We significantly replicated four CpG signals (cg06690548, cg11376147, cg22304262, cg14476101) for GGT and one CpG signal (cg06690548) for ALT (Table 2).

A meta-analysis combining the two data sets resulted in six new CpG sites significantly associated with GGT, and one for AST (cg23734418) (Supplementary Table 2). We repeated the analyses in a second model further adjusted for BMI, smoking history and alcohol consumption to test for potential confounding. The effect estimates did not change substantially between the two models.

Table 1. Characteristics of Rotterdam Study participants

Variable	Discovery (N= 731)	Replication (N= 719)
Age (years)*	59.6 (46.4 - 89.3)	67.5 (51.5 - 79.9)
Female, N (%)	395 (54)	437 (57)
AST (U/L)	24.9 (9 - 596)	25.4 (10 - 91)
ALT(U/L)	27.8 (20 - 90)	20.9 (1 - 121)
GGT(U/L)	32.2 (2 - 197)	30.2 (4 - 188)
BMI (kg/m^2)	27.4(10 - 50.2)	27.7 (17.6 - 47.6)
Smoking history (%)		
Current smoking	195 (27)	77 (11)
Former smoker	319 (44)	393 (55)
Never smoker	210 (29)	246 (34)
Alcohol consumption (%)		
Never	69 (10)	99 (14)
1 time per month	97 (13.3)	95 (13)
2-4 times per month	97 (13.3)	95 (13)
2-3 times per week	171 (23.3)	133 (18)
4 or more times per week	295 (40)	294 (41)
Hepatic Steatosis	-	280 (40)
Coffee intake (g/day)	-	406 (0 - 1276)
Fasting glucose (mmol/l)	5.4 ± 1.21	5.4 ± 1.15
HDL-cholesterol (mmol/l)	1.4 ± 0.40	1.5 ± 0.44
Triglycerides (mmol/l)	1.3 (0.9 - 1.8)	1.4 ± 0.85
Hypertensive medication	315 (50)	310 (43)

Abbreviations: ALT, Alanine aminotransferases; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma-glutamyl transferase; N, number. Data are mean ± SD or median (interquartile range) or N (%).

Association with hepatic steatosis

The CpGs associated with liver enzymes were tested for an association with hepatic steatosis as diagnosed by ultrasound measurements in the replication dataset at Bonferroni corrected threshold P-value < 0.012. Three models were used and cg06690548, located in SLC7A11 gene, showed an association with hepatic steatosis in all three models (model 3: OR (95% CI) = 0.69 (0.55 - 0.87); P- value < 2.2 × 10-3) (Table 3).

Integration of EWAS, genetic variation, and expression data

The top four CpG sites were further explored in association with expression levels (eQTM) or genetic variants (meQTL) in near-by or far-away genes (Supplementary Table 3). Except for SLC7A11, all other CpGs showed to be associated with genetic variants of near-by genes. Two SNPs located on chromosome 2 revealed genetic effects with

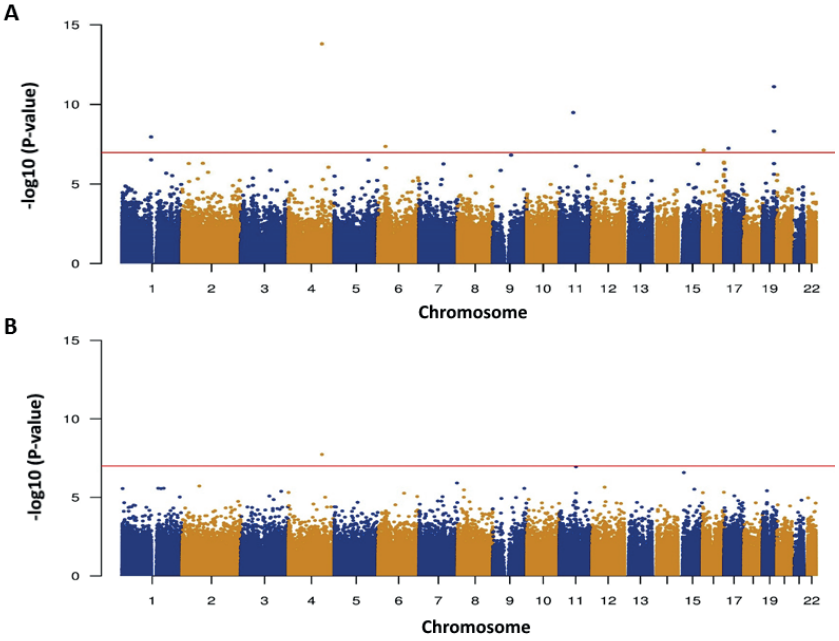

Figure 2 A). Manhattan plot of epigenome-wide results testing for association between and the methylation status of GGT levels. The x-axis displays the chromosome on which the CpG is located, and the y-axis displays $-\log_{10}$ (*P*-value). The red horizontal line represents the Bonferroni-adjusted p value threshold.
Figure 2 B). Manhattan plot of epigenome-wide results testing for association between and the methylation status of ALT levels. The x-axis displays the chromosome on which the CpG is located, and the y-axis displays $-\log_{10}$ (*P*-value). The red horizontal line represents the Bonferroni-adjusted p value threshold.

Table 2. Epigenome-wide association between DNA methylation and liver enzymes (GGT, ALT and AST)

CpG	Chr	Gene	Position	Discovery				Replication			
				Effect	SE	*P*-value	N	Effect	SE	*P*-value	N
GGT											
cg06690548*	4	*SLC7A11*	139162808	-0.011	0.001	1.5×10^{-14}	709	-0.015	0.002	**3.9×10^{-9}**	699
cg02711608	19	*SLC1A5*	47287964	-0.015	0.002	7.6×10^{-12}	722	-0.004	0.002	0.045	714
cg11376147*	11	*SLC43A1*	57261198	-0.011	0.001	3.2×10^{-10}	722	-0.004	0.001	**0.004**	714
cg22304262*	19	*SLC1A5*	47287778	-0.011	0.002	4.8×10^{-9}	722	-0.008	0.002	**0.001**	713
cg14476101*	1	*PHGDH*	120255992	-0.017	0.003	1.1×10^{-8}	722	-0.017	0.003	**6.7×10^{-6}**	714
cg04095776	6	*PSORS1C1*	31106941	-0.009	0.001	4.3×10^{-8}	722	0.002	0.002	0.31	713
cg11024682	17	*SREBF1*	17730094	0.009	0.001	5.7×10^{-8}	722	0.003	0.001	0.09	713
cg03497652	16	*ANKS3*	4751569	0.016	0.003	7.5×10^{-8}	722	0.008	0.003	0.007	714
ALT											
	4	*SLC7A11*	139162808	-0.013	0.0023	1.8×10^{-8}	705	-0.012	0.0035	0.0004	703

Abbreviations: Chr, chromosome; SE, standard error; N, number of participants. *CpG with association confirmed by replication dataset; significance threshold: 1.1×10^{-7} (discovery cohort), 0.0063 (replication cohort). Effect represent the change in methylation proportion per unit change in liver enzyme level (log(U/L)).

Table 3. Association of differential DNA methylation at the top CpGs with hepatic steatosis.

			Hepatic Steatosis (n= 280)					
			Model 1		Model 2		Model 3	
Gene	CpG	Chr	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
SLC7A11	cg06690548	4	0.66 (0.49 - 0.82)	$\mathbf{1.09 \times 10^{-6}}$	0.68 (0.55 - 0.85)	$\mathbf{7.7 \times 10^{-4}}$	0.69 (0.55 - 0.87)	$\mathbf{2.2 \times 10^{-3}}$
SLC43A1	cg11376147	11	0.78 (0.62 - 0.95)	$\mathbf{4.38 \times 10^{-3}}$	0.81 (0.66 - 1.01)	0.06	0.81 (0.65 - 1.00)	0.05
PHGDH	cg14476101	1	0.75 (0.58 - 0.91)	$\mathbf{6.87 \times 10^{-4}}$	0.80 (0.65 - 0.99)	0.04	0.78 (0.62 – 0.97)	0.02
SLC1A5	cg22304262	19	0.72 (0.55 - 0.88)	$\mathbf{9.58 \times 10^{-5}}$	0.77 (0.62 - 0.94)	0.01	0.80 (0.65 - 1.00)	0.05

Abbreviations: Chr, chromosome; OR 95% CI, odds ratio 95% confidence interval; n, number of cases; SE, standard error; *significant: level of significance *P*-value < 0.012

Model 1 adjusted for age, sex, granulocytes, lymphocytes, monocytes, cohort.

Model 2 adjusted as model 1 plus alcohol consumption, smoking history, coffee, BMI, hypertension, triglycerides, HDL, glucose levels.

Model 3 adjusted as model 2 plus liver enzyme levels

methylation levels at SLC1A5. Using data from the Rotterdam study, we found a suggestive association between rs41276626 in PHGDH and GGT levels [rs41276626: beta (se): 0.03 (0.01); P-value= 0.008)] (Supplementary Table 4). Adjusting the model for this SNP did not affect the association between GGT levels and DNA methylation at PHGDH. Cg14476101 (PHGDH) was the only CpG associated with expression levels of nearby genes (P-value= 2.05 × 10-55) (Supplementary Figure 1).

Using information provided by the Illumina array manufacturer, we functionally annotated the top hits and found that four of them (cg06690548, cg11376147, cg14476101, cg22304262) are located in the respective gene bodies. One of them (cg11376147) represents an enhancer element (bioinformatically determined) and another (cg14476101) is located within a reprogramming-specific differentially methylated region.

Liver enzymes related genes

We analyzed 781 probes representing genes identified by genome-wide association studies associated with GGT and 46 probes representing ALT-related GWAS genes. Collectively, the methylation probes were enriched for association with GGT (Fisher combined probability P-value = 0.0024), but not for ALT (P-value = 0.3).

Mendelian Randomization approach

In reference to Supplementary Table 5, the predicted and observed estimates were in opposing directions for SLC43A1 and PHGDH. For SLC7A11, the estimates were consistent but not significantly different (P value =0.07). This analysis suggests supportive evidence of our hypothesis that methylation at SLC7A11 might be a cause of GGT, but not for the other two genes.

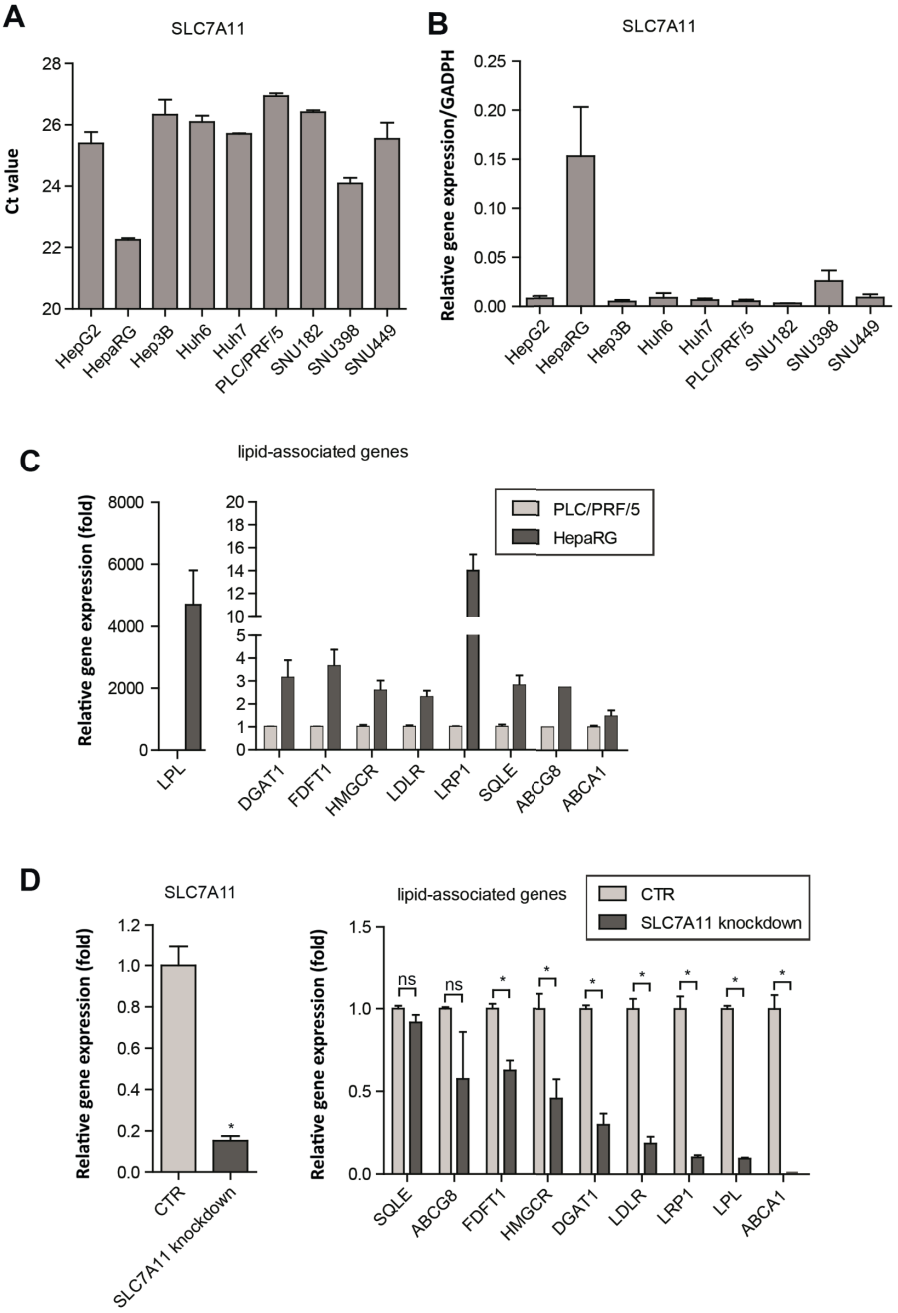

Figure 3 A). Expression levels of *SLC7A11* in 9 different human liver cell lines. The expression level of SLC7A11 was quantified by qRT-PCR. The Ct values of SLC7A11 are shown in the Y axes of the figure (lower Ct value indicates higher level of gene expression). The results were presented as mean ± SEM (n=3).

Figure 3 B). The relative expression levels of *SLC7A11* against *GAPDH* (reference gene) are shown (n=3). HepaRG cells have the highest level of *SLC7A11* expression relative to GAPDH.
Figure 3 C). The expression levels of *SLC7A11* and 9 lipid-associated genes in PLC/PRF/5 and HepaRG cell lines are shown. The relative gene expression levels were quantified by qRT-PCR (n=3). This figure shows that compared with PLC/PRF/5 cells, HepaRG cells express much higher levels of the lipid-associated genes, indicating a positive correlation between *SLC7A11* and lipid-associated genes, in particular LPL. *GAPDH* serves as a reference gene, and the gene expression levels in PLC/PRF/5 set as 1.
Figure 3 D). Knockdown of SLC7A11 gene by lentiviral shRNA vectors in HepaRG cells results in significant decrease in expression of 7 out of the 9 lipid-associated genes. The experiments were repeated 5 times and the results were presented as mean ± SEM. *GAPDH* serves as a reference gene, and the gene expression levels in CTR cells set as 1. Comparisons between groups were performed with Mann-Whitney test. Differences were considered significant at *P*-value < 0.05.

As shown in Supplementary Table 6, the predicted and observed beta estimates are opposing for SLC7A11 suggesting that it's not likely that methylation at the CpG is affected by GGT levels. Notably, for SLC43A1 and PHGDH the expected and observed effect estimates are consistent and not significantly different (P = 0.55 and P = 0.11), supporting the hypothesis that methylation at these CpG sites might be a consequence of GGT levels.

The potential role of *SLC7A11* in lipid metabolism

To experimentally show the importance of SLC7A11 in liver function, we first examined the expression levels of this gene in 9 different human liver cell lines as shown in Figure 3A. This experiment showed that SLC7A11 has relatively high expression levels in all these cell lines with an exceptional abundance in HepaRG cell line (Figure 3B). We further selected two of these cell lines, HepaRG and PLC/PRF/5, with opposite profiles of the expression levels of SLC7A11, specifically, high and low expression levels, and compared with the relative expression levels of a number of important lipid-associated genes reported in previous studies (23, 24). Compared with PLC/PRF/5 cells, HepaRG cells expressed much higher levels of the lipid-associated genes indicating a positive correlation between expression of SLC7A11 and the lipid-associated genes (Figure 3C). In particular, the expression level of LPL was highly correlated with the SLC7A11 expression, approximately 5000-fold ($\Delta\Delta CT = -12.2$) higher in HepaRG than PLC/PRF/5. To have more insight into the correlation between SLC7A11 and the lipid-associated genes, we next knocked down SLC7A11 in HepaRG cells. After successfully silencing SLC7A11, we observed a significant decrease in expression levels of 7 out of 9 lipid-associated genes, specially ABCA1 and LPL (Figure 3D). These experiments strongly suggest SLC7A11 to be involved in lipid metabolism that may take place through inducing the expression of lipid-related genes.

DISCUSSION

In this study, we conducted the first EWAS of DNA methylation in whole blood for liver enzymes levels (GGT, ALT, and AST) in the Rotterdam Study. We found that differential methylation of CpG sites within SLC7A11, SLC43A1, SLC1A5, PHGDH were associated with GGT levels. Methylation differences at SLC7A11 were associated with ALT levels as well. In addition, we demonstrated that cg06690548 (SLC7A11) was directly associated with hepatic steatosis. Finally, we showed that SLC7A11 is abundantly expressed in human liver cells and its expression is positively correlated with lipid-associated genes such as LPL. Collectively, our findings provide evidence in support of cross talk between genetic and epigenetic features and liver enzymes at the identified genetic loci.

The most significant replicated CpG is located at SLC7A11 gene and was found to be associated with GGT, ALT levels and hepatic steatosis. DNA methylation is one of the epigenetic mechanisms that cells use to control gene expression, indicating that the identified CpG may alter SLC7A11 expression. Our knockdown experiment showed that reduced expression of SLC7A11 in liver cells resulted in alterations in lipid metabolism as assessed by a significant decrease of expression levels of lipid-associated genes. These findings suggest the potential role of SLC7A11 in respect to lipid homeostasis in the liver and consequently, liver related phenotypes such as hepatic steatosis. As an amino acid transporter of cysteine and glutamate, SLC7A11 might be implicated in hepatic oxidative stress through GGT enzyme. GGT has pro-oxidative properties that may promote lipid peroxidation followed by activation of inflammatory response stellate cells that lead to fibrogenesis in hepatic steatosis (25). Our EWAS results, supported by literature and our experimental findings, suggest that DNA methylation at SLC7A11 plays a role in lipid metabolism in the general population. We found an inverse strong association between cg06690548 and serum triglycerides levels in the Rotterdam Study (meta-analysis results in 1,485 individuals (Effect=-0.008, P-value= $3.9 \times 10\text{-}8$) (26), and the association was further confirmed by a recent epigenome wide association study of lipid levels in 3,187 individuals (27). Additionally, cg06690548 was implicated in metabolic risk; DNA methylation changes at the site were reported to be inversely associated with diabetes (Effect= 0.16, P-value= 0.005) and insulin resistance as measured by HOMA-IR (Effect=-0.16, P-value= $8.1 \times 10\text{-}7$)(28). Altogether, we present both molecular and genetic association evidence to support the conclusion that the function of SLCA11 is important for liver enzymes and hepatic steatosis as a key molecular regulator of lipid-associated genes.

Our second top hit, PHGDH, encodes the phosphoglycerate dehydrogenase enzyme, which catalyzes the first step in the phosphorylation pathway of serine biosynthesis. In fact, serine deficiency has been reported in a reconstructed genome-scale metabolic model of hepatocytes among patients with non-alcoholic fatty liver disease (29). Similarly, serine was demonstrated to ameliorate alcoholic fatty liver by accelerating

serine–dependent homocysteine metabolism in mice and rats (30). Methylation of the same locus has been linked to body mass index and waist circumference in recent EWAS studies (31, 32). PHGDH represents an interesting gene for further research as methylation and cis gene expression has been reported to be strongly associated not only in blood, as we have shown with our data, but also in liver tissue (32).

Two other replicated loci for GGT, SLC43A1 and SLC1A5, are coding genes for protein transporters including amino acids such as glutamine and asparagine, glucose, bile salts, organic acids and metal ions in intestine, liver and kidney (33, 34). SLC1A5 is glutamate-transporter reported to facilitate the uptake of glutamine in tumor cells, which in turn, serves multiple metabolic functions within cells depending on the tumor oncogenotype and microenvironment (35). Drugs such as Tamoxifene and Raloxifene targeting SLC1A5, are used to suppress the proliferation of estrogen receptor-negative cells through inhibition of glutamine uptake (36). While SLC1A5 role in oncogenesis is well-characterized, our findings warrant further examination of its role in cardiometabolic risk profile. Previously, SLC7A11, SLC1A5, PHGDH and SLC43A1 have been reported to be associated with adiposity (32). An elegant work by Wahl et al. on epigenome wide association study of adiposity measured by body mass index, reported three of our top hits SLC7A11, SLC43A1 and PHGDH to be significantly associated with BMI levels as well. Methylation at SLC7A11 showed consistent direction of association between blood, liver and adipose tissue, whereas for SLC43A1 and PHGDH, consistency was shown only between blood and liver tissue suggesting that the relationship between methylation marks are highly likely to be shared across tissues. In another study, SLC7A11, SLC1A5, PHGDH were reported to be linked to blood concentrations of metabolites related to steroid hormones that are upregulated in obesity (37, 38). However, the results are suspected to be driven by external common factors such as smoking and BMI seem to be an important mediator. To investigate whether our findings are confounded by such factors, we further adjusted for smoking history, body mass index and alcohol consumption in a second model. Although a decline in beta estimates indicate a potential confounding effect of these factors, the significance of the results imply that other pathways might be linked with these genes and liver enzymes.

Assessing direction of causality remains an important issue to be explored in epigenetics. In an attempt to investigate the nature of the association by employing Mendelian Randomization approach, we found suggestive evidence for methylation at SLC7A11 to be a cause of altered GGT levels, whereas differential methylation at SLC43A1 and PHGDH might be a consequence of GGT. However, these results should be interpreted with caution and further well-powered studies with adequate sample size should confirm these findings.

The combined analysis of the discovery and replication panel identified further loci including ANKS3, ABCG1, and CPT1A highlighting a continued benefit of the EWAS

approach by using larger sample size studies to infer new biology. While ANKS3 is a protein coding gene with unknown function, DNA methylation in ABCG1 has recently been linked to glycemic traits and type 2 diabetes (39, 40). More specifically, ABCG1 is involved in macrophage cholesterol and phospholipid transport, the dysregulation of which might be involved in the pathogenesis of cardiovascular and malignant diseases (41). CPT1A is a protein coding gene important in the mitochondrial transport of carnitine resulting in decreased rate of fatty acid beta-oxidation. DNA methylation in this gene has been recently reported to be associated with lipids and metabolic syndrome (42, 43). GGT is known to be involved in pathophysiology of cardio-metabolic outcomes including hepatic steatosis, type 2 diabetes and metabolic syndrome (44). The genes identified contributing mostly to the modulation of cholesterol and lipid metabolism could play a key role in the pathway from altered liver enzyme levels to the development of complex metabolic diseases.

We identified a protective association between cg06690548 (SLC7A11) and hepatic steatosis: DNA methylation levels of cg06690548 are inversely associated with steatosis of the liver compared to healthy individuals. Except for SLC7A11, the associations in the other loci did not remain independent after controlling for several cardio-metabolic risk factors. Over the recent years, it has become clear that epigenetic features might hinder a high predictive value for disease diagnosis, prognosis or risk stratification (45). Other studies are encouraged to investigate whether methylation differences could enable future large-scale screens for candidate epigenetic biomarkers important to predict future risk of hepatic steatosis, resulting in earlier detection of new cases and introducing new potential therapeutic interventions

The interpretation of our results should be made in light of strengths and limitation of the current study. The strengths of this study include the large sample size of healthy adults with available data on DNA methylation with internal replication, the use of complementary data such as expression levels and genetic variation, further integration with clinical outcome data and finally, validation with in vitro experiments for the top identified gene. Our study, however, has some limitations. We used whole blood samples for the quantification of DNA methylation in relation to liver enzymes and therefore, might have missed CpGs important to other relevant tissues such as liver. While these identified CpGs represent only a subset of epigenetic markers associated with liver enzymes, that does not affect their validity. Additionally, fat accumulation for the diagnosis of hepatic steatosis was done using ultrasound, which is a qualitative technique and is less precise than quantitative imaging techniques such as MRI in diagnosis of liver fat, however, it is a widely accepted modality in large population-based studies. Methylation levels and liver enzymes were measured at the same time point, making it challenging to understand whether specific DNA methylation marks are affecting or being affected

by liver enzymes or their determinants. Finally, although we adjusted for several cardiometabolic risk factors, we cannot rule out residual confounding.

In conclusion, this is the first epigenome-wide association study of liver enzymes in whole blood. We identified four differentially methylated sites for liver enzymes including SLC7A11, SLC43A1, SLC1A5 and PHGDH of which SLC7A11 was independently associated with hepatic steatosis. We provide experimental evidence suggesting that SLC7A11 play a role in lipid metabolism through regulating the expression of known lipid genes. While the current study sheds light on the epigenetic and genetic mechanisms associated with liver enzymes, future efforts in larger different population based studies could lead to identification of new differentially methylated sites.

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Chapter 4.4

A peripheral blood DNA methylation signature of hepatic fat reveals a potential causal pathway for non-alcoholic fatty liver disease

Jiantao Ma[1]*, Jana Nano[2,3]*, Jingzhong Ding[4]*, Yinan Zheng[5]*, Rachel Hennein[1], Chunyu Liu[1,6], Elizabeth K. Speliotes[7], Tianxiao Huan[1], Ci Song[1,8], Michael M. Mendelson[1,9], Roby Joehanes[1], Michelle T. Long[10], Liming Liang[11, 12], Jennifer A. Smith[13], Lindsay Reynolds[4], Mohsen Ghanbari[2,14], Taulant Muka[2], Joyce van Meurs[2,15], Louise J.M. Alferink[16], Oscar H. Franco[2], Abbas Dehghan[2,17], Scott Ratliff[13], Wei Zhao[13], Lawrence Bielak[13], Sharon LR Kardia[13], Patricia A Peyser[13], Hongyan Ning[18], Lisa B. VanWagner[18,19], Donald M Lloyd-Jones[18], John Jeffrey Carr[20], Philip Greenland[18], Alice H. Lichtenstein[21], Frank B. Hu[22], Yongmei Liu[4], Lifang Hou[5], Sarwa Darwish Murad[16], Daniel Levy[1]

[1] Population Sciences Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland and the Framingham Heart Study, Framingham, Massachusetts, USA
[2] Department of Epidemiology, Erasmus University Medical Center, Rotterdam, the Netherlands
[3] Helmholtz Zentrum München, German Research Center for Environmental Health, Institute of Epidemiology, Neuherberg, Germany
[4] Department of Epidemiology and Prevention, Wake Forest School of Medicine, North Carolina, USA
[5] Center for Population Epigenetics, Robert H. Lurie Comprehensive Cancer Center and Department of Preventive Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA
[6] Department of Biostatistics, Boston University, Boston, Massachusetts, USA
[7] University of Michigan Medical School, Ann Arbor, Michigan, USA
[8] Department of Medical Sciences, Molecular epidemiology, Uppsala University, Sweden
[9] Department of Cardiology, Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts, USA
[10] Section of Gastroenterology, Department of Medicine, Boston University School of Medicine, Boston, Massachusetts, USA
[11] Department of Epidemiology, Harvard T.H. Chan School of Public Health
[12] Department of Biostatistics, Harvard T.H. Chan School of Public Health
[13] Department of Epidemiology, School of Public Health, University of Michigan, MI, USA
[14] Department of Genetics, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[15] Department of Internal Medicine, Erasmus University Medical Center, Rotterdam, the Netherlands
[16] Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, the Netherlands
[17] Department of Biostatistics and Epidemiology, MRC-PHE Centre for Environment and Health, School of Public Health, Imperial College London, London, UK
[18] Department of Preventive Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA
[19] Division of Gastroenterology & Hepatology, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA
[20] Department of Radiology & Radiological Sciences, Vanderbilt University Medical Center, Nashville, Tennessee, USA
[21] Cardiovascular Nutrition Laboratory, USDA Human Nutrition Research Center on Aging at Tufts University, Boston, Massachusetts, USA
[22] Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA
* These authors contributed equally

(Submitted)

ABSTRACT

Background and Aims

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease. Methylation patterns of leukocyte DNA may reveal biomarkers and therapeutic targets to address the rising epidemic of NAFLD. We aimed to identify the peripheral blood DNA methylation signature of hepatic fat.

Methods

We conducted an epigenome-wide association study of hepatic fat in 3,400 European ancestry (EA) participants from four population-based cohort studies. Hepatic fat was measured using computed tomography or ultrasound imaging and DNA methylation was assessed at over 400,000 cytosine-guanine dinucleotides (CpGs) in whole blood or CD14+ monocytes using the Illumina BeadChip. Additionally, we implemented epigenome-wide association studies in 401 participants of Hispanic ancestry (HA) and 724 participants of African ancestry (AA).

Results

We identified 22 CpGs associated with hepatic fat in EA participants at a false discovery rate <0.05 (corresponding $p=6.9\times10^{-6}$) and replication at Bonferroni corrected $p<8.6\times10^{-4}$. Mendelian randomization analyses supported a causal contribution of hypomethylation of cg08309687 (*LINC00649*) on NAFLD ($p=1.1\times10^{-7}$). Hypomethylation at the same locus, cg08309687, was also putatively causal for increased fasting glucose (p=0.04). One of the 22 replicated CpGs in EA participants, cg19693031 (*TXNIP*) was associated with liver fat in HA participants ($p=1.7\times10^{-4}$) and none of the CpGs were significant in AA participants, after correcting for multiple testing.

Conclusion

Our study demonstrates that a peripheral blood derived DNA methylation signature is robustly associated with hepatic fat accumulation. The potentially causal CpGs may represent attractive biomarkers and therapeutic targets for NAFLD. Future studies are warranted to explore underlying mechanisms and to examine DNA methylation signatures of NAFLD across racial/ethnic groups.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) includes a spectrum of histologic features ranging from hepatic fat accumulation (steatosis) to inflammation and/or fibrosis (steatohepatitis) to end-stage cirrhosis; though steatosis is the most common phenotype [1]. The current estimated global prevalence of NAFLD is 24%, which has increased substantially along with the increasing rates of obesity worldwide [2]. NAFLD is considered to be the hepatic manifestation of metabolic syndrome due to its strong correlation to type 2 diabetes and cardiovascular disease [3] and it is currently the second leading contributor to hepatic failure necessitating transplantation [4].

A prior study in three family-based cohorts estimated the heritability of steatosis to be 27%; however, common genetic variants from genome-wide association studies (GWAS) account for less than five percent of inter-individual variance in hepatic fat [5]. Epigenetics may explain part of the inter-individual variance of steatosis. DNA methylation is the most widely studied epigenetic phenomenon and several studies have demonstrated altered DNA methylation profiles in liver biopsy samples collected from individuals with NAFLD [6, 7] . One study showed that whole blood derived DNA hypermethylation at one cytosine-guanine dinucleotide (CpG; cg06690548) located at intron of gene *SLC7A11* may be associated with a lower risk of steatosis [8]. However, in general, these studies are limited by small sample sizes to discover DNA methylation sites associated with hepatic fat accumulation.

To fill this knowledge gap, we examined the ethnicity-specific epigenome-wide association between DNA methylation at over 400,000 CpGs and hepatic fat in European ancestry (EA), African ancestry (AA), and Hispanic ancestry (HA) participants from five population-based cohort studies with hepatic fat measurements derived from noninvasive imaging. For hepatic fat-associated CpGs, we further examined their relations to genetic variants, gene expression, and regulatory functions and potential causal relations to NAFLD and impaired glycemic traits.

METHODS

Study population. The present study included multiethnic participants from five population-based cohorts including the Coronary Artery Risk Development in Young Adults (CARDIA) Study (n=757), the Framingham Heart Study (FHS; n=1,496), the Genetic Epidemiology Network of Arteriopathy (GENOA; n=150), the Multi-Ethnic Study of Atherosclerosis (MESA; n=1,256), and the Rotterdam Study (RS; n=866). We excluded participants with missing DNA methylation and hepatic fat measurements and those who reported that they consumed a high amount of alcohol, equivalent to ≥21 drinks/week in men or ≥14 drinks/week in women [1]. Depending on data availability, we excluded participants who had history of myocardial infarction and stroke, cancer (except

for non-melanoma skin cancer), or bariatric surgery. We also excluded those who used medication (e.g., tamoxifen, steroids, or amiodarone) or have diseases (e.g., hepatitis C) that could cause secondary hepatic steatosis. Cohort-specific exclusion is detailed in the Supplemental Methods. Due to potential differences in DNA methylation patterns between different ethnicities [9], we analyzed the association between DNA methylation and hepatic fat separately in EA (n=3,400), HA (n=401), and AA (n=724) participants. The protocol for each study was approved by the Institutional Review Board in each cohort. All participants provided written informed consent.

Study design. The study design flow chart is presented in Figure 1. We first conducted the epigenome-wide association studies of hepatic fat among EA participants, including both discovery and replication. We then examined differential DNA methylation in relation to hepatic fat in the HA and AA participants. We further examined the functional and regulatory annotations for the replicated CpGs and tested the potential causal associations of the identified CpGs with NAFLD and glycemic traits.

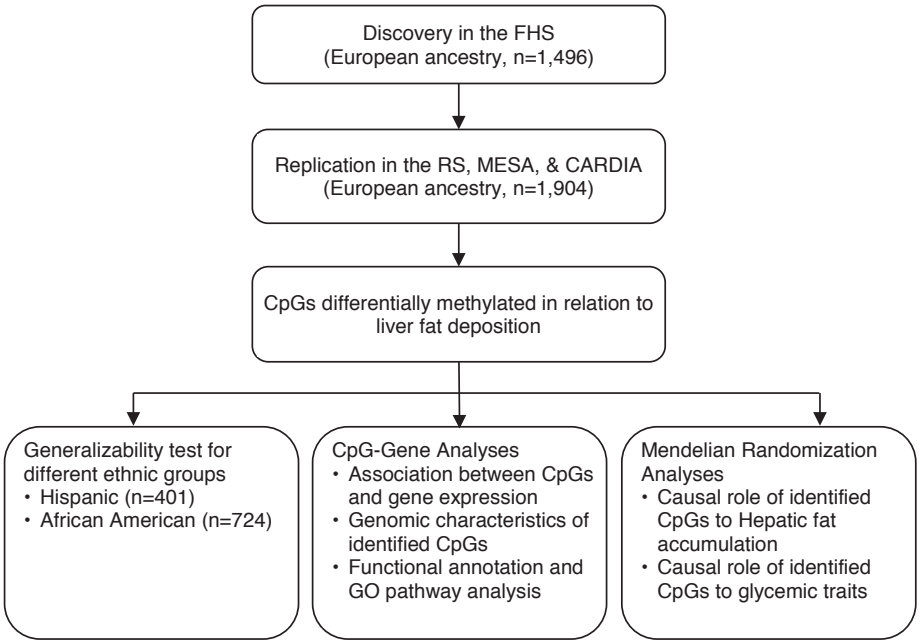

Figure 1. Study design flow chart.
Footnote: FHS=Framingham Heart Study. RS=Rotterdam Study. MESA=Multi-Ethnic Study of Atherosclerosis study. CARDIA=Coronary Artery Risk Development in Young Adults study. GO=Gene Ontology. CpG=DNA methylation site.

Hepatic fat assessment. Detailed description for hepatic fat assessment in each cohort is presented in the Supplemental Methods. The RS used ultrasound to estimate hepatic fat and diagnosed steatosis on a dichotomized scale. The other cohorts used computed tomography (CT) to quantify hepatic fat on a continuous scale by using either mean Hounsfield units of the liver image or the ratio of the Hounsfield units of the liver image to that of a control.

DNA methylation profiling. Methylation profiles were measured using DNA derived from all leucocytes in peripheral blood in the FHS, CARDIA, GENOA, and RS and from CD14+ monocytes in the MESA (Supplemental Table 1). In the FHS, GENOA, MESA, and RS cohorts, DNA methylation was assayed using the Infinium HumanMethylation 450 BeadChip, which contains 480,000 CpG sites. In the CARDIA, DNA methylation was measured using the Infinium Methylation EPIC BeadChip, which contains the majority of 450 BeadChip CpGs. Details for DNA preparation, bisulfite conversion, methylation profiling, and quality control procedures in each cohort are described in the Supplemental Methods. Raw methylation signals were normalized using various schemes, primarily the DASEN option of the *WateRmelon* R package [10]. We analyzed either methylation signal M values (in the CARDIA study; calculated as logit transformation of β values) or β values (in all other cohorts; calculated as methylated signals divided by the sum of methylated and unmethylated signals). Non-autosomal probes were excluded from the present study. For quality control purposes, we excluded study samples if they had missing more than 1-5% of methylation probes, poor SNP matching, or outliers identified by multidimensional scaling techniques. We also excluded cross-hybridizing probes and previously identified single nucleotide polymorphism (SNP) probes as described in the Supplemental Methods.

Epigenome-wide association study of hepatic fat. We conducted the discovery epigenome-wide association study in FHS and interrogated the differentially methylated CpGs at false discovery rate (FDR) <0.05 in the replication cohorts (EA samples in CARDIA, MESA, and RS). Because hepatic fat was measured using different scales, we meta-analyzed the p-values in the replication cohorts using logit method based on the general fixed effect model using *metap* R package. We also extracted and reported the direction of the association in each cohort. Linear regression models or linear mixed models with consideration of family structures were conducted to examine directionality and calculate p-values in each cohort. The statistical significance in the replication analysis was determined using the Bonferroni corrected p-value threshold, defined as 0.05 divided by the number of significant CpGs in the discovery phase. We used sex- and age-adjusted models (model 1) in the discovery and replication analyses. Estimated leukocyte composition [11] and technical variables were also adjusted for in a cohort-specific manner

(Supplemental Methods). Included in the sensitivity analyses, we conducted global meta-analyses in all EA participants to examine the impact of potential confounders. We performed the same sex- and age-adjusted model (model 1). We additionally adjusted for lifestyle factors including smoking status, physical activity levels, and alcohol intake in model 2. We further adjusted for BMI in model 3. We also performed a discovery and replication analysis using model 3 in EA participants. Covariates are assessed using cohort-specific methods (Supplemental Methods).

We conducted similar epigenome-wide association studies with adjustment for same covariates to identify hepatic fat related CpGs in MESA HA participants and AA participants in the CARDIA, GENOA, and MESA studies. Similar meta-analyses of p-values were performed for AA participants. We first tested whether the replicated CpGs in EA participants were also significant in separate analyses of HA and AA participants (at Bonferroni corrected p-value threshold). Additionally, we examined whether the significant CpGs in HA or AA participants (FDR <0.05) could be replicated in the global meta-analysis in EA participants (at Bonferroni corrected p-value threshold).

Methylation Quantitative Trait Loci (meQTL). To determine meQTLs, defined as DNA sequences that affect methylation levels at CpG sites, we analyzed the association of SNPs and DNA methylation in 4,170 FHS participants. We obtained SNP data in the FHS using Affymetrix 550K Array and imputed with the 1,000 Genomes Project reference panel [12]. We first calculated the residuals for DNA methylation using linear regression models with adjustment for age, sex, and technical covariates. We then regressed the residuals on SNPs. We defined *cis*-meQTLs as SNPs associated with DNA methylation at nearby CpGs (±500 kilobases (kb) from CpG, MAF >0.01, imputation r^2 >0.5, p-value $<1\times10^{-4}$).

Expression Quantitative Trait Loci (eQTL). We conducted eQTL analysis in 5,256 participants in FHS as previously described [13]. We excluded eQTLs (SNPs) with MAF ≤0.01, imputation r^2 ≤0.5, and p-value $\geq1\times10^{-4}$. We defined *cis*-eQTLs as SNPs residing within 500kb of a nearby gene.

Gene expression association analysis. In FHS, we profiled whole blood derived mRNA expression using the Affymetrix Human Exon 1.0 ST GeneChip platform, which contains more than 5.5 million probes for 17,873 genes [13]. We examined the associations between gene expression and DNA methylation and between gene expression and hepatic fat in FHS. To prioritize genes in these analyses, we selected Illumina-annotated genes. For CpGs without annotated genes, we identified a set of genes by overlapping *cis*-meQTLs (p-value threshold $<5\times10^{-7}$) with *cis*-eQTLs (p-value threshold $<5\times10^{-7}$). In addition, we examined the association between CpGs and nearby genes (±500kb of the CpG site).

The association between DNA methylation and gene expression was analyzed in 4,561 participants in the FHS as previously described [14]. Briefly, we first calculated the residuals for gene expression using linear regression models after adjusting for sex, age, technical covariates, and blood cell counts. We then calculated statistics by regressing residuals of gene expression on residuals of DNA methylation using linear mixed models to account for family structure. For genes significantly associated with CpGs (p-value $<5\times10^{-7}$), we further examined their association with hepatic fat using similar statistical procedures in 2,317 FHS participants. In the association analysis between selected genes and hepatic fat, we applied Bonferroni correction to account for multiple testing, i.e., 0.05 divided by the number of genes associated with CpGs. For genes that associated with both CpGs and hepatic fat, we conducted mediation tests to estimate the proportion of mediation by gene expression on the association of CpGs and hepatic fat. In this mediation analysis, we used sex- and age-adjusted linear mixed models as described above and used the quasi-Bayesian Monte Carlo method with 1000 simulations to calculate confidence intervals [15].

Mendelian randomization (MR) analysis. We conducted MR analyses (Supplemental Figure 1) to test the potential causal association from the replicated CpGs to NAFLD. Due to the well documented association between NAFLD and type 2 diabetes [16], we also examined whether the replicated CpGs were causally associated with glycemic traits including fasting glucose, fasting insulin, and type 2 diabetes. We performed MR analysis according to the procedure of the two-sample MR, which analyzed summary statistics from instrument-exposure and instrument-outcome association analyses [17]. We used independent *cis*-meQTLs (n=4,170), defined as pair-wised linkage disequilibrium (LD) r^2 <0.1, as instrumental variables (IVs). Using *TwoSampleMR* R package, we performed the primary analysis using the inverse variance weighted (IVW) method and sensitivity analysis using the MR-Egger method. The effect sizes and standard errors for IVs-CpG were obtained in FHS as described above and the effect sizes and standard errors for IVs-NAFLD were obtained from the meta-analysis of GWASs in the GOLD consortium (n=7,176) [5]. We used effect sizes and standard errors derived from previous GWASs conducted by the Meta-analyses of Glucose and Insulin-related Traits Consortium (MAGIC) [18] for IVs-fasting glucose (n up to 133,010) and IVs-fasting insulin (n up to 108,557) and by the Diabetes Genetics Replication and Meta-analysis Consortium (DIAGRAM) for IVs-type 2 diabetes (n up to 158,200) [19].

Functional and regulatory annotation. We conducted hypergeometric tests with Bonferroni correction to examine genomic characteristics of the replicated CpGs using the Infinium HumanMethylation 450 BeadChip annotation files. We queried *cis*-meQTLs in the platform of Functional Mapping and Annotation of Genome-Wide Association Studies (FUMA GWAS) [20]. Using this platform, we examined the overlap between *cis*-meQTLs

with signals in the NHGRI-EBI Catalog of published GWAS [21]. We also studied genes using differentially expressed genes measured in human whole blood and liver samples in the Genotype-Tissue Expression (GTEx v6) database [22] and visualized the genomic region for *cis*-meQTLs. To assess the relevance of the identified peripheral blood-derived CpGs in liver, we compared DNA methylation levels in whole blood with that in liver using data deposited in the Gene Expression Omnibus (GEO Series accession number GSE48472) [23]. Gene ontology (GO) biological process enrichment analysis was performed using the GO Consortium website (http://www.geneontology.org/; accessed on June 8, 2018).

Note: Other supplementary Figures/Tables can be provided on request.

RESULTS

Cohort characteristics of EA participants

The clinical characteristics of study participants are shown in Supplemental Table 1. The discovery analysis included 1,496 EA participants (mean age 59 years; 48% women) in FHS and the replication analysis included 1,904 EA participants (mean age 63 years; 52% women) from three cohort studies: RS, MESA, and CARDIA. In the additional analyses, HA participants were from MESA (n=401; mean age 68 years; 50% women) and AA participants from MESA, CARDIA, and GENOA cohorts (n=724; mean age 60 years; 61% women).

Epigenome-wide association study of hepatic fat in European ancestry participants

Of the 400,129 CpGs analyzed in age- and sex-adjusted models, 58 CpGs were significantly associated with hepatic fat in the discovery cohort (FHS) at FDR <0.05 (corresponding p-value = 6.9×10^{-6}; Supplemental Table 2; Manhattan plot is displayed in Supplemental Figure 2; QQ plot with lambda is displayed in Supplemental Figure 3). The t-statistics for the 58 CpGs were correlated between the FHS and each of the replication cohorts (CARDIA, MESA, and RS EA samples; Supplemental Figure 4). In CARDIA, hepatic fat was measured at the year 20 examination. The t-statistics calculated between hepatic fat and DNA methylation measured at the year 20 examination were highly correlated with those calculated using DNA methylation measured at the year 15 examination (Supplemental Figure 5). Twenty-four (41%) CpGs were replicated (Bonferroni corrected p-value $<0.05/58=8.6\times10^{-4}$) in the meta-analysis of the replication cohorts (n=1,904; Table 1). We removed two CpGs from the replication analysis because they were highly correlated ($|r| \geq 0.7$) and close to other CpGs with lower p-values in the association analysis (Supplemental Table 3). The two CpGs were cg16246545 (51 bases upstream of cg14476101 on chromosome 1; annotated to PHGDH; r = 0.89) and cg03068497 (76 bases downstream of cg21429551 on chromosome 7; annotated to GARS; r = 0.90). Pairwise correlations among the remaining sentinel CpGs located in the same chromosome were low to moderate ($|r|$ ranging from 0.09 to 0.54; Supplemental Table 3).

Sensitivity analysis

In the global meta-analysis of all EA participants, compared with the sex- and age-adjusted models, additional adjustment for lifestyle factors including smoking status, physical activity levels, and alcohol consumption did not materially change the association between DNA methylation levels and hepatic fat (Figure 2). Further adjusting for BMI, which is correlated with liver fat (Spearman r = 0.45 in FHS), reduced the strength of the associations (Figure 2); however, all 22 CpGs remained nominally associated with hepatic fat (p-value <0.05; Table 1). After adjusting for sex, age, lifestyle factors, and BMI, two CpGs, cg06690548 (SLC7A11) and cg19693031 (TXNIP), remained significant in FHS at FDR <0.05 (corresponding p-value = 1.2×10^{-7}; Supplemental Table 4) and in the replication samples (Bonferroni corrected p-value <0.025). Both CpGs were among the replicated CpGs in the sex- and age-adjusted analysis. Leave-one-cohort-out analysis in EA participants showed p-values in the global meta-analysis with exclusion of one cohort were highly correlated with those in all samples, r ranging from 0.83 to 0.87 (Supplemental Figure 6).

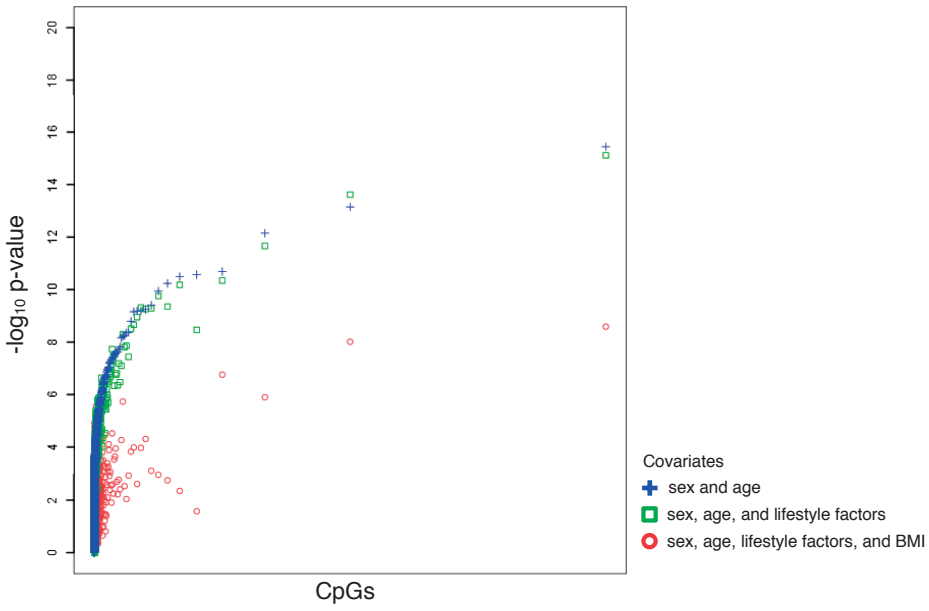

Figure 2. Comparisons of sequential adjustment models in European ancestry participants. Y-axis values are observed -log10(p-values) in the global meta-analysis of all EA participants. X-axis are sorted by CpGs (symbols at same vertical position are same CpG). Blue cross represents sex- and age-adjusted model. Green square represents model with additional adjustment for lifestyle factors (smoking, physical activity, and alcohol intake). Red circle represents the fully adjusted model including sex, age, smoking, physical activity, alcohol intake, and BMI.

Table 1. Significant CpGs in the epigenome-wide association study for hepatic fat in European ancestry participants

CpG	CHR	Position	Gene	Has *cis*-meQTLs	Primary discovery-replication analysis			Global meta-analysis		
					Discovery p-value	Replication p-value	Direction	p-value [1]	p-value [2]	p-value [3]
cg09469355	1	2161886	SKI	Yes	2.93E-08	4.07E-04	-,-,-,-	6.66E-09	7.60E-08	5.14E-05
cg17901584	1	55353706	DHCR24	Yes	4.76E-08	2.10E-04	-,-,-,-	5.28E-09	1.48E-08	2.94E-03
cg03725309	1	109757585	SARS	Yes	1.37E-06	1.29E-06	-,-,-,-	6.21E-10	4.60E-10	1.02E-04
cg14476101	1	120255992	PHGDH	Yes	7.54E-08	1.10E-05	-,+,-,-	6.67E-10	2.10E-09	9.84E-05
cg19693031	1	145441552	TXNIP	No	1.33E-12	3.29E-04	-,+,-,-	1.96E-11	4.32E-11	1.66E-07
cg06690548	4	139162808	SLC7A11	No	1.78E-15	5.27E-06	-,-,-,-	6.77E-14	2.30E-14	9.25E-09
cg05119988	4	166251189	SC4MOL	Yes	6.91E-06	7.75E-05	-,-,-,-	5.50E-08	2.06E-07	8.51E-04
cg03957124	6	37016869		No	9.08E-08	9.80E-05	-,-,-,-	4.25E-09	1.31E-08	9.10E-03
cg18120259	6	43894639		Yes	3.35E-06	3.06E-04	-,+,-,-	1.12E-07	1.22E-06	7.93E-03
cg17501210	6	166970252	RPS6KA2	Yes	1.30E-07	3.38E-07	-,-,-,-	5.53E-11	4.23E-10	1.76E-03
cg21429551	7	30635762	GARS	Yes	7.29E-09	1.85E-04	-,+,-,-	1.53E-09	2.94E-09	1.42E-04
cg11376147	11	57261198	SLC43A1	Yes	4.88E-06	8.76E-05	-,+,-,-	4.87E-08	7.11E-08	2.70E-03
cg00574958	11	68607622	CPT1A	No	3.27E-10	9.93E-06	-,-,-,-	3.05E-11	6.28E-11	4.46E-03
cg26894079	11	122954435	ASAM	Yes	5.58E-06	8.57E-04	-,-,-,-	3.94E-07	2.81E-06	3.83E-03
cg11024682	17	17730094	SREBF1	Yes	6.58E-07	2.68E-07	+,+,+,+	1.10E-10	1.67E-10	1.08E-03
cg14020176	17	72764985	SLC9A3R1	Yes	2.36E-06	7.97E-04	+,+,+,+	2.05E-07	2.73E-07	3.36E-05
cg19016694	17	80821826	TBCD	Yes	9.49E-09	2.42E-05	-,-,-,-	3.74E-10	4.92E-10	7.63E-04
cg15860624	19	3811194	ZFR2	Yes	3.46E-07	5.12E-05	+,+,+,+	5.73E-09	4.74E-09	1.76E-06
cg02711608	19	47287964	SLC1A5	Yes	2.76E-09	1.21E-04	-,+,-,-	6.23E-10	1.06E-09	2.44E-03
cg08309687	21	35320596		Yes	2.48E-07	3.57E-06	-,-,-,-	5.37E-10	5.29E-10	4.65E-05
cg27243685	21	43642366	ABCG1	Yes	1.15E-13	1.14E-05	+,+,+,+	6.77E-13	2.08E-12	1.20E-06
cg06500161	21	43656587	ABCG1	Yes	1.95E-16	3.35E-09	+,+,+,+	3.45E-16	7.33E-16	2.45E-09

The discovery-replication analysis used sex- and age-adjusted models. The global meta-analysis was conducted in all European ancestry participants. 1. Model adjusted for sex and age; 2. Model adjusted for sex, age and lifestyle covariates including smoking, physical activity, and alcohol; 3. Model adjusted for sex, age, lifestyle covariates, and BMI. '+' sign represents hypermethylation is associated with increased hepatic fat and '-' sign represents hypomethylation is associated with increased hepatic fat. CHR: chromosome; cis-meQTL: cis-methylation quantitative trait loci

Variation in hepatic fat explained by differentially methylated CpGs

In CARDIA, the 22 replicated CpGs captured 10% of interindividual variation (i.e., the adjusted R-squared) in hepatic fat after accounting for sex and age (p-value = 1.4×10^{-7}) and 4.3% of interindividual variation after additionally accounting for lifestyle factors, and BMI (p-value = 0.001). The proportion of variation explained by the 22 replicated CpGs is similar to that observed in the discovery cohort (FHS). In the FHS, the 22 replicated CpGs explained 4.6% of interindividual variation in hepatic fat after accounting for sex, age,

lifestyle factors, BMI, serum alanine transaminase, serum aspartate transaminase, and a genetic risk score for NAFLD (p-value = 1.4×10^{-12}).

DNA methylation profiles in HA and AA participants

For the 22 CpGs that replicated in the EA participants, one CpG (cg19693031; annotated to TXNIP) remained significant in the sex- and age-adjusted model in HA participants after Bonferroni correction for multiple testing (p-value $<2.3\times10^{-3}$; Supplemental Table 5). Additionally, of the 22 CpGs that replicated in the EA participants, four CpGs were nominally significant in HA participants (p-value <0.05; Supplemental Table 5) and three CpGs were nominally significant in the meta-analysis of AA participants (p <0.05; Supplemental Table 6). No CpG was detected at FDR <0.05 in HA participants. We discovered 26 CpGs at FDR <0.05 in the meta-analysis of AA participants (Supplemental Table 7), of which, two CpGs were nominally significant (p-value = 0.02 and 0.04, respectively) in the global meta-analysis of EA participants.

Functional and regulatory annotation of hepatic fat-associated CpGs

Compared to all analyzed CpGs on the microarray, the 22 hepatic fat-associated CpGs in EA participants were more likely to reside in the south shore (0 – 2kb downstream of CpG island; p-value = 5.5×10^{-4}) or south shelf (2 – 4kb downstream of CpG islands; p-value = 4.1×10^{-4}), in DNase I hypersensitivity sites (p-value = 1.7×10^{-3}), in reprogramming-specific differentially methylated regions (p-value = 3.8×10^{-4}), and in gene body regions (p-value = 2.4×10^{-4}).

The mean DNA methylation levels of the 22 replicated CpGs in EA participants measured in whole blood were moderately correlated with those measured in liver tissue [23] (Supplemental Figure 7; Pearson r = 0.59; p-value = 0.004). This suggests that whole blood derived DNA methylation markers may be useful proxies for the corresponding DNA methylation patterns in the liver.

The 22 hepatic fat-associated CpGs were annotated to 18 unique genes (Table 1; gene function described in Supplemental Table 8). A heatmap for average expression of the 18 genes in 53 specific tissue types included in GTEx [22] is provided in Supplemental Figure 8. Several genes were moderately to highly expressed in liver and adipose tissues. Based on liver-specific differentially expressed genes in GTEx, the 18 annotated genes were enriched with genes that are up-regulated in the liver, including DHCR24, SLC43A1, CPT1A, SREBF1, SC4MOL, and SLC9A3R1 (Bonferroni corrected p-value =0.005; Supplemental Table 9). Gene ontology (GO) pathway analysis for these annotated genes showed enrichment for 18 biological processes (Fisher's exact with FDR corrected p <0.05; Supplemental Table 10). The most significant enriched pathway was positive regulation of the cholesterol biosynthetic process (GO:0045542; >100-fold enrichment;

FDR adjusted p-value = 0.02), which included two known lipid-metabolism related genes, ABCG1 and SREBF1[24, 25].

GWAS analysis

We were able to identify 3,737 cis-meQTL (i.e. SNPs associated with CpGs) variants for 18 of the 22 hepatic fat-associated CpGs (Table 1) in FHS. By overlapping cis-meQTL variants with GWAS results in the NHGRI-EBI GWAS Catalog [21], we found that cis-meQTLs or strong proxies of cis-meQTLs (LD R^2 >0.8) for nine CpGs were associated with 26 unique traits in GWAS (Supplemental Table 11). For example, rs637868 for cg14476101 (PHDGH) was associated in GWAS with alanine aminotransferase (ALT) levels [26] and rs2834288 for cg08309687 (LINC00649) was associated in GWAS with abundance of gut microbiota [27].

Three-way association and mediation analysis of CpGs, gene expression, and liver fat

In FHS, seven of the 22 CpGs replicated in EA participants were associated with whole blood derived expression of six annotated genes (cg19693031 with TXNIP, cg17901584 with DHCR24, cg14476101 with PHGDH, cg06690548 with SLC7A11, cg00574958 with CPT1A, cg06500161 with ABCG1, and cg27243685 with ABCG1; Supplemental Table 12) at a p-value threshold of $<5\times10^{-7}$. In addition, cg17501210 (annotated to RPS6KA2) was associated with expression of one non-annotated cis-gene, RNASET2 (transcription start site residing 301kb downstream from cg17501210; p-value = 9.4×10^{-11}; Supplemental Table 12). Among these seven genes, expression levels of ABCG1 and CPT1A were significantly associated with liver fat in FHS (p-value = 1.2×10^{-30} and 2.0×10^{-17}, respectively). Overall, hypermethylation of cg06500161 and cg27243685 were associated with decreased gene expression of ABCG1 and increased hepatic fat, whereas, hypomethylation of cg00574958 was associated with increased gene expression of CPT1A and increased hepatic fat (Supplemental Figure 9). In addition, expression of ABCG1 and CPT1A mediated the association between corresponding CpGs and hepatic fat by ~20% and 10%, respectively (Supplemental Figure 9).

Two CpGs, cg08309687 (LINC00649) and cg18120259 (LOC100132354), reside in intergenic non-protein coding regions and had no annotated genes in the Illumina annotation database. We overlapped their cis-meQTLs (p-value $<5\times10^{-7}$) with whole-blood derived cis-eQTLs (p-value $<5\times10^{-7}$) and identified eight cis-genes for cg08309687 and one cis-gene for cg18120259 (regional plots in Supplemental Figure 10A and 10B). None of the CpG-gene pairs was significantly associated at the predefined threshold (p-value $<5\times10^{-7}$); however, five of these pairs were nominally significant (p-value <0.05; Supplemental Table 12). Among the five genes, whole blood derived expression of TMEM50B was directly associated with DNA methylation level of cg08309687 (p-value = 6.3×10^{-4}) and inversely associated with hepatic fat (p-value = 4.1×10^{-5}) in FHS (Figure 3). Expression of TMEM50B mediated 8% (95% CI: 2-16%; p-value $<2.2\times10^{-16}$) of the association

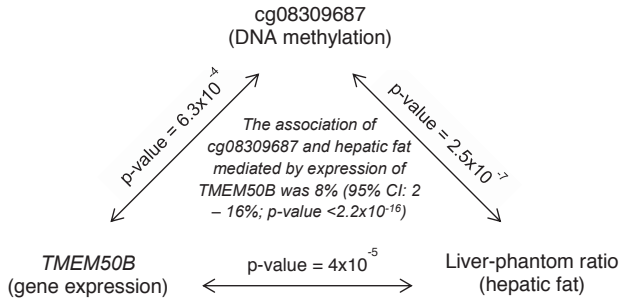

Figure 3. Three-way association of whole-blood derived DNA methylation level at cg08309687, whole-blood derived gene expression for *TMEM50B*, and CT-derived liver-phantom ratio (hepatic fat) in the Framingham Heart Study.

between cg08309687 and hepatic fat. We lacked expression data for LINC00649 in our study sample; however, in GTEx whole blood samples [22], expression level of LINC00649 was associated with expression of TMEM50B (Spearman r = 0.76, p-value $<2.2\times10^{-16}$).

MR analysis for a potential causal role of DNA methylation on NAFLD

We conducted MR analyses to identify CpGs that may be causal for NAFLD using cis-meQTLs as IVs. The IVW analysis (Supplemental Table 13) showed that hypomethylation at cg08309687 (LINC00649) was significantly associated with NAFLD (Figure 4; p-value = $1.1\text{x}10^{-7}$). In addition, hypomethylation at cg14476101 (PHDGH) was nominally associated with NAFLD (Supplemental Figure 11; p-value = 0.02). Neither CpG was significant in the sensitivity analysis using the MR-Egger method (p-value = 0.25 and 0.91, respectively). No horizontal pleiotropy effect was detected (p-value = 0.11 and 0.27).

MR analyses for CpGs in relation to glycemic traits

As depicted in Supplemental Table 14, although no CpG sites showed significant causal association with glycemic traits at the Bonferroni-corrected p-value threshold, hypermethylation of cg27243685 (ABCG1) and hypomethylation of cg08309687 (LINC00649) were nominally associated with fasting glucose concentrations (p-value = 0.01 and 0.04, respectively). Additionally, hypermethylation of cg21429551 (GARS) and cg14020176 (SLC9A3R1) and hypomethylation of cg02711608 (SLC1A5) were nominally associated with increased fasting insulin concentrations (p-value =0.02, 0.049, 0.02, respectively). Lastly, hypomethylation of cg14020176 (SLC9A3R1) was nominally associated with increased risk of type 2 diabetes (p-value =0.01).

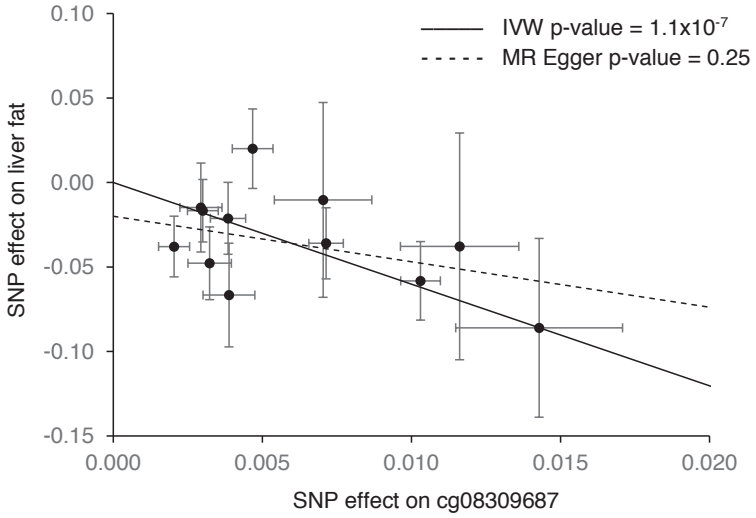

Figure 4. Potential causal association of cg08309687 to non-alcoholic fatty liver disease using Mendelian randomization (MR) analysis. Plot depicts IVW (solid line) and MR Egger estimate (dashed line). No horizontal pleiotropy effect was detected (p-value= 0.11). SNP=single nucleotide polymorphism; IVW=inverse variance weighted.

DISCUSSION

We found that differential methylation of peripheral blood derived DNA at 22 CpG sites was associated with hepatic fat in 3,400 EA participants in four population-based cohort studies. These CpGs reside at several loci regulating key biological processes relevant to the development of steatosis. Findings from the MR analyses are consistent with a potential causal role of differential DNA methylation in relation to fat accumulation and implicate steatosis as a potential causal factor for altered glucose metabolism. For example, hypomethylation at cg08309687 was associated with increased hepatic fat and higher fasting glucose concentrations. Taken together, our study demonstrates a unique peripheral blood derived DNA methylation signature for hepatic fat accumulation and provides insights into how environmental exposures may cause NAFLD and its downstream consequences mediated by DNA methylation.

Previous studies using array-based methods have examined epigenome-wide DNA methylation patterns in the liver samples of individuals with biopsy-proven NAFLD [6, 7]. While these studies showed a strong contrast between DNA methylation profiles of individuals with nonalcoholic steatohepatitis (NASH) compared with controls (i.e., individuals without steatosis or steatohepatitis), differences in DNA methylation patterns of individuals with steatosis (fatty liver alone) versus controls were less obvious [6, 7]. In contrast to prior studies using liver biopsies, we used non-invasive imaging to assess hepatic fat, which is a safe and cost-effective screening tool in population-based

studies. We therefore had a much larger sample size and more statistical power to detect epigenetic signals associated with elevated hepatic fat.

Two recent large epigenome-wide association studies identified CpGs associated with BMI [14, 28]. The majority (17 of the 22) of the replicated CpGs in the present study were also observed in at least one of the two large-scale BMI-DNA methylation studies. Several CpGs associated with both liver fat and BMI are annotated to key genes involved in lipid metabolism pathways, namely *SREBF1*, *CPT1A*, *ABCG1*, and *DHCR24* [24, 25]. These genes are in a top gene network associated with general adiposity identified in a previous analysis in MESA – a participating cohort in the present study [29]. Therefore, the overlap between liver fat associated CpGs with those linked to BMI supports a key role of adiposity and adiposity-related pathways in the pathogenesis of steatosis.

Furthermore, two hepatic fat-associated CpGs (cg14476101 and cg21429551) are annotated to *PHGDH* and *SARS*, which are key regulators involved in L-serine metabolism and have been previously related to the severity of steatosis [30]. *PHGDH* (*phosphoglycerate dehydrogenase*) encodes a key enzyme for serine synthesis. One study using genomic-scale metabolic models showed that patients with NASH had low expression levels of *PHGDH* and serine deficiency [30]. Consistent with those findings, the present study observed that hypermethylation of blood-derived DNA at cg14476101 was associated with downregulated blood-derived gene expression of *PHGDH* and increased hepatic fat accumulation. Additionally, a similar association between cg14476101 and expression levels of *PHGDH* was observed in a study comparing liver biopsy samples obtained from suspected NAFLD patients with normal control samples [28].

Our MR analyses implicated cg08309687 as a potential causal factor for NAFLD. This CpG is located in a long intergenic non-coding region (LINC00649) that may play a role in transcription regulation relevant to steatosis. We could not examine if LINC00649 mediated the observed association between cg08309687 and hepatic fat because we lacked expression data for LINC00649 in our study cohorts. However, we observed that *cis*-meQTL variants for cg08309687 coincide with *cis*-eQTLs of several nearby genes. We also established three-way associations of DNA methylation levels at cg08309687, hepatic fat, and gene expression levels for a nearby gene, *TMEM50B* (Figure 3). Together with data from GTEx, our analysis indicates DNA methylation at cg08309687 may affect LINC00649 and subsequently alter expression of nearby genes (e.g., *TMEM50B*) and impact hepatic fat accumulation. In addition, our data suggest that cg08309687 may be involved in the regulation of the gut microbiome, which has been postulated to play a critical role in the development of NAFLD [31]. Future studies are needed to explore pathways underlying the observed association between DNA hypomethylation at cg08309687 and NAFLD.

Using models with additional adjustment for BMI, we showed that two CpGs, cg06690548 (*SLC7A11*) and cg19693031 (*TXNIP*), are independently associated with

hepatic fat in the EA population. Additionally, cg19693031 replicated in HA participants, and the association was also independent of BMI (p-value = 0.03). Investigators from the RS (one of the contributing cohorts in the present study) previously knocked down *SLC7A11* in human hepatocytes (HepaRG cells) and observed up-regulation of multiple essential lipid metabolism genes, suggesting that *SLC7A11* may play an important role in hepatic lipid metabolism [8]. Although cg19693031 (*TXNIP*) has not been previously linked to obesity-related traits, several studies have shown that hypomethylation at cg19693031 (*TXNIP*) was associated with increased risk of type 2 diabetes [32]. As NAFLD is strongly associated with type 2 diabetes [16], these observations are in accordance with our finding that DNA hypomethylation at this locus is associated with increased hepatic fat.

Information regarding other CpGs highlighted in our study is limited. The DNA methylation site cg05119988 (*SC4MOL*) has been associated with blood low-density lipoprotein cholesterol concentrations [33]. The annotated gene for this CpG, *SC4MOL,* is among a key gene network associated with BMI [29]. A GWAS study showed that a genetic variant annotated to *SC4MOL* was associated with fasting insulin and glucose concentrations in Africans [34]. Experimental studies also support the role of *SC4MOL* in lipid metabolism [35, 36]. Nevertheless, additional studies are needed to examine the functions of the replicated CpGs in the present study.

To our knowledge, this is the first epigenome-wide association study investigating peripheral blood derived DNA methylation signatures of hepatic fat in the general population. However, some limitations deserve mention. The present analyses included participants from multiple ancestries. Differences in DNA methylation patterns associated with hepatic fat were observed between ethnic groups. Such variability is consistent with other observations that DNA methylation levels differ by race and/or ethnicity [9]. However, the sample size in the present study may not be sufficient for robust comparisons among the AA and HA participants. The MR analyses in the present study may be limited for a few reasons. First, the effect sizes and standard errors for the IVs were estimated using relatively small scale GWASs. Second, effect sizes and standard errors for instrument-exposure and instrument-outcome associations were estimated in partially overlapped study samples, which may lead to instrument bias [37]. Further, limitations exist due to differences in data collection methodologies among cohorts. However, we observed that DNA methylation signals were correlated among the cohorts of EA participants. As demonstrated in the CARDIA study, DNA methylation signals were relatively stable over time (Supplemental Figure 5). In addition, CpGs measured in monocytes were correlated well with those measured using whole blood samples (i.e. all leucocytes). This finding is consistent with observations from one prior BMI-DNA methylation study in which the observed associations between DNA methylation and BMI were shared across different cell subsets [28].

In conclusion, the present study demonstrates a unique DNA methylation pattern related to hepatic fat in EA participants. The unique epigenetic signature is potentially useful for predicting NAFLD. Although the mechanisms are not fully understood, our study showed that DNA methylation at several CpG sites may play causal roles in hepatic fat accumulation. These findings may be useful to design better strategies for the diagnosis of NAFLD as well as aid in its prevention and treatment. Future studies with larger and more ethnically diverse sample sizes are needed to validate our findings and to explore the biological role of DNA methylation in the development and progression of NAFLD.

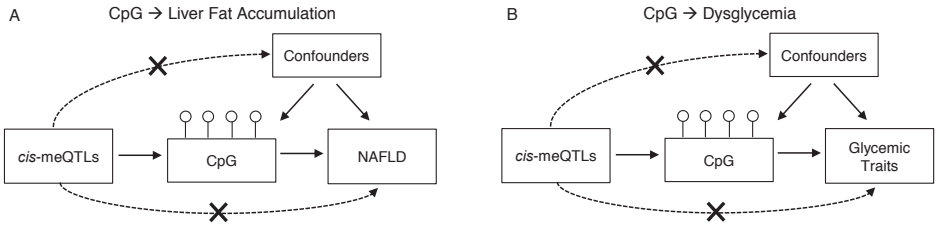

Figure S1. Mendelian randomization approach to analyze potential causal association for A) CpG to non-alcoholic fatty liver disease (NAFLD) and B) CpG to dysglycemic traits including fasting glucose concentrations, fasting insulin concentrations, and type 2 diabetes in the Framingham Heart Stduy. *cis*-meQTLs: single nucleotide polymorphisms (SNPs) associated with DNA methylation at CpG site.

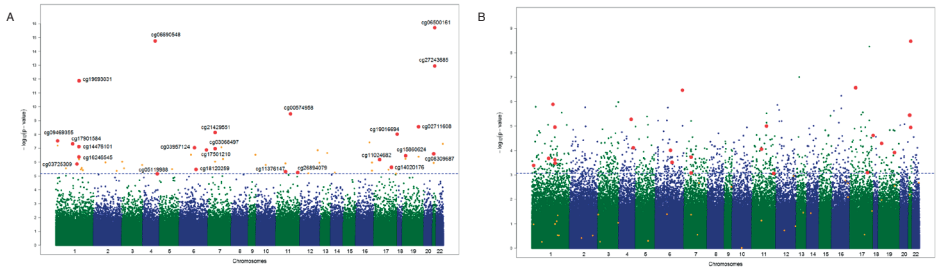

Figure S2. Manhattan plots generated using the sex- and age-adjusted model.
Plot 1A represents the discovery analysis in the FHS with a p-value threshold of 6.9×10^{-6} (dotted line) corresponding to FDR < 0.05. Plot 1B represents the replication meta-analysis of EA participants in the RS, CARDIA, and MESA using the Bonferroni corrected p-value threshold of 8.6×10^{-4}. Orange dots are significant CpGs in the FHS and red dots are replicated CpGs. FDR=false discovery rate. EA=European ancestry. FHS=Framingham Heart Study. RS=Rotterdam Study. MESA=Multi-Ethnic Study of Atherosclerosis study. CARDIA=Coronary Artery Risk Development in Young Adults study. CpG=DNA methylation site.

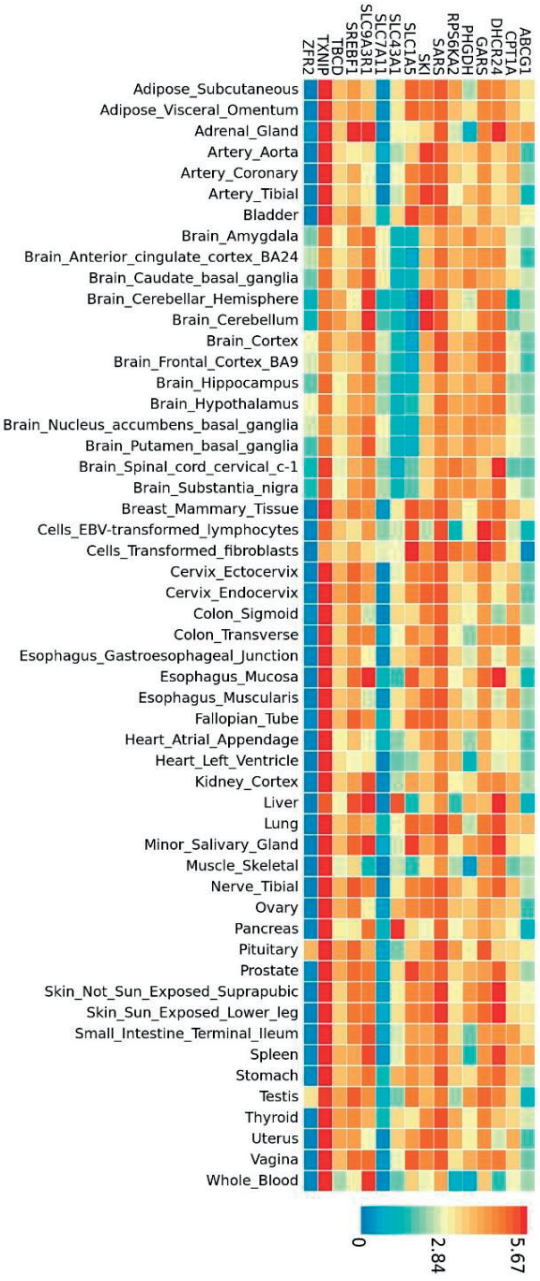

Figure S8. Heatmap for average expression of the 19 genes in 53 specific tissue types.
Colors represents average expression value (log2 transformed Reads Per Kilobase per Million per tissue per gene, winsorization at 50). Higher expression is represented using darker red and lower expression is represented by darker blue. Figure downloaded from GTEx through FUMA GWAS (www. fuma.ctglab.nl/). GTEx= Genotype-Tissue Expression. FUMA GWAS=Functional Mapping and Annotation of Genome-Wide Association Studies.

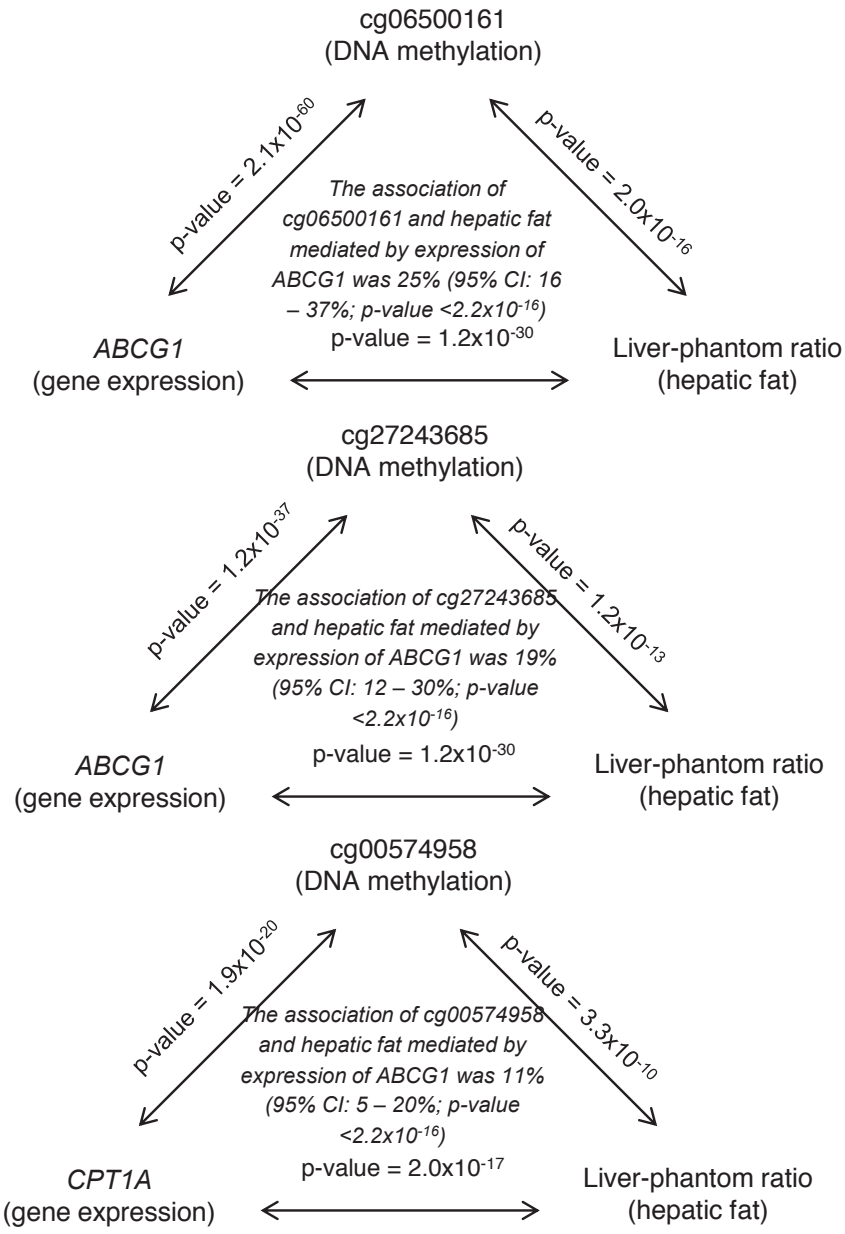

Figure S9. Three-way association of whole-blood derived DNA methylation level at cg06500161, cg27243685, and cg00574958 and whole-blood derived gene expression level for *ABCG1* and *CPT1A*, and CT-derived liver-phantom ratio (hepatic fat) in the FHS. CT=computed tomography. FHS=Framingham Heart Study

Table S1. Participant characteristics and methods for measurements of DNA methylation and Hepatic fat.

Cohort	N	Age (years)	Women (%)	Race/ Ethnicity	BMI (kg/m^2)	Smoking status (%, current/ former/none)	Physical activity score	Alcohol (drinks/day)
FHS	1496	58.8 (10.9)	47.90%	EA	28.2 (5.2)	10.3/58.1/31.6	35.9 (6.0)	4.0 (4.8)
RS	866	65.4 (7.3)	57.00%	EA	27.6 (4.1)	6/61/33	62.7 (0.25 - 430.35)*	41% more than 4 times per week
MESA	583	70.2 (9.5)	48.37%	EA	28.3 (5.4)	8.7/54.2/37	2504 (2683)	0.65 (1.31)
	401	68.4 (9.4)	50.37%	HA	30.0 (5.3)	7/49.9/43.1	2833 (3314)	0.29 (1.15)
	272	70.0 (9.0)	59.93%	AA	30.6 (5.7)	13/45/42	3630 (6614)	0.29 (0.77)
CARDIA	455	45.9 (3.3)	48.50%	EA	28.1 (6.0)	10.9/24.7/63.3	363.8 (255)	0.6 (0.9)
	302	44.3 (3.7)	53.90%	AA	30.7 (6.6)	23.4/12.8/63.5	341.9 (309)	0.4 (0.7)
GENOA	150	71.6 (6.0)	77.30%	AA	31.4 (6.8)	7.3/36/56.7	inactive/ active (119/31)	0.35 (1.35)

Data are mean (SD), unless otherwise indicated. Physical actiivty score is based on cohort-specific definition. EA: European ancestry. HA: Hispanic ancestry. AA: African ancestry. FHS=Framingham Heart Study. RS=Rotterdam Study. MESA=Multi-Ethnic Study of Atherosclerosis study. CARDIA=Coronary Artery Risk Development in Young Adults study. GENOA=Genetic Epidemiology Network of Arteriopathy. BMI=body mass index. CT=computed tomography. HU=Hounsfield units. *Rotterdam Study other subsample had a median score of 58 (0.75 - 351.35) for physical activity.

DNA Methylation		Hepatic Fat		Time interval between liver fat & blood draw (years, min, P20, mean, median, P75, max)	Family Cohort	White blood cell counts
Tissue source	Array	Assessment	Liver fat proxy			
Whole blood	Illumina 450K	CT	Ratio of HU of liver to HU to an external calcium control	0 0.2 2.5 3.0 3.9 5.8	Yes	Estimated using Houseman method
Whole blood	Illumina 450K	Ultrasound	Yes/No	At the same time	No	blood cell counts
CD14+ monocytes	Illumina 450K	CT	Average of three mean liver attenuation measures (Hounsfield unit)	8.0, 9.1, 9.3, 9.6,11.0	No	Estimated from Gene expression signatures measured in same samples
Whole blood	Illumina 850K	CT			No	
Whole blood	Illumina 450K	CT	Ratio of HU of liver to HU to an external calcium control (control roc ~ 100mg Ca)	9.5, 10.9, 11.7, 11.8, 12.5, 14.0	Sibships (but unrelated sample for this analysis)	Estimated using Houseman method

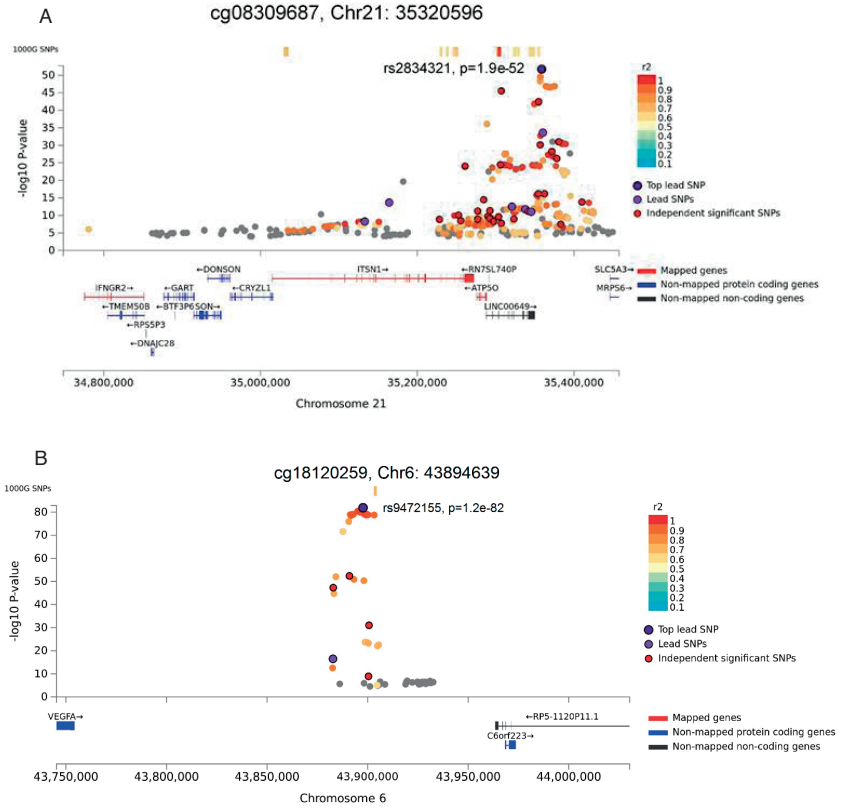

Figure S10. Regional plots of *cis*-meQTLs for two CpG sites.
Analyses overlapped cis-meQTLs (p-value < $5x10^{-7}$) with eQTLs (p-value < $5x10^{-7}$). Plot 10A depicts seven genes (*ATP50*, *ITSN1*, *TMEM50B*, *MRPS6*, *SLC5A3*, and *IFNGR2*) and one long intergenic non-protein coding RNA (LINC00649) for cg08309687. Plot 10B depicts one gene (*VEGFA*) for cg18120259. P-values were obtained from the FHS. *cis*-meQTLs=*cis*-methylated quantitative trait loci. eQTL=expression quantitative trait loci. FHS=Framingham Heart Study.

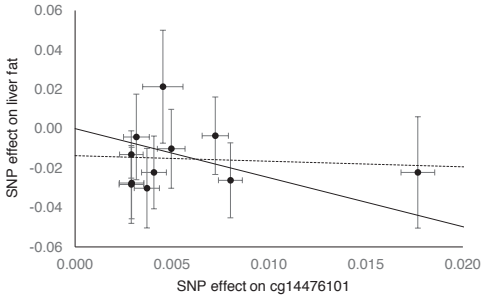

Figure S11. Potential causal association of cg14476101at *PHGDH* locus to hepatic fat accumulation using MR analyses.
P-value-IVW=0.02, P-value-Egger=0.91, and P-value-Egger-intercept=0.27. MR=Mendelian randomization. IVW=inverse variance weighted. SNP=single nucleotide polymorphism. CpG=DNA methylation site.

Table S2. Significant CpGs in the discovery phase of the epigenome-wide association study for hepatic fat using sex- and age-adjusted Model.

CpG	FHS				RS				CARDIA				MESA					
	Beta	SE	P	N	Beta	SE	P	N	Beta	SE	P	N	Beta	SE	P	N	P for meta-analysis	Direction
cg06500161	-6.2E-03	7.4E-04	2.0E-16	1496	9.6E-03	2.1E-03	5.6E-06	865	-1.1E+01	2.2E+00	1.5E-06	455	-2.0E-04	8.6E-05	2.2E-02	583	3.4E-09	+,+,+,+
cg06690548	1.1E-02	1.3E-03	1.8E-15	1496	-1.1E-03	2.6E-03	6.7E-01	847	2.6E+00	9.7E-01	8.2E-03	440	1.0E-03	2.1E-04	1.4E-06	583	5.3E-06	-,-,-,-
cg27243685	-4.7E-03	6.4E-04	1.2E-13	1496	5.7E-03	1.5E-03	1.3E-04	866	-5.2E+00	1.8E+00	4.0E-03	454	-8.5E-05	5.3E-05	1.1E-01	583	1.1E-05	+,+,+,+
cg19693031	8.0E-03	1.1E-03	1.3E-12	1496	1.7E-03	3.5E-03	6.3E-01	866	3.9E+00	1.2E+00	1.2E-03	452	5.4E-04	1.8E-04	2.9E-03	583	3.3E-04	-,+,-,-
cg00574958	2.6E-03	4.2E-04	3.3E-10	1496	-3.7E-03	1.5E-03	1.2E-02	865	3.4E+00	7.5E-01	6.2E-06	452	2.7E-05	3.4E-05	4.3E-01	583	9.9E-06	-,-,-,-
cg02711608	4.9E-03	8.3E-04	2.8E-09	1496	5.0E-04	2.4E-03	8.3E-01	866	5.0E+00	1.5E+00	5.8E-04	454	4.9E-04	1.4E-04	5.3E-04	583	1.2E-04	-,+,-,-
cg21429551	9.7E-03	1.7E-03	7.3E-09	1496	8.0E-04	4.9E-03	8.7E-01	865	2.3E+00	6.7E-01	6.7E-04	455	1.2E-03	3.3E-04	5.8E-04	583	1.9E-04	-,+,-,-
cg19016694	4.0E-03	6.9E-04	9.5E-09	1496	-1.0E-02	2.6E-03	1.3E-04	865	4.4E+00	2.4E+00	6.6E-02	454	3.2E-04	1.4E-04	2.0E-02	583	2.4E-05	-,-,-,-
cg09469355	4.4E-03	7.9E-04	2.9E-08	1496	-3.8E-03	2.4E-03	1.2E-01	866	2.4E+00	2.0E+00	2.3E-01	455	4.2E-04	1.1E-04	1.9E-04	583	4.1E-04	-,-,-,-
cg08305942	4.6E-03	8.4E-04	3.8E-08	1496	-8.5E-03	2.3E-03	2.2E-04	866	2.0E+00	1.8E+00	2.7E-01	455	1.5E-04	1.6E-04	3.7E-01	583	1.7E-03	-,-,-,-
cg09349128	4.3E-03	7.9E-04	4.8E-08	1496	9.0E-04	2.2E-03	6.9E-01	866	7.3E+00	1.7E+00	2.8E-05	455	-4.6E-05	6.9E-05	5.1E-01	583	2.1E-03	-,+,+,-
cg17901584	6.2E-03	1.1E-03	4.8E-08	1496	-5.1E-03	3.3E-03	1.2E-01	866	3.8E+00	1.3E+00	4.6E-03	455	5.4E-04	1.9E-04	4.8E-03	583	2.1E-04	-,-,-,-
cg05603985	3.8E-03	7.1E-04	6.3E-08	1496	8.0E-04	1.9E-03	6.7E-01	866	8.6E-01	1.9E+00	6.5E-01	455	2.4E-04	8.5E-05	5.8E-03	583	1.1E-01	-,+,-,-
cg14476101	8.1E-03	1.5E-03	7.5E-08	1496	2.1E-03	4.3E-03	6.3E-01	866	3.0E+00	1.2E+00	1.1E-02	455	1.1E-03	2.3E-04	3.4E-06	583	1.1E-05	-,+,-,-
cg17540192	-3.7E-03	6.9E-04	8.4E-08	1496	-3.0E-04	2.1E-03	9.0E-01	863	-2.5E+00	9.7E-01	9.4E-03	452	-6.0E-05	1.4E-04	6.6E-01	583	2.8E-01	+,-,+,+
cg03957124	4.5E-03	8.5E-04	9.1E-08	1496	-1.0E-02	2.8E-03	3.9E-04	866	7.4E+00	3.0E+00	1.5E-02	455	1.7E-04	1.2E-04	1.7E-01	583	9.8E-05	-,-,-,-
cg03068497	9.8E-03	1.8E-03	1.1E-07	1496	1.0E-03	5.1E-03	8.5E-01	865	2.4E+00	6.9E-01	5.5E-04	454	8.7E-04	3.2E-04	6.2E-03	583	8.2E-04	-,+,-,-
cg17501210	5.8E-03	1.1E-03	1.3E-07	1496	-5.6E-03	2.8E-03	4.5E-02	866	4.7E+00	1.0E+00	4.4E-06	454	6.3E-04	2.0E-04	2.0E-03	583	3.4E-07	-,-,-,-
cg20494738	-4.9E-03	9.4E-04	1.4E-07	1496	2.9E-03	1.4E-03	3.8E-02	865	-2.0E+00	1.1E+00	8.1E-02	452	-2.4E-04	9.1E-05	7.8E-03	583	1.1E-03	+,+,+,+
cg03185794	4.5E-03	8.7E-04	2.2E-07	1496	-2.3E-03	3.0E-03	4.5E-01	866	4.0E+00	2.0E+00	4.4E-02	454	2.6E-04	1.5E-04	8.3E-02	583	3.5E-02	-,-,-,-
cg08309687	6.9E-03	1.3E-03	2.5E-07	1496	-1.7E-02	4.0E-03	2.8E-05	866	3.0E+00	1.3E+00	1.8E-02	450	4.4E-04	1.9E-04	2.5E-02	583	3.6E-06	-,-,-,-

Table S2. (continued)

CpG	FHS				RS				CARDIA				MESA					
	Beta	SE	P	N	Beta	SE	P	N	Beta	SE	P	N	Beta	SE	P	N	P for meta-analysis	Direction
cg05973262	-4.1E-03	7.9E-04	2.9E-07	1496	4.4E-03	2.5E-03	7.9E-02	866	-2.6E+00	1.8E+00	1.6E-01	454	-1.8E-04	1.4E-04	2.0E-01	583	4.0E-02	+,+,+,+
cg13876222	-4.9E-03	9.5E-04	3.0E-07	1496	8.6E-03	3.1E-03	5.1E-03	866	-1.7E+00	6.6E-01	9.9E-03	452	-1.7E-05	4.0E-05	6.7E-01	583	3.0E-03	+,+,+,+
cg15860624	-5.7E-03	1.1E-03	3.5E-07	1496	8.8E-03	3.2E-03	6.6E-03	865	-4.2E+00	1.4E+00	3.4E-03	455	-4.1E-04	1.8E-04	2.2E-02	583	5.1E-05	+,+,+,+
cg07626482	3.9E-03	7.8E-04	4.1E-07	1496	1.3E-03	2.2E-03	5.4E-01	866	4.6E+00	2.0E+00	2.2E-02	455	3.0E-04	1.0E-04	3.5E-03	583	2.8E-03	-,+,-,-
cg16246545	5.7E-03	1.1E-03	4.2E-07	1496	1.5E-03	3.6E-03	6.7E-01	866	3.6E+00	1.4E+00	7.4E-03	455	7.1E-04	1.9E-04	2.4E-04	583	2.4E-04	-,+,-,-
cg24000650	-4.7E-03	9.5E-04	5.7E-07	1496	4.1E-03	3.0E-03	1.7E-01	865	-2.3E+00	1.2E+00	4.5E-02	455	-3.6E-04	1.9E-04	5.5E-02	583	1.1E-02	+,+,+,+
cg22103219	5.2E-03	1.0E-03	5.9E-07	1496	-9.5E-03	3.1E-03	2.0E-03	866	3.7E+00	1.8E+00	3.5E-02	454	1.1E-04	1.5E-04	4.8E-01	583	2.2E-03	-,-,-,-
cg19266329	4.1E-03	8.3E-04	6.2E-07	1496	2.4E-03	2.7E-03	3.7E-01	866	2.5E+00	1.7E+00	1.3E-01	454	1.9E-04	1.4E-04	1.8E-01	583	1.0E-01	-,+,-,-
cg11024682	-3.7E-03	7.4E-04	6.6E-07	1496	1.1E-02	2.2E-03	6.4E-07	865	-3.8E+00	2.2E+00	8.0E-02	455	-2.9E-04	1.0E-04	5.2E-03	583	2.7E-07	+,+,+,+
cg23068772	4.2E-03	8.6E-04	9.2E-07	1496	-6.0E-04	2.2E-03	7.8E-01	865	4.3E-01	1.6E+00	7.9E-01	454	4.8E-04	1.3E-04	3.5E-04	583	4.2E-02	-,-,-,-
cg23032421	4.5E-03	9.3E-04	9.3E-07	1496	-1.1E-02	2.7E-03	6.8E-05	866	1.9E-01	1.5E+00	9.0E-01	455	1.1E-05	7.6E-05	8.8E-01	583	4.2E-02	-,-,-,-
cg03147185	3.5E-03	7.2E-04	1.1E-06	1496	-3.2E-03	2.3E-03	1.6E-01	865	3.0E+00	2.8E+00	2.7E-01	454	1.7E-05	9.2E-05	8.5E-01	583	3.8E-01	-,-,-,-
cg07021906	-4.9E-03	1.0E-03	1.1E-06	1496	6.9E-03	3.1E-03	2.4E-02	865	-2.9E+00	1.3E+00	1.9E-02	454	-2.7E-04	1.7E-04	1.2E-01	583	2.2E-03	+,+,+,+
cg26725076	4.4E-03	9.1E-04	1.1E-06	1496	2.5E-03	2.4E-03	3.0E-01	865	4.5E+00	1.8E+00	1.3E-02	454	-2.8E-05	1.4E-04	8.4E-01	583	1.2E-01	-,+,+,-
cg27037013	6.4E-03	1.3E-03	1.1E-06	1496	-1.4E-02	4.1E-03	1.1E-03	866	2.4E-01	9.2E-01	7.9E-01	455	2.6E-04	1.4E-04	6.5E-02	583	6.4E-03	-,-,-,-
cg25281677	4.6E-03	9.4E-04	1.2E-06	1496	-3.0E-03	2.4E-03	2.2E-01	866	-1.1E+00	1.8E+00	5.2E-01	449	2.3E-04	1.1E-04	3.5E-02	583	7.4E-02	-,-,-,+
cg03725309	3.7E-03	7.6E-04	1.4E-06	1496	-1.6E-03	2.8E-03	5.7E-01	865	2.9E+00	1.0E+00	3.8E-03	455	5.1E-04	1.0E-04	6.0E-07	583	1.3E-06	-,-,-,-
cg19939077	3.8E-03	7.8E-04	1.6E-06	1496	0.0E+00	2.1E-03	1.0E+00	865	1.0E+00	1.5E+00	5.0E-01	454	2.2E-05	1.1E-04	8.4E-01	583	9.8E-01	-,+,-,-
cg02504211	-3.6E-03	7.6E-04	1.6E-06	1496	4.6E-03	2.3E-03	5.1E-02	863	-7.6E-01	1.3E+00	5.4E-01	453	-1.5E-04	1.2E-04	2.0E-01	583	9.1E-02	+,+,+,+
cg14020176	-3.2E-03	6.8E-04	2.4E-06	1496	8.4E-03	2.3E-03	3.2E-04	866	-2.0E+00	1.6E+00	1.9E-01	450	-1.6E-04	1.2E-04	1.9E-01	583	8.0E-04	+,+,+,+
cg12593793	3.9E-03	8.4E-04	2.6E-06	1496	-5.0E-03	2.4E-03	3.4E-02	866	5.0E-01	1.0E+00	6.3E-01	454	1.7E-04	9.6E-05	7.5E-02	583	4.5E-02	-,-,-,-

Table S2. (continued)

CpG	FHS				RS				CARDIA				MESA					
	Beta	SE	P	N	Beta	SE	P	N	Beta	SE	P	N	Beta	SE	P	N	P for meta-analysis	Direction
cg02081905	-1.7E-03	3.5E-04	2.8E-06	1496	6.0E-04	8.0E-04	4.5E-01	863	-9.9E-01	1.6E+00	5.3E-01	450	-2.1E-05	3.8E-05	5.9E-01	583	5.4E-01	+,+,+,+
cg19677267	7.0E-03	1.5E-03	2.8E-06	1496	-2.5E-03	4.6E-03	5.9E-01	866	2.6E-01	7.6E-01	7.3E-01	455	2.8E-04	2.5E-04	2.7E-01	583	5.5E-01	-,-,-,-
cg13795986	5.0E-03	1.1E-03	3.0E-06	1496	-1.0E-04	3.1E-03	9.7E-01	865	3.5E+00	1.3E+00	7.3E-03	449	-1.5E-04	1.9E-04	4.1E-01	583	2.9E-01	-,-,+,-
cg23205886	3.8E-03	8.2E-04	3.2E-06	1496	9.0E-04	2.7E-03	7.5E-01	866	1.4E+00	2.1E+00	4.9E-01	455	2.0E-04	1.7E-04	2.6E-01	583	5.0E-01	-,+,-,-
cg18120259	3.9E-03	8.4E-04	3.4E-06	1496	4.2E-03	2.6E-03	1.1E-01	866	4.9E+00	2.0E+00	1.3E-02	455	4.2E-04	1.4E-04	3.2E-03	583	3.1E-04	-,+,-,-
cg24678869	-2.5E-03	5.4E-04	3.4E-06	1496	3.6E-03	1.6E-03	2.4E-02	865	-1.7E+00	2.8E+00	5.4E-01	455	-8.9E-05	8.8E-05	3.1E-01	583	7.9E-02	+,+,+,+
cg05418719	4.0E-03	8.7E-04	3.5E-06	1496	-2.5E-03	2.7E-03	3.6E-01	865	3.7E+00	2.1E+00	7.8E-02	453	-3.4E-05	1.2E-04	7.8E-01	583	2.8E-01	-,-,+,-
cg24691964	3.7E-03	8.0E-04	3.8E-06	1496	-5.3E-03	2.0E-03	7.7E-03	866	2.4E-01	1.7E+00	8.9E-01	451	2.9E-05	1.0E-04	7.7E-01	583	3.0E-01	-,-,-,-
cg02203067	-3.4E-03	7.3E-04	4.1E-06	1496	5.7E-03	2.4E-03	1.6E-02	865	-3.1E+00	2.0E+00	1.2E-01	454	-2.0E-04	1.4E-04	1.5E-01	583	8.1E-03	+,+,+,+
cg02640489	2.9E-03	6.3E-04	4.3E-06	1496	-2.3E-03	2.9E-03	4.2E-01	866	-1.1E+00	2.0E+00	5.8E-01	452	1.6E-04	1.2E-04	1.8E-01	583	3.0E-01	-,-,-,+
cg11376147	3.1E-03	6.8E-04	4.9E-06	1496	6.0E-04	1.9E-03	7.6E-01	866	3.7E+00	1.4E+00	8.7E-03	449	5.2E-04	1.2E-04	3.7E-05	583	8.8E-05	-,+,-,-
cg18819791	5.1E-03	1.1E-03	5.6E-06	1496	5.0E-04	2.7E-03	8.5E-01	866	2.7E+00	1.2E+00	2.7E-02	455	2.7E-04	1.2E-04	2.2E-02	583	3.7E-02	-,+,-,-
cg26894079	4.9E-03	1.1E-03	5.6E-06	1496	-7.7E-03	2.8E-03	6.8E-03	864	3.4E+00	1.5E+00	2.7E-02	453	2.4E-04	1.4E-04	9.5E-02	583	8.6E-04	-,-,-,-
cg22876908	3.4E-03	7.6E-04	6.4E-06	1496	2.4E-03	2.2E-03	2.8E-01	866	2.0E+00	2.0E+00	3.2E-01	454	1.3E-04	1.1E-04	2.6E-01	583	1.9E-01	-,+,-,-
cg11969813	-5.0E-03	1.1E-03	6.8E-06	1496	9.1E-03	3.4E-03	7.1E-03	866	-1.6E+00	1.3E+00	2.3E-01	455	2.4E-05	4.0E-05	5.5E-01	583	3.0E-02	+,+,-,+
cg05119988	5.5E-03	1.2E-03	6.9E-06	1496	-6.7E-03	3.1E-03	3.0E-02	865	3.5E+00	1.2E+00	3.2E-03	454	3.1E-04	1.2E-04	8.5E-03	583	7.8E-05	-,-,-,-

Analysis was conducted in the EA participants. Meta-analysis p-values were calculated in RS, CARDIA, and MESA. In the RS, a positive beta value means that hypermethylation was associated with increased hepatic fat and a negative beta value means that hypermethylation was associated with decreased hepatic fat. Whereas, in the FHS, CARDIA, and MESA, a negative beta value means that hypermethylation was associated with increased hepatic fat and a positive beta value means that hypermethylation was associated with decreased hepatic fat. Regarding the direction, the '+' sign represents elevated DNA methylation (hypermethylation) is associated with increased hepatic fat and the '-' sign represents decreased DNA methylation (hypomethylation) is associated with increased hepatic fat. FHS=Framingham Heart Study. RS=Rotterdam Study. MESA=Multi-Ethnic Study of Atherosclerosis study. CARDIA=Coronary Artery Risk Development in Young Adults study. CpG=DNA methylation site. CHR=chromosome. SE=standard error.

Table S4. Epigenome-wide association study of liver fat using fully adjusted models.

IlmnID	Discovery				Replication													
	FHS				RS				CARDIA-EA				MESA-EA				P for meta-analysis in replication cohorts	Direction
	Beta	SE	P	N	Beta	SE	P	N	Beta	SE	P	N	Beta	SE	P	N		
cg06690548	8.3E-03	1.4E-03	2.6E-09	1496	1.0E-04	2.8E-03	9.7E-01	847	3.0E+00	1.0E+00	3.6E-03	386	9.2E-04	2.2E-04	4.0E-05	580	0.004	-,+,-,-
cg19693031	6.5E-03	1.2E-03	1.2E-07	1496	1.2E-03	3.9E-03	7.7E-01	866	1.5E+00	9.2E-01	1.1E-01	376	4.4E-04	1.9E-04	2.1E-02	580	0.006	-,+,-,-

Model adjusted for sex, age, smoking, physical activity, alcohol, and BMI. Analysis was conducted in the European ancestry (EA) participants. Discovery significance p-value 1.2E-07 (FDR < 0.05); replication significance p-value < 0.025. Meta-analysis p-values were calculated in RS, CARDIA, and MESA. In the RS, a positive beta represents hypermethylation was associated with increased hepatic fat and a negative beta represents hypermethylation was associated with decreased hepatic fat. Whereas, in the FHS, CARDIA, and MESA, a negative beta represents hypermethylation was associated with increased hepatic fat and a positive beta represents hypermethylation was associated with decreased hepatic fat. Regarding the direction, the '+' sign represents elevated DNA methylation (hypermethylation) is associated with increased hepatic fat and the '-' sign represents decreased DNA methylation (hypomethylation) is associated with increased hepatic fat. FHS=Framingham Heart Study. RS=Rotterdam Study. MESA=Multi-Ethnic Study of Atherosclerosis study. CARDIA=Coronary Artery Risk Development in Young Adults study. EA=European ancestry. CpG=DNA methylation site. SE=standard error.

Table S5. Replication of the 22 signficant CpGs identified in the EA participants in the MESA HA cohort (N=401).

IlmnID	Model 1			Direction in HA participants	Direction in EA participants
	Beta	SE	P		
cg19693031	8.7E-04	2.3E-04	1.7E-04	-	-,+,-,-
cg03957124	4.1E-04	1.4E-04	2.8E-03	-	-,-,-,-
cg06500161	-2.9E-04	9.8E-05	3.0E-03	+	+,+,+,+
cg17901584	6.1E-04	2.2E-04	6.3E-03	-	-,-,-,-
cg19016694	4.1E-04	1.7E-04	1.6E-02	-	-,-,-,-
cg21429551	6.1E-04	3.4E-04	7.4E-02	-	-,+,-,-
cg14020176	-2.0E-04	1.4E-04	1.5E-01	+	+,+,+,+
cg26894079	2.6E-04	1.8E-04	1.5E-01	-	-,-,-,-
cg00574958	6.0E-05	4.4E-05	1.7E-01	-	-,-,-,-
cg27243685	-8.7E-05	6.3E-05	1.7E-01	+	+,+,+,+
cg11024682	-1.9E-04	1.4E-04	1.8E-01	+	+,+,+,+
cg08309687	3.3E-04	2.5E-04	1.8E-01	-	-,-,-,-
cg03725309	1.5E-04	1.3E-04	2.6E-01	-	-,-,-,-
cg06690548	1.3E-04	1.2E-04	2.9E-01	-	-,-,-,-
cg18120259	1.8E-04	1.7E-04	2.9E-01	-	-,+,-,-
cg05119988	9.6E-05	1.3E-04	4.6E-01	-	-,-,-,-
cg15860624	-1.3E-04	2.3E-04	5.8E-01	+	+,+,+,+
cg09469355	4.7E-05	1.3E-04	7.1E-01	-	-,-,-,-
cg14476101	8.3E-05	2.5E-04	7.4E-01	-	-,+,-,-
cg11376147	3.8E-05	1.2E-04	7.4E-01	-	-,+,-,-
cg17501210	6.0E-05	2.7E-04	8.3E-01	-	-,-,-,-
cg02711608	-5.5E-06	1.6E-04	9.7E-01	+	-,+,-,-

Model adjusted for sex and age. Bonferroni-corrected p-value threshold is 0.002 (0.05/22 CpGs). A negative beta value means that hypermethylation was associated with increased hepatic fat and a positive beta value means that hypermethylation was associated with decreased hepatic fat. Regarding the direction, the '+' sign represents elevated DNA methylation (hypermethylation) is associated with increased hepatic fat and the '-' sign represents decreased DNA methylation (hypomethylation) is associated with increased hepatic fat. MESA=Multi-Ethnic Study of Atherosclerosis study. HA=Hispanic ancestry. EA=European ancestry. CpG=DNA methylation site. CHR=chromosome. SE=standard error.

Table S6. Replication of the 22 signficant CpGs identified in the EA participants in AA participants in the MESA, CARDIA, and GENOA cohorts (N=721).

	Model 1														
	GENOA				MESA				CARDIA					Direction in AA participants	Direction in EA participants
CpG	Beta	SE	P	N	Beta	SE	P	N	Beta	SE	P	N	Meta-analysis P		
cg14476101	-1.4E-02	5.7E-02	8.1E-01	150	6.0E-04	2.8E-04	3.6E-02	272	3.7E+00	1.3E+00	3.5E-03	304	1.1E-02	+, -, -	-,+,-,-
cg00574958	1.4E-02	2.0E-02	4.9E-01	150	1.1E-04	4.9E-05	2.5E-02	272	1.9E+00	9.0E-01	3.4E-02	296	1.4E-02	-, -, -	-,-,-,-
cg08309687	-5.4E-02	5.1E-02	2.9E-01	150	6.5E-04	2.6E-04	1.4E-02	272	1.2E+00	1.1E+00	2.7E-01	303	2.7E-02	+, -, -	-,-,-,-
cg27243685	-2.9E-02	1.4E-02	3.7E-02	150	-2.8E-05	6.8E-05	6.9E-01	272	-3.2E+00	1.8E+00	6.9E-02	299	5.2E-02	+, +, +	+,+,+,+
cg21429551	9.5E-02	5.6E-02	9.5E-02	150	5.8E-04	4.0E-04	1.4E-01	272	2.6E-01	8.0E-01	7.5E-01	303	1.7E-01	-, -, -	-,+,-,-
cg03725309	5.6E-03	2.3E-02	8.1E-01	150	1.9E-04	1.4E-04	1.7E-01	272	1.9E+00	1.0E+00	6.5E-02	302	1.8E-01	-, -, -	-,-,-,-
cg17901584	-6.6E-02	4.5E-02	1.5E-01	150	2.6E-04	2.9E-04	3.6E-01	272	7.9E-01	9.6E-01	4.1E-01	302	1.9E-01	+, -, -	-,-,-,-
cg26894079	-7.7E-03	4.1E-02	8.5E-01	150	9.0E-05	1.8E-04	6.2E-01	272	4.1E+00	1.5E+00	7.7E-03	302	1.9E-01	+, -, -	-,-,-,-
cg17501210	1.2E-01	7.0E-02	8.6E-02	150	3.9E-04	3.8E-04	3.1E-01	272	3.3E-01	8.1E-01	6.9E-01	303	2.2E-01	-, -, -	-,-,-,-
cg06690548	1.9E-02	2.0E-02	3.4E-01	150	1.5E-04	1.2E-04	2.3E-01	272	8.6E-01	1.1E+00	4.2E-01	301	2.4E-01	-, -, -	-,-,-,-
cg06500161	2.5E-02	3.2E-02	4.4E-01	150	-2.3E-04	1.3E-04	8.1E-02	272	-8.3E-01	2.2E+00	7.1E-01	304	2.8E-01	-, +, +	+,+,+,+
cg19693031	2.5E-02	4.5E-02	5.8E-01	150	7.5E-04	2.9E-04	1.0E-02	272	-1.1E-01	1.1E+00	9.2E-01	304	2.8E-01	-, -, +	-,+,-,-
cg03957124	3.2E-02	3.0E-02	2.9E-01	150	-4.6E-05	1.7E-04	7.9E-01	272	3.5E+00	2.3E+00	1.3E-01	304	3.1E-01	-, +, -	-,-,-,-
cg14020176	-1.5E-02	2.3E-02	5.3E-01	150	3.4E-05	1.7E-04	8.4E-01	272	2.4E+00	1.4E+00	8.2E-02	301	4.1E-01	+, -, -	+,+,+,+
cg15860624	-6.6E-02	5.1E-02	2.0E-01	150	-7.5E-05	2.9E-04	8.0E-01	272	8.9E-01	1.1E+00	4.2E-01	304	4.5E-01	+, +, -	+,+,+,+
cg09469355	-6.5E-04	3.8E-02	9.9E-01	150	1.9E-04	1.5E-04	2.1E-01	272	3.6E+00	1.9E+00	6.0E-02	303	5.3E-01	+, -, -	-,-,-,-
cg11376147	-5.2E-04	1.6E-02	9.7E-01	150	2.0E-04	1.3E-04	1.3E-01	272	1.7E+00	1.3E+00	2.1E-01	302	5.6E-01	+, -, -	-,+,-,-
cg18120259	2.8E-02	3.3E-02	4.0E-01	150	2.3E-05	2.0E-04	9.1E-01	272	2.6E+00	2.9E+00	3.8E-01	301	6.8E-01	-, -, -	-,+,-,-
cg02711608	-5.3E-03	4.0E-02	9.0E-01	150	2.7E-04	2.0E-04	1.8E-01	272	2.7E-01	7.9E-01	7.4E-01	302	7.0E-01	+, -, -	-,+,-,-
cg05119988	-8.2E-03	3.9E-02	8.4E-01	150	8.3E-05	1.6E-04	6.1E-01	272	1.2E+00	1.4E+00	4.1E-01	304	7.1E-01	+, -, -	-,-,-,-
cg19016694	1.0E-02	3.5E-02	7.6E-01	150	5.8E-05	2.1E-04	7.8E-01	272	-6.5E-01	1.8E+00	7.1E-01	303	8.6E-01	-, -, +	-,-,-,-
cg11024682	4.2E-04	3.4E-02	9.9E-01	150	1.3E-06	1.6E-04	9.9E-01	272	-3.8E+00	2.1E+00	6.7E-02	304	9.8E-01	-, -, +	+,+,+,+

Model adjusted for sex and age. Bonferroni-corrected p-value threshold is 0.002 (0.05/22 CpGs). A negative beta value means that hypermethylation was associated with increased hepatic fat and a positive beta value means that hypermethylation was associated with decreased hepatic fat. Regarding the direction, the '+' sign represents elevated DNA methylation (hypermethylation) is associated with increased hepatic fat and the '-' sign represents decreased DNA methylation (hypomethylation) is associated with increased hepatic fat. MESA=Multi-Ethnic Study of Atherosclerosis study. CARDIA=Coronary Artery Risk Development in Young Adults study. GENOA=Genetic Epidemiology Network of Arteriopathy. EA=European ancestry. AA=African ancestry. CpG=DNA methylation site. SE=standard error.

Table S8. Functional description for the 18 Illumina annotated genes.

Annotated gene	CHR	Full Name	Gene type	Description
ABCG1	21	ATP binding cassette subfamily G member 1	protein coding	The protein this gene encodes is involved in macrophage cholesterol and phospholipids transport; may regulate cellular lipid homeostasis
ASAM	11	CXADR like membrane protein	protein coding	Expression of this gene in white adipose tissue is implicated in adipocyte maturation and development of obesity.
CPT1A	11	carnitine palmitoyltransferase 1A	protein coding	CPT I is the key enzyme in the carnitine-dependent transport across the mitochondrial inner membrane and the rate of fatty acid beta-oxidation.
DHCR24	1	24-dehydrocholesterol reductase	protein coding	This gene encodes a flavin adenine dinucleotide-dependent oxidoreductase which is involved in cholesterol biosynthesis.
GARS	7	glycyl-tRNA synthetase	protein coding	This gene encodes glycyl-tRNA synthetase, one of the aminoacyl-tRNA synthetases that charge tRNAs with their cognate amino acids.
PHGDH	1	phosphoglycerate dehydrogenase	protein coding	This gene encodes the enzyme which is involved in the early steps of L-serine synthesis in animal cells and other amino acid synthesis.
RPS6KA2	6	ribosomal protein S6 kinase A2	protein coding	This gene encodes a member of the ribosomal S6 kinase family of serine/threonine kinases, which has been implicated in controlling cell growth and differentiation.
SARS	1	seryl-tRNA synthetase	protein coding	This gene belongs to the class II amino-acyl tRNA family. The encoded enzyme catalyzes the transfer of L-serine to tRNA (Ser) and is related to bacterial and yeast counterparts.
SC4MOL	4	methylsterol monooxygenase 1	protein coding	Sterol-C4-mehtyl oxidase-like protein is localized to the endoplasmic reticulum membrane and is believed to function in cholesterol biosynthesis.
SKI	1	SKI proto-oncogene	protein coding	This gene encodes the nuclear protooncogene protein homolog of avian sarcoma viral (v-ski) oncogene. It functions as a repressor of TGF-beta signaling, and may play a role in neural tube development and muscle differentiation

Table S8. (continued)

Annotated gene	CHR	Full Name	Gene type	Description
SLC1A5	19	solute carrier family 1 member 5	protein coding	The SLC1A5 gene encodes a sodium-dependent neutral amino acid transporter that can act as a receptor for RD114/type D retrovirus
SLC43A1	11	solute carrier family 43 member 1	protein coding	SLC43A1 belongs to the system L family of plasma membrane carrier proteins that transports large neutral amino acids
SLC7A11	4	solute carrier family 7 member 11	protein coding	This gene encodes a member of a heteromeric, sodium-independent, anionic amino acid transport system that is highly specific for cysteine and glutamate.
SLC9A3R1	17	SLC9A3 regulator 1	protein coding	This gene encodes a sodium/hydrogen exchanger regulatory cofactor.
SREBF1	17	sterol regulatory element binding transcription factor 1	protein coding	This gene encodes a basic helix-loop-helix-leucine zipper transcription factor that binds to the sterol regulatory element-1, which is a motif that is found in the promoter of the low density lipoprotein receptor gene and other genes involved in sterol biosynthesis.
TBCD	17	tubulin folding cofactor D	protein coding	Cofactor D is one of four proteins involved in the pathway leading to correctly folded beta-tubulin from folding intermediates. Cofactor D is believed to play a role in capturing and stabilizing beta-tubulin intermediates in a quasi-native confirmation.
TXNIP	1	thioredoxin interacting protein	protein coding	This gene encodes a thioredoxin-binding protein that is a member of the alpha arrestin protein family. Thioredoxin is a thiol-oxidoreductase that functions as a regulator of cellular redox signaling, cellular metabolism, endoplasmic reticulum stress, and tumor suppression
ZFR2	19	zinc finger RNA binding protein 2	protein coding	unknown

Gene information is taken from NCBI Genes. CHR=chromosome.

Supplemental Table 10. The Enriched Biological Processes for Genes Mapped to the 18 Illumina annotated genes.

GO biological process complete	Ref List	Observed	Observed Genes	Expected	Representation	Fold Enrichment	P-value	FDR
positive regulation of cholesterol biosynthetic process (GO:0045542)	5	2	ABCG1, SREBF1	0	+	> 100	1.61E-05	1.94E-02
positive regulation of sterol biosynthetic process (GO:0106120)	5	2	ABCG1, SREBF1	0	+	> 100	1.61E-05	1.80E-02
positive regulation of cholesterol metabolic process (GO:0090205)	6	2	ABCG1, SREBF1	0.01	+	> 100	2.15E-05	2.24E-02
amyloid precursor protein catabolic process (GO:0042987)	9	2	ABCG1, DHCR24	0.01	+	> 100	4.22E-05	3.67E-02
L-alpha-amino acid transmembrane transport (GO:1902475)	41	3	SLC1A5, SLC43A1, SLC7A11	0.04	+	81.03	8.05E-06	1.26E-02
serine family amino acid metabolic process (GO:0009069)	42	3	SARS, GARS, PHGDH	0.04	+	79.11	8.62E-06	1.23E-02
L-amino acid transport (GO:0015807)	61	3	SLC1A5, SLC43A1, SLC7A11	0.06	+	54.47	2.50E-05	2.45E-02
amino acid transmembrane transport (GO:0003333)	72	3	SLC1A5, SLC43A1, SLC7A11	0.07	+	46.14	4.03E-05	3.71E-02
carboxylic acid transmembrane transport (GO:1905039)	112	4	CPT1A, SLC1A5, SLC43A1, SLC7A11	0.1	+	39.55	3.18E-06	2.48E-02
organic acid transmembrane transport (GO:1903825)	112	4	CPT1A, SLC1A5, SLC43A1, SLC7A11	0.1	+	39.55	3.18E-06	1.66E-02
cholesterol metabolic process (GO:0008203)	113	4	ABCG1, SREBF1, DHCR24, SC4MOL	0.1	+	39.2	3.29E-06	1.29E-02
secondary alcohol metabolic process (GO:1902652)	119	4	ABCG1, SREBF1, DHCR24, SC4MOL	0.11	+	37.23	4.01E-06	8.96E-03
sterol metabolic process (GO:0016125)	132	4	ABCG1, SREBF1, DHCR24, SC4MOL	0.12	+	33.56	5.98E-06	1.04E-02
organic acid transport (GO:0015849)	269	5	CPT1A, SLC9A3R1, SLC1A5, SLC43A1, SLC7A11	0.24	+	20.59	3.60E-06	1.13E-02

Supplemental Table 10. (continued)

GO biological process complete	Ref List	Observed	Observed Genes	Expected	Representation	Fold Enrichment	P-value	FDR
carboxylic acid transport (GO:0046942)	269	5	CPT1A, SLC9A3R1, SLC1A5, SLC43A1, SLC7A11	0.24	+	20.59	3.60E-06	9.38E-03
organic anion transport (GO:0015711)	414	6	ABCG1, CPT1A, SLC9A3R1, SLC1A5, SLC43A1, SLC7A11	0.37	+	16.05	1.32E-06	2.07E-02
anion transport (GO:0006820)	534	6	ABCG1, CPT1A, SLC9A3R1, SLC1A5, SLC43A1, SLC7A11	0.48	+	12.44	5.64E-06	1.10E-02
small molecule metabolic process (GO:0044281)	1836	8	ABCG1, SARS, SREBF1, SC4MOL, GARS, CPT1A, PHGDH, DHCR24	1.66	+	5.43	1.22E-05	1.59E-02

Ref List quanitifies the number of genes in the database for the GO analysis. The 'Observed' column quantifies the number of genes in the testing set. The 'Expected' column depicts the expected number of genes for the testing set. When comparing the observed versus expected number of genes, '+' denotes over-representation while '-' denotes under-representation. Raw p-values are reported along with the FDR threshold. GO=gene ontology. FDR=false discovery rate.

Supplemental Table 12. Triangular association of CpGs, gene expression, and hepatic fat in the FHS.

CpG	Gene	CHR	CpG position	TSS	P-value for CpG and gene association	P-value for gene and liver fat association	Direction for CpG and liver fat association	Direction for CpG and gene association	Direction for gene and liver fat association
cg06500161	ABCG1	21	43656587	43619809	2.1E-60	1.2E-30	+	-	-
cg27243685	ABCG1	21	43642366	43619809	1.2E-37	1.2E-30	+	-	-
cg00574958	CPT1A	11	68607622	68522090	1.9E-20	2.0E-17	-	-	+
cg17901584	DHCR24	1	55353706	55302242	6.3E-17	1.2E-01	-	-	+
cg06690548	SLC7A11	4	139162808	139060406	6.1E-14	3.7E-01	-	-	+
cg14476101	PHGDH	1	120255992	120202441	3.7E-12	3.2E-02	-	-	+
cg17501210	RNASET2	6	166970252	167271494	9.4E-11	7.7E-02	-	-	-
cg19693031	TXNIP	1	145441552	145438489	8.2E-08	7.8E-03	-	-	-
cg08309687	ATP5O	21	35320596	35273997	2.2E-04	8.7E-01	-	+	+
cg08309687	TMEM50B	21	35320596	34804794	6.3E-04	4.1E-05	-	+	-
cg08309687	ITSN1	21	35320596	35014716	6.5E-03	2.3E-02	-	+	+
cg08309687	SLC5A3	21	35320596	35467498	1.4E-02	2.0E-01	-	-	-
cg08309687	MRPS6	21	35320596	35322421	4.5E-02	5.5E-01	-	-	+

Gene expression levels were derived from whole blood in the FHS. Regarding direction, '+' sign represents a positive association and '-' sign represents an inverse association. CpG=DNA methylation site. FHS=Framingham Heart Study. CHR=chromosome. TSS=transcription start site.

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Chapter 4.5

An epigenome-wide association study (EWAS) of obesity-related traits

Klodian Dhana[1,2*], Kim V.E. Braun[1,2*], Jana Nano[1], Trudy Voortman[1], Ellen W. Demerath[3], Weihua Guan[4], Myriam Fornage[5,6], Joyce B.J. van Meurs[7], Andre G. Uitterlinden[1,7], Albert Hofman[1,8], Oscar H. Franco[1], Abbas Dehghan[1,9]

[1] Department of Epidemiology, Erasmus MC, University Medical Center, Rotterdam, the Netherlands.
[2] Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, MA, USA.
[3] Division of Epidemiology and Community Health, University of Minnesota, Minneapolis, MN, USA.
[4] Division of Biostatistics, University of Minnesota School of Public Health, Minneapolis, MN, USA.
[5] Human Genetics Center, School of Public Health, University of Texas Health Sciences Center at Houston, Houston, TX, USA.
[6] Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX, USA.
[7] Department of Internal Medicine, Erasmus University Medical Center, Rotterdam, the Netherlands.
[8] Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, MA, USA.
[9] Department of Epidemiology, Imperial College London, London, UK.
* *These authors contributed equally.*

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ABSTRACT

We conducted an epigenome-wide association study (EWAS) on obesity-related traits. We used data from two prospective, population-based cohort studies: the Rotterdam Study (RS) and the Atherosclerosis Risk in Communities (ARIC) Study. We used RS (n=1,454) as the discovery panel and ARIC (n=2,097) as replication panel. Linear mixed-effect models were used to assess the cross-sectional association between genome-wide DNA methylation in leukocytes with body mass index (BMI) and waist circumference (WC) adjusting for sex, age, smoking, leukocyte proportions, array number and position on array. The two latter were modelled as random effects. Fourteen CpGs were associated with BMI and 26 CpGs with WC in RS after Bonferroni-correction ($P < 1.07 \times 10\text{-}7$), of which 12 and 14 CpGs replicated in ARIC Study, respectively. The most significant novel CpGs were located at *MSI2* (cg21139312) and *LARS2* (cg18030453) and were associated both with BMI and WC. CpGs at *BRDT*, *PSMD1*, *IFI44L*, *MAP1A*, and *MAP3K5* were associated with BMI. CpGs at *LGALS3BP*, *MAP2K3*, *DHCR24*, *CPSF4L*, and *TMEM49* were associated with WC. We report novel associations of methylation at *MSI2* and *LARS2* with obesity-related traits. These results provide further insight in mechanisms underlying obesity-related traits, which can enable identification of new biomarkers in obesity-related chronic diseases.

INTRODUCTION

Obesity is an important risk factor for cardiovascular disease, diabetes, some cancers, and musculoskeletal disorders (1-3). Evidence suggests that obesity is not only dependent on lifestyle factors, but is a result of interactions between genes and lifestyle (4, 5). Epigenetics has been proposed as a molecular mechanism that can affect the expression of genes by environmental influences and potentially could describe further the link between obesity and its complications (6). Nevertheless, unlike genetics, DNA methylation is dynamic overtime, therefore change in DNA methylation could also be a consequence of obesity.

Epigenetics is the study of heritable variation in gene function that is not a result of a change in DNA sequence (7). One of the best studied epigenetic mechanisms is DNA methylation, the attachment of a methyl group to a cytosine nucleotide of CpG dinucleotides. DNA methylation has varying functions at different locations in the human genome, including regulation of gene expression (8). To date, epigenome-wide association studies (EWAS) have identified several differentially methylated CpG regions related to body mass index (BMI) - the most widely used measure of obesity - and waist circumference (WC) (9-11). These few studies were performed in either patient populations, specific ethnic groups, or young adults. However, information among the older adults from population-based studies are scarce. In older adults and elderly, biological mechanisms involved in body weight and body composition may be different compared to younger adults (12). Therefore, it is crucial to explore the relationship of obesity to epigenetic variation in older adults.

We performed a cross-sectional EWAS of DNA methylation in blood leukocytes for BMI and WC in subjects from the Rotterdam Study (RS), and replicated our findings in the Atherosclerosis Risk in Communities (ARIC) Study.

METHODS

Study population

The RS is a large prospective, population-based cohort study aimed at assessing the occurrence of and risk factors for chronic diseases (cardiovascular, endocrine, hepatic, neurological, ophthalmic, psychiatric, dermatological, oncological, and respiratory) in the elderly (13). The study comprises 14,926 subjects in total, living in the well-defined Ommoord district in the city of Rotterdam in the Netherlands. In 1989, the first cohort, Rotterdam Study-I (RS-I), was established and comprised of 7,983 subjects with age 55 years or above. In 2000, the second cohort, Rotterdam Study-II (RS-II) was included with 3,011 subjects who had reached an age of 55 years since 1989. In 2006, the third cohort, Rotterdam Study-III (RS-III) was further included with 3,932 subjects with age 45 years and above. The discovery panel for the current analysis consisted of a random sample

of 1,454 participants from the first and second visit of the third cohort (RS-III-1, RS-III-2) and third visit of the second cohort (RS-II-3). We sought replication of the identified CpG sites in the ARIC Study, which is described in detail elsewhere (14). Briefly, the ARIC Study is a prospective cohort study of cardiovascular disease in adults. Between 1987 and 1989, 7,082 men and 8,710 women aged 45-64 were recruited from four US communities. Methylation data was available in a subset of 2,097 African American participants (10). RS and ARIC study protocols were approved by Institutional Review Boards at each participating university and all participants provided written informed consent.

Anthropometric measures and covariates

Height and weight were measured with the participants standing without shoes and heavy outer garments. WC 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. Hip circumference was recorded as the maximum circumference over the buttocks. BMI was calculated as weight divided by height squared (kg/m2), and WHR was calculated as WC divided by hip circumference (15). Information on current and past smoking behavior was acquired from questionnaires.

DNA methylation data

DNA was extracted from whole peripheral blood (stored in EDTA tubes) by standardized salting out methods. Genome-wide DNA methylation levels were measured using the Illumina Human Methylation 450K array (16). In short, samples (500ng of DNA per sample) were first bisulfite treated using the Zymo EZ-96 DNA-methylation kit (Zymo Research, Irvine, CA, USA). Next, samples were hybridized to the arrays according to the manufacturers' protocol. The methylation percentage of a CpG site was reported as a beta-value ranging between 0 (no methylation) and 1 (full methylation). The data preprocessing was additionally performed in both datasets using an R programming pipeline which is based on the pipeline developed by Tost & Toulemat (17), which includes additional parameters and options to preprocess and normalize methylation data directly from idat files. We excluded probes which had a detection p-value >0.01 in >95% of samples. 11,648 probes at X and Y chromosomes were excluded to avoid gender bias. The raw beta values were then background corrected and normalized using the DASEN option of the WateRmelon R-package (18). Per individual probe, participants with methylation levels higher than three times the inter-quartiles range (IQR) were excluded.

Statistical analyses

The characteristics of the discovery and replication population are presented as mean for continuous variables and proportion for the categorical variables. In the discovery

stage, we modeled cross-sectional associations between Dasen normalized beta-values of the CpG sites as outcome and BMI or WC as exposure using linear mixed effect models adjusting for age, sex, smoking, white blood cell proportions, array number (65 arrays) and position on array (12 positions; a combination of row number and column number). We performed an independent analysis in individuals from RS-III-1, from RS-III-2, and from RS-II-3. We then performed a fixed effects meta-analysis on the estimates of these three cohorts using the inverse-variance weighted method implemented in METAL combining RS-III-1 with RS-III-2 and RS-II-3 (19). Technical covariates (array number and position on array) were modeled as random effects. For the RS-III-1 we estimated leukocyte proportions (B-cells, CD4+ T-cells, CD8+ T-cells, granulocytes, monocytes and NK-cells) by a formula developed by Houseman and implemented in the *minfi* package in R (20, 21). For RS-II-3 and RS-III-2 we used white blood cell counts (WBC), i.e. lymphocytes, monocytes, and granulocytes, which were assessed with a Coulter AcT diff2 Hematology Analyzer. We corrected for multiple testing using a robust Bonferroni corrected *P*-value of 1.07×10^{-7} as the threshold for significance (0.05/463,456 probes). The probes identified in the discovery analysis were tested for replication in the independent samples from the ARIC study. A Bonferroni corrected *P*-value of 0.05 divided by the number of significant findings in the discovery study was used as a threshold of significant replication. Finally, we checked all identified CpG sites for cross-reaction or polymorphism (22). A CpG site was considered polymorphic when a SNP with a minor allele frequency of >0.01 resided at the position of the cytosine or guanine nucleotide, or within 10 bp from the CpG site within the probe binding site (23).

Methylation risk score

A methylation risk score was calculated based on CpG sites that were associated with the phenotypes. The effect estimates were used to build the methylation risk score using data from the discovery panel. Linear regression analyses were performed in using BMI or WC as outcome variable and the included CpG sites as exposure variables. With the use of linear regression models we calculated the lipids variance explained by the methylation risk score.

Note: Supplementary Material/Appendix can be found in the website of the published journal or can be provided on request.

RESULTS

Table 1 summarizes the characteristics of participants in the studies. RS is entirely comprised of Europeans, whereas the ARIC study included only African Americans. Compared to RS (mean age 63.7 (8.1)), the participants in ARIC were on average younger (mean age 56.2 (5.7)) and comprised more women (63% in ARIC vs. 55% in RS)). The

Table 1. Characteristics of study populations

		RS (N=1,450)	ARIC (N=2,097)
Age, years		63.7 (8.1)	56.2 (5.7)
Gender, women		55.9	63.6
Race, %			
	European	100	0
	African American	0	100
BMI (kg/m2)		27.7 (4.4)	30.1 (6.1)
BMI status			
	Normal weight	28.9	17.6
	Overweight	46.5	37.6
	Obese	24.6	43.8
WC (cm)		93.7 (12.9)	101.3 (15.1)
Smoking status			
	Current smoker	18.8	24.4
	Current nonsmoker	81.2	75.6
Diabetes, %		11	26

Values are mean (SD) or percentage. ARIC, Atherosclerosis Risk in Communities; BMI, body mass index; RS, Rotterdam Study; WC, waist circumference.

Table 2. CpG methylation sites associated with BMI in RS at level of genome-wide significance ($P < 1.08 \times 10^{-7}$) and successfully replicated at ARIC ($P < 3.57 \times 10^{-3}$)

ProbeID	Chr	Gene	Mean (SD) methylation	Discovery Panel (RS)		Replication Panel (ARIC)		%Variance explained
				Beta	*P*	Beta	*P*	
cg00574958	11	*CPT1A*	0.19 (0.04)	-0.0011	6.2×10^{-15}	-0.0029	3.2×10^{-12}	1.9
cg00851028	1	NA	0.72 (0.04)	0.0010	5.4×10^{-08}	0.0038	9.0×10^{-04}	0.4
cg03421440	1	*BRDT*	0.71 (0.07)	-0.0015	3.2×10^{-08}	-0.0043	1.3×10^{-03}	0.3
cg06096336	2	*PSMD1*	0.64 (0.05)	0.0016	4.3×10^{-08}	0.0058	5.5×10^{-04}	1.0
cg06500161	21	*ABCG1*	0.71 (0.03)	0.0011	1.7×10^{-09}	0.0081	1.5×10^{-13}	0.3
cg06872964	1	*IFI44L*	0.62 (0.06)	0.0015	4.8×10^{-08}	0.0100	4.3×10^{-07}	0.4
cg11024682	17	*SREBF1*	0.55 (0.04)	0.0013	6.6×10^{-15}	0.0068	9.6×10^{-09}	0.1
cg15159104	15	*MAP1A*	0.48 (0.05)	0.0010	3.2×10^{-08}	0.0048	5.1×10^{-06}	0.0
cg15903032	10	NA	0.57 (0.04)	0.0010	7.6×10^{-08}	0.0037	2.8×10^{-03}	0.2
cg18030453	3	*LARS2*	0.72 (0.04)	0.0009	4.5×10^{-09}	0.0028	1.7×10^{-03}	0.1
cg21139312	17	*MSI2*	0.89 (0.03)	0.0009	4.5×10^{-10}	0.0028	1.2×10^{-06}	2.0
cg21506299	6	*MAP3K5*	0.23 (0.06)	-0.0010	3.5×10^{-08}	-0.0019	2.8×10^{-03}	1.1

Betas shows the regression coefficients based on linear mixed models and reflect differences in methylation beta values per increase in BMI unit.

Models are adjusted for age, gender, current smoking, leukocyte proportions, array number, and position on array.

ARIC, Atherosclerosis Risk in Communities; BMI, Body Mass Index; NA, Not Annotated; RS, Rotterdam Study

Table 3. CpG methylation sites associated with WC in RS at level of genome-wide significance ($P < 1.08 \times 10^{-7}$) and successfully replicated at ARIC ($P < 1.92 \times 10^{-3}$)

ProbeID	Chr	Gene	Mean (SD) methylation	Discovery Panel (RS)		Replication Panel (ARIC)		%Variance explained
				Beta	*P*	Beta	*P*	
cg00574958	11	*CPT1A*	0.19 (0.04)	-0.0005	1.2×10^{-17}	-0.0034	5.8×10^{-17}	3.3
cg00851028	1	*NA*	0.72 (0.04)	0.0004	6.0×10^{-09}	0.0043	1.2×10^{-04}	0
cg04927537	17	*LGALS3BP*	0.57 (0.05)	0.0006	7.0×10^{-08}	0.0093	7.0×10^{-08}	1.6
cg05899984	12	*NA*	0.84 (0.03)	0.0003	8.1×10^{-08}	0.0038	5.7×10^{-06}	2.9
cg06500161	21	*ABCG1*	0.71 (0.03)	0.0005	2.4×10^{-12}	0.0096	4.4×10^{-19}	0.8
cg11024682	17	*SREBF1*	0.55 (0.04)	0.0005	2.9×10^{-15}	0.0080	3.5×10^{-12}	1.2
cg13139542	2	*NA*	0.89 (0.02)	0.0002	6.0×10^{-08}	0.0029	4.7×10^{-06}	0
cg15416179	17	*MAP2K3*	0.14 (0.03)	-0.0002	9.1×10^{-08}	-0.0019	2.6×10^{-04}	3.6
cg17901584	1	*DHCR24*	0.68 (0.07)	-0.0005	1.7×10^{-08}	-0.0080	8.3×10^{-08}	2.0
cg18030453	3	*LARS2*	0.72 (0.04)	0.0003	8.8×10^{-08}	0.0029	8.6×10^{-04}	0
cg18772573	17	*CPSF4L*	0.85 (0.03)	0.0003	7.3×10^{-08}	0.0039	2.8×10^{-05}	0
cg21139312	17	*MSI2*	0.89 (0.03)	0.0004	5.9×10^{-12}	0.0028	6.1×10^{-07}	8.2
cg24174557	17	*TMEM49*	0.38 (0.07)	-0.0005	1.1×10^{-08}	-0.0059	5.3×10^{-05}	0

Betas shows the regression coefficients based on linear mixed models and reflect differences in methylation beta values per increase in WC unit.

Models are adjusted for age, gender, current smoking, leukocyte proportions, array number, and position on array.

ARIC, Atherosclerosis Risk in Communities; NA, Not Annotated; RS, Rotterdam Study; WC, waist circumference.

respective mean values of BMI in RS and ARIC study were 27.6 kg/m2 and 30.1 kg/m2. The mean values of WC were 93.7 cm and 101.3 cm in RS and ARIC, respectively.

Table 2 and Table 3 present the CpG sites associated with BMI and WC in both populations. Using the Bonferroni-corrected statistical significance level of 1.07 × 10-7 we identified 14 CpG sites associated with BMI (Supplementary Table 1) and 26 CpG sites with WC (Supplementary Table 2) in RS. In the ARIC Study we successfully replicated 12

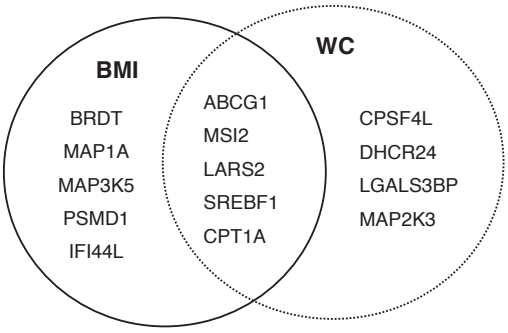

Figure 1. Successfully replicated CpGs for BMI and WC and their overlap

out of 14 BMI related CpG sites (P <3.57 × 10-3) (Table 2) and 14 out of 26 WC related CpG sites (P <1.92 × 10-3) (Table 3). Among these, eight BMI-related CpG sites and 11 WC-related CpG sites were novel. The most significant novel CpG sites were located at MSI2 (cg21139312) and LARS2 (cg18030453) both for BMI and WC. For MSI2 methylation, we observed an increase of 0.0009 (P 4.5 × 10-10) and 0.0004 (P 5.9 × 10-12) with every increase of BMI (kg/m2) and WC (cm), respectively. For LARS2 methylation, an increase of 0.0009 (P 4.5 × 10-10) was observed with every unit increase of BMI, and an increase of 0.0003 (P 8.8 × 10-08) with every unit increase of WC. Additionally, for BMI other novel CpGs were located in the BRDT (cg03421440) and MAP1A (cg15159104) genes. For WC, the other top novel CpG sites were located in TMEM49 (cg24174557) and LGALS3BP (cg04927537) genes. In addition to these novel findings, we confirmed previous reported CpG sites including CPT1A, ABCG1 and SREBF1 associated with BMI and WC. The scatterplots of the association between the replicated CpG sites are shown in Supplementary Figure 1 and Supplementary Figure 2. Figure 1 summarizes successfully replicated findings for BMI and WC and highlights the overlapping loci including ABCG1, MSI2, LARS2, SREBF1, and CPT1A. To test for genomic inflation, we calculated the lambda for the EWAS on BMI and WC, and created QQ-plots. The lambda was 1.487 and 1.556 for the EWAS on BMI and WC, respectively. The QQ-plot are shown in Supplementary Figure 3 and Supplementary Figure 4.

We calculated a methylation risk score based on the 12 CpG sites for BMI and 14 CpG sites for WC that were identified and replicated in the current study. For BMI, 2.0% of the variance was explained by the methylation risk score, whereas for WC the variance explained was 6.4%.

DISCUSSION

This study used an EWAS approach to identify novel differentially methylated genes for obesity-related traits in older adults. The EWAS analysis in RS provided numerous novel loci associated with BMI and WC, of which many findings successfully replicated in ARIC. Our most significant CpG sites associated with both BMI and WC were located at the *MSI2* and *LARS2* genes. Additionally, CpG sites at *BRDT* and *MAP1A* were associated with BMI, and CpG sites at *TMEM49* and *LGALS3BP* were associated with WC. Moreover, we confirmed previous findings that methylation at *CPT1A*, *ABCG1*, and *SREBF1* are associated with BMI and WC.

Previous EWAS on obesity traits were conducted in population-based studies including ARIC and Genetics of Lipid Lowering Drugs and Diet Network Study (GOLDN) (9, 10), and in individuals with history of myocardial infarction or healthy blood donors from the Cardiogenics Consortium (11). Similar to our findings, ARIC and GOLDN reported an inverse association between CpG site at *CPT1A* and BMI (9, 10) and positive associa-

tions of CpGs at *ABCG1* and *SREBF1* with BMI and WC (10). The Cardiogenics Consortium, however, reported only a positive association between three CpG sites at *HIF3A* with BMI in both blood and adipose tissue DNA in European adults (11). These CpGs sites at *HIF3A* did not achieve the threshold for statistical significance in our study. However, CpGs sites at *HIF3A* were replicated by ARIC in DNA blood (10). This discrepancy may be due to difference in the prevalence of obesity and comorbidities in our study (25% obese, 11% diabetes, 7 % CHD) compared with ARIC (44% obese, 26% diabetes) and Cardiogenics Consortium (4% diabetes, 52% MI).

The known loci, *CPT1A, ABCG1,* and *SREBF1* are involved in regulation of lipids, lipoprotein metabolism and insulin sensitivity (24-26). Specifically, the *CPT1A* gene encodes for carnitine palmitoytransferase-1, which is a mitochondrial protein involved in fatty acid metabolism (27) and lipoprotein subfraction (25). The *ABCG1* gene encodes for ATP-binding cassette sub-family G member 1 protein and is involved in the transport of cholesterol and phospholipids in macrophages (28). Finally, the *SREBF1* gene encodes for sterol regulatory element-binding transcription factor 1, which is known to promote adipocyte differentiation and signaling of insulin action (26). Although it has been shown previously that these loci are associated with obesity-related traits, it is still important to replicate these findings across different study population. Since the EWAS approach is hypothesis-free, findings are prone to be false-positive. By replicating previously reported results, we can say with more certainty that these CpG sites are true-positive findings.

In addition to confirming these previously identified loci, we have identified and replicated novel CpG sites located in the gene body of the *MSI2* (cg21139312) and *LARS2* (cg18030453) gene, which were associated with both BMI and WC. The CpG site at *MSI2* gene explained 2.2% of variation in BMI and 8.2% of variation in WC. *MSI2* encodes RNA-binding proteins and plays a central role in posttranscriptional gene regulation (29). A genome-wide association study in pigs suggested that *MSI2* is associated with eating behaviors, including number of visits to feeder per day (30). Moreover, another study performed in mice reported that *MSI2* is linked with the proliferation and maintenance of stem cells in the central nervous system (31). This study suggested that during neurogenesis *MSI2* expression persisted in a subset of neuronal lineage cells, such as parvalbumin-containing GABA neurons in the neocortex (29, 31). GABA receptors are involved in controlling feeding behavior, reinforcing the role of *MSI2* in obesity. The other novel locus associated with both BMI and WC, *LARS2,* encodes an enzyme that catalyzes aminoacylation of mitochondrial tRNALeu (32). A previous post-mortem study showed that *LARS2* expression (human leucyl-tRNA synthetase 2, mitochondrial NM015340) was increased in brain tissue of patients with bipolar disorder compared to controls (33). Considering that bipolar disorder is associated with obesity, overweight, and abdominal obesity (34), methylation of *MSI2* and *LARS2* could play a role in disturbances in eating

behaviors, and consequently BMI and WC. However, further studies are warranted to establish the temporality and the pathway of the associations. Even though previous studies have investigated the association between DNA methylation and anthropometrics, this study is the first report an association between DNA methylation of several CpG sites, including *MSI2* and *LARS2*, with BMI and WC. One possible explanation of discrepancies between findings of our current study compared to previous similar studies, is the difference in population characteristics. Study populations from previous studies consisted of mixed ethnic groups, participants of younger age, or at high disease risk (11, 35-37). Considering that our discovery cohort consisted of an ethnic homogenous group of older adults from the general population of Rotterdam, underlying mechanisms may differ from other population groups.

In this study we conducted an EWAS in a European population and replicated the findings in African Americans. Epidemiologic studies have reported large disparities across racial/ethnic groups in the development of obesity (38). For example, in the current study the rates of obesity were significantly lower in Europeans (24.6%) than in African Americans (43.8%). However, despite the differences in ethnicity and prevalence of obesity between our studies, most of our CpGs sites (86.7%) successfully replicated in ARIC. This may indicate that, in contrast to genetic studies where replication across ethnic groups is challenging due to differences in LD pattern, epigenetic findings could more easily be translated across ethnic groups.

The strength of the current study includes the large sample size with available data on DNA methylation and the ability to replicate our findings in different ethnic population. However, the results of this study must be interpreted in light of several limitations. We used whole blood samples for the quantification of DNA methylation, whereas adipose tissue may be a more relevant tissue in examining obesity. In this case, important CpG sites may not have been identified in our study. Unlike in genetic studies, unraveling the direction of the association between DNA methylation and phenotypes in epigenetic epidemiology remains challenging. Due to the cross-sectional design and the nature of our variables, which are responsive to the environment and dynamic over time, a temporal direction of the association between DNA methylation and anthropometric measures cannot be determined. As previous studies have shown that change in DNA methylation is a consequence of BMI for the majority of CpG sites, this may be the most likely direction for the associations observed in the current study as well (37). However, longitudinal studies are required to confirm the direction of the associations between DNA methylation and anthropometrics. Another possibility is that our findings could be explained by third common factors. For instance, associations may be confounded by differences in cell type proportion. In order to avoid this source of confounding, all analyses were adjusted for cell type proportions. However, as in any observational study residual confounding, due to various lifestyle factors, still remains an issue. Another possibility is

that our findings could be explained by third common factors. For instance, associations may be confounded by differences in cell type proportion. In order to avoid this source of confounding, all analyses were adjusted for cell type proportions. However, as in any observational study residual confounding, due to various lifestyle factors, still remains an issue. Furthermore, the QQ plots showed a high genomic inflation. Many EWAS studies have reported high genomic inflation (39). Adjustment for potential confounders such as technical covariates could decrease the inflation. The correlation between CpGs and the large number of findings in EWAS studies are suggested to explain the residual inflation (40). In this study we did adequate adjustment for technical covariates. Moreover, the replication of our results in an independent population provides further evidence for the robustness of our findings.

In conclusion, we have reported a novel association of increased methylation at the *MSI2* and *LARS2* genes with increased BMI and WC in older adults. Moreover, we confirmed three previously identified methylation loci (*CPT1A*, *ABCG1* and *SREBF1*) suggested to be associated with obesity. Further investigations using repeatedly measured genome-wide DNA methylation and obesity-related traits are needed to assess causality and to further evolve the growing field of epigenetic epidemiology toward novel therapeutic and preventative approaches of obesity and non-communicable related disorders.

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CHAPTER 5

Diabetes Adverse Outcomes

Chapter 5.1

Type 2 diabetes and dementia risk: a mendelian randomization study

Jana Nano[1,2,3], Frank Wolters[1,2], Yuan Ma[2], Taulant Muka[2], Oscar H. Franco[2], Abbas Deghan[4], Arfan Ikram[2], Albert Hofman[1,2]

[1] Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, USA
[2] Department of Epidemiology, Erasmus MC, Rotterdam, The Netherlands.
[3] Institute of Epidemiology, Helmholtz Zentrum München, Neuherberg, Germany; German Center for Diabetes Research, Germany
[4] Department of Epidemiology and Biostatistics, Imperial College London, London, UK

(In preparation)

ABSTRACT

Background

In observational studies, type 2 diabetes has been associated with an increased risk of dementia, but the causal direction and magnitude of the association remains uncertain. We aimed to appraise the causal relevance by using a Mendelian Randomization approach.

Method

We used data from a prospective population-based cohort study, comprising of 9354 individuals without dementia at baseline. Cox proportional hazard models were used to investigate the association of prediabetes and type 2 diabetes with dementia risk. A Mendelian Randomization (MR) study was performed using genetic variants recently identified in a genome wide association study (GWAS) of type 2 diabetes among Europeans. Associations with dementia were investigated within the Rotterdam study and large-scale GWAS.

Results

During follow-up (median of 8.7 years), 861 participants were diagnosed with dementia. The predicted hazard ratio of prediabetes on dementia risk was 1.11 (95% Confidence Interval (CI): 0.95, 1.29), whereas for diabetes, we found a hazard ratio of 1.31 (95%CI: 1.08, 1.60) after adjusting for multiple confounders. The genetic risk score was associated with increased risk of diabetes (per log odds ratio of diabetes=0.10 (0.008), P value= 2 × 10-16), explaining 3.1% of the observed variation. Multiple instrumental variable analysis using genetic associations from previous GWA studies did not provide evidence for a causal role of diabetes on dementia risk.

Conclusions

Previously observed association between type 2 diabetes and dementia risk in observational studies might not be causal. Likely, results from the observational studies can be explained by reverse causation and/or residual confounding.

INTRODUCTION

Prospective observational studies have reported that type 2 diabetes increases the risk of dementia by ~2 fold even after adjusting for other cardiometabolic risk factors (1-3). Dementia prevalence is rising dramatically, making it a leading cause of dependence and disability worldwide (4, 5). Targeting modifiable risk factors such as type 2 diabetes might help to further reduce incidence of dementia. It has been postulated that the common pathogenesis between these disease entities is multifactorial, including systemic inflammation, oxidative stress, insulin resistance, and advances glycation end products (6-8). Worldwide, ~3% of dementia cases are attributable to diabetes (9). However, this suggestion is predicated on the risk factor having causal effects on dementia risk, which is currently uncertain. While implementing randomized trial of risk factor modification might not be feasible, alternative approaches such as Mendelian Randomization (MR) offer an opportunity to investigate causality of associations, and therefore help prioritization of prevention strategies.

The Mendelian Randomization approach, which mimics the random allocation of individuals to the placebo and intervention arms of a randomized clinical trial, is based on the fact that alleles are allocated randomly during gamete formation and consequently, genetic variants are inherited independently of potential confounding (10). The aim of this study was to conduct a Mendelian Randomization study, using diabetes related genetic variants reported in the recent genome wide association study (GWAS) of type 2 diabetes in Europeans, to help clarify the nature of the association between diabetes and dementia risk (11). We investigated the associations between prediabetes and diabetes with the risk of dementia in a large prospective population-based cohort study of participants aged ≥ 45 years. Further, we checked the MR assumption in our population and utilized summary level consortia data to test our hypothesis.

METHODS

Study population

The Rotterdam Study is a prospective cohort study which started since 1990 in the Ommoord district, in the city of Rotterdam, The Netherlands. 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. The third cohort was formed in 2006 and included 3932 participants aged 45 years and older (RS-III). There were no eligibility criteria to enter the Rotterdam Study cohorts except the minimum age and residential area based on postal codes. Participants have been re-examined every 3–4 years, and have been followed up for a variety of diseases. A more detailed description of the Rotterdam Study

can be found elsewhere (12). 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. We used the third visit of the first cohort (1997–99) and the first center visit for both the second cohort (2000–01) and the third cohort (2006–08) as baseline. Figure 1 represent the study flowchart with respective exclusion criteria for each step.

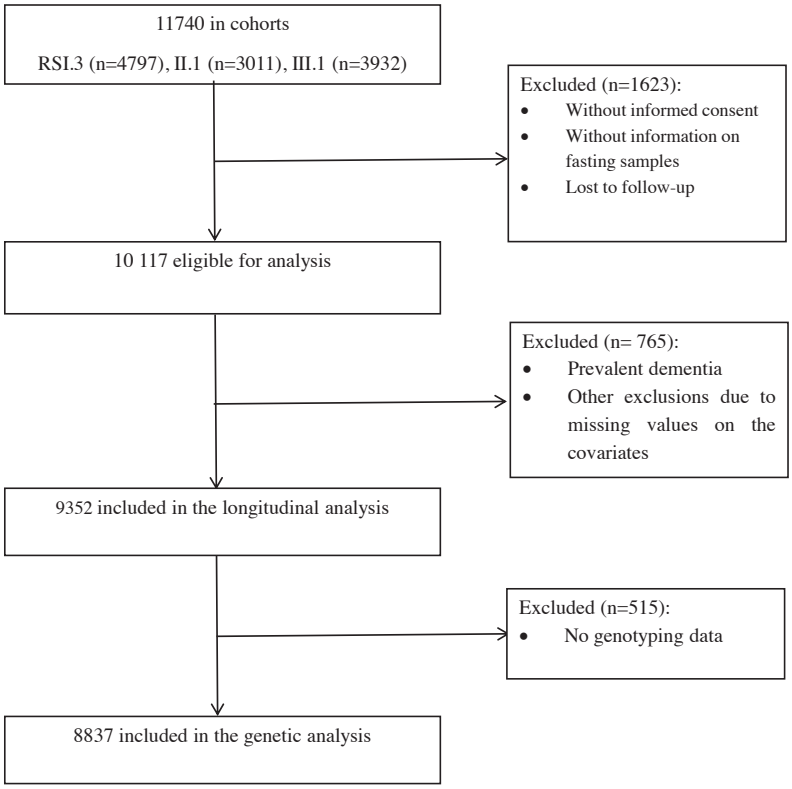

Figure 1. Flowchart for the selection of study participants in the Rotterdam Study.

Assessment of prediabetes and type 2 diabetes

The participants were followed from the date of baseline center visit onwards. At baseline and during follow-up, cases of prediabetes and type 2 diabetes were ascertained through active follow-up using general practitioners' records, hospital discharge letters, pharmacy dispensing data, and serum fasting glucose measurements taken from the Rotterdam Study visits. According to the WHO guidelines, prediabetes was defined as a fasting blood glucose between 6.0 mmol/L and 7.0 mmol/L and type 2 diabetes was

defined as a fasting blood glucose > 7.0 mmol/L, or the use of blood glucose lowering medication (13). Information regarding the use of blood glucose lowering medication was derived from both structured home interviews and linkage to pharmacy records. At baseline, more than 95% of the Rotterdam Study population was covered by the pharmacies in the study area. All potential events of prediabetes and type 2 diabetes were independently adjudicated by two study physicians. In case of disagreement, consensus was sought with a specialist.

Ascertainment of incident dementia

The method for dementia screening and diagnosis in the Rotterdam Study has been described in more detail previously (14). In brief, participants were screened for all-cause dementia (which includes Alzheimer disease (AD), vascular dementia, and Parkinson disease dementia) using a 3-step protocol. In the first step, participants underwent a Mini-Mental State Examination (MMSE) and Geriatric Mental Schedule (GMS) at baseline and during follow-up examinations (15). Screen-positive participants (MMSE ,26 or GMSE .0) were invited for the second step, which consisted of a physician interview using the Cambridge Examination for Mental Disorders in the Elderly (CAMDEX). Besides these examinations, all participants are continuously monitored for dementia using digital linkage of the study database with medical records from general practitioners in the area and the Regional Institute for Outpatient Mental Health Care. Final diagnosis was made by a consensus panel, led by a neurologist, according to the standard criteria for dementia (DSM-III-R) and AD (National Institute of Neurological and Communicative Disorders and Stroke– Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA)) (15). Follow-up for incident dementia was near complete (98.0% of potential person-years) until January 1st, 2015.

Other measurements

Information on medical history and medication use was obtained from questionnaires in combination with medical records. During home interviews, participants provided information on smoking habits, alcoholic consumption and education. Smoking habits were categorized as current, former and never smoking. Education was defined as low (primary education), intermediate (secondary general or vocational education), or high (higher vocational education or university). Body mass index was calculated as weight in kilograms divided by squared height in meters. Total cholesterol, high-density lipoprotein cholesterol was measured at the baseline visit. At home interview, participants self-reported if they were using statins or anti-hypertensive medications. Blood pressure was measured in the sitting position on the right arm and calculated as the mean of two measurements using a random-zero sphygmomanometer. Physical activity was assessed with an adapted version of the Zutphen Physical Activity Questionnaire Every activity

mentioned in the questionnaire was attributed a MET-value according to the 2011 and therefore, providing total physical activity in MET hours/week (16). APOE genotype was determined using PCR on coded DNA samples. The assessment of cardiovascular disease including coronary heart disease and stroke was done through active follow-up, as described previously (17), using general practitioner records and hospital discharge letters

Genotyping

Genotyping was conducted, in self-reported white participants in all three cohorts using the Illumina Infinium HumanHap550K Beadchip in RS-I and RS-II and the Illumina Infinitum HumanHap 610 Quad chip in RS-III at the Genetic Laboratory of the Erasmus MC, Department of Internal Medicine, Rotterdam, the Netherlands. Participants were excluded if they had excess autosomal heterozygosity, mismatch between called and phenotyping sex, or recognized as being outlier with identical-by-state clustering analysis. Before imputation, SNPs with minor allele frequency (MAF) < 0.01, call rate < 95% and departure from Hardy-Weinberg equilibrium cut off P-value 1×10-6 were excluded. SNPs were imputed based on the 1000 Genomes cosmopolitan phase 1 version 3 reference.

Construction of genetic risk score

In this study, we selected 70 independent single-nucleotide polymorphisms (SNPs) recently reported in the GWA of diabetes conducted in European populations (11). The SNPs characteristics are shown in Supplementary Table 1. The effect allele (coded 0-2) was the diabetes risk-raising allele. We then calculated the genetic risk score by multiplying the number of risk alleles at each locus by corresponding reported beta coefficient and summed the products.

Statistical analysis

Continuous variables were reported as mean ± SD unless otherwise indicated and categorical variables were presented as percentages. For the observational association between prediabetes, type 2 diabetes and the risk of dementia, we estimated hazard ratios (HRs) in two adjusted Cox proportional hazard models. Model 1 was adjusted for age, sex and study cohort whereas model 2, was further adjusted for body mass index, systolic and diastolic blood pressure, use of blood pressure lowering medication, statins use, cholesterol levels, high density lipoprotein, C-reactive protein, smoking, alcohol intake, physical activity, education, APOE genotype, history of heart failure, history of stroke and history of coronary heart disease. We verified that the proportional hazard assumption was met.

Associations of individual SNPs and genetic risk score with diabetes and prediabetes (combined prevalent and incident cases) were assessed using logistic regression analysis among participants in the Rotterdam Study. The same was performed for all dementia

cases. SNPs were modelled per diabetes-increasing allele (additive model). Estimates in the Rotterdam Study represent log odds ratios (ORs) per genetically predicted unit difference in log-odds of having the relevant exposure.

MR using data from International Genomics of Alzheimer's Project (IGAP)

To maximize the statistical power, we examined the association of diabetes –related genetic variants with dementia using data from the largest GWAS meta-analyses of Alzheimer Disease in individuals of European ancestry, IGAP (Figure 2). In stage 1, IGAP used genotyped and imputed data on 7,055,881 SNPs to meta-analyze four previously published GWAS datasets consisting of 17,008 AD cases and 37,154 controls (18). We extracted individual diabetes SNP effect estimates and accompanying standard errors in the IGAP database in order to calculate a combined effect of the individual genetic instrumental variable on dementia by means of an inverse-variance weighted (IVW) approach (19, 20). An important assumption of MR is that each SNPs must only influence risk of the outcome through the exposure of interest; inclusion of SNPs that contribute through pleiotropic pathways could bias the results. Type 2 diabetes SNPs are likely to lay in genes of different biological effect that may, or may not, influence dementia risk independent of diabetes. To account for the potential inclusion of invalid genetic instrumental variables, we performed sensitivity analysis using MR-Egger regression and weighted median estimator analyses. As opposed to IVW, MR-Egger regression allows an intercept distinct from the origin providing evidence for pleiotropic effects (21, 22). Additionally, the slope of the MR-Egger regression can provide pleiotropy-corrected causal estimates; however, this estimate of slope is underpowered unless the SNPs combine to explain a large proportion of the variance in the exposure with varying effect sizes. An important condition of this approach is that a SNP's association with the exposure variable must be independent of its direct effects upon the outcome (previously described as the Instrument Strength Independent of Direct Effect or InSIDE assumption), which

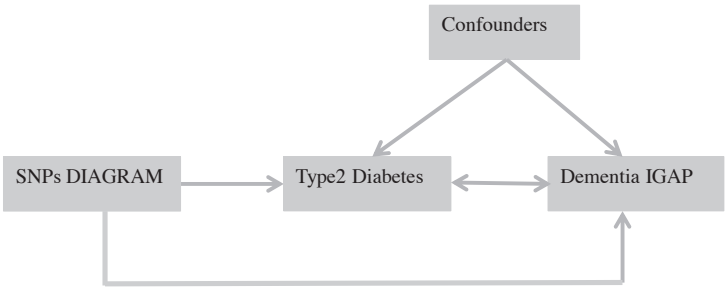

Figure 2. Schematic representation of the Mendelian Randomization analysis in Consortia. SNPs associated with diabetes from DIAGRAM consortia are selected and the corresponding effect estimates of these SNPs on dementia were retrieved from IGAP consortia of dementia.

may not always be satisfied in cases when all pleiotropic effects can be attributed to a single confounder. Nonetheless, the MR-Egger method can provide unbiased estimates even if all the chosen SNPs are invalid (21). On the other hand, in the weighted median approach, MR estimates are ordered and weighted by the inverse of their variance. The median MR estimate should remain unbiased as long as greater than 50% of the total weight comes from SNPs without pleiotropic effects. The weighted median approach offers some important advantages over MR-Egger because it improves precision and is more robust to violations of the InSIDE assumption. Therefore, we employed both methods as sensitivity analyses to assess whether pleiotropy had influenced our results (23).

In an additional analysis, we used sets of variants with similar patterns of diabetes related quantitative trait associations such as insulin secretion, insulin resistance and body mass index/lipids in association with dementia risk. The sets of variants were identified through linkage hierarchical clustering methods of diabetes-related metabolic phenotypes as previously described (11, 24).

Power calculations were performed using a publically available power calculator (http://cnsgenomics.com/shiny/mRnd/). Using data of the IGAP consortium, we had 100% power to detect a causal association with an effect estimate of 1.3 from the observational result.

Statistical analyses were performed using R version 2.15.1 (R Foundation for Statistical Computing, Vienna, Austria) and in particular, "MRCIEU/TwoSampleMR" package. Missing data for these covariates were imputed (n=5 imputations) by using 'mice' package in R.

RESULTS

Baseline characteristics of the population use for analysis in the Rotterdam Study are presented in Table 1. The mean age of the study population was 64.6 years with 57.3% being women. In our population, the prevalence of prediabetes was 25.6% (2388 cases out of 9328 individuals) and for type 2 diabetes was 11.7% (1100 cases out of 9328 individuals). Among those who had diabetes, 50.9% (560) did not use antidiabetic medication, 34% (383) used diabetes medication (specifically, 44% (491) used oral medication and 13% (146) used insulin). During a median follow-up of 8.7 years (IQR: 7.0 – 14.3), 861 participants were diagnosed with dementia.

Association between prediabetes, type 2 diabetes and dementia risk

Presence of prediabetes was not associated with the risk of dementia neither in the age and sex adjusted model nor after controlling for a wide range of confounders [hazard ratio (HR): 1.11 (95% Confidence Interval (95%CI): 0.95, 1.29)] (Table 2). For type 2 diabetes, we found an increased risk of dementia in the first model (HR: 1.28, 95%CI: 1.06, 1.54), which was reinforced in the fully adjusted model (HR: 1.31, 95%CI: 1.08, 1.60). In the

Table 1. Baseline characteristics of study participants

N	9352
Gender (Women)	6259 (57.3)
Age (years)	64.6 (9.9)
Waist circumference (cm)	93.72 (12.1)
Body Mass Index (kg/m2)	27.26 (4.2)
Glucose (mmol/L)	5.82 (1.4)
Insulin (pmol/L)	73.00 [50.0, 106.0]
HOMA-IR (units)	2.58 [1.7, 4.0]
Systolic blood pressure (mm Hg)	137.38 (21.3)
Diastolic blood pressure (mm Hg)	78.40 (11.8)
Triglycerides (mmol/L)	1.54 (0.8)
LDL cholesterol (mmol/L)	3.64 (0.9)
Total cholesterol (mmol/L)	5.71 (1.0)
HDL cholesterol (mmol/L)	1.40 (0.4)
C-reactive protein (mg/L)	1.60 [0.6, 3.5]
Physical activity (MET-hours/week)	74.49 (50.2)
Alcohol intake(grams/day)	3.75 [0.5, 11.4]
History of coronary heart disease	766 (7.2)
History of stroke	228 (2.2)
History of heart failure	231 (2.3)
Use of hypertensive medication	2584 (24.4)
Use of statins	1406 (13.3)
Highest level of education	
Primary	1310 (12.4)
Lower/intermediate	4321 (40.8)
Intermediate / higher	3081 (29.1)
Higher	1876 (17.7)
Smoking history	
Never	3223 (30.8)
Ever	2598 (24.8)
Current	4643 (44.4)
APOE genotype	
0	6875 (71.6)
1	2504 (26.1)
2	219 (2.3)

Data are n (%), mean(SD), or median [IQR, interquartile range; for characteristics with skewed distributions]
HOMA-IR: homeostatic model assessment –insulin resistance; HDL- high density lipoprotein; LDL- low density lipoprotein.

sensitivity analysis, censoring for all dementia cases occurring in the first four years of the follow-up and excluding comorbidities such as history of cardiovascular disease and heart failure, revealed similar results (Supplementary Table 2). However, in an attempt to investigate the association between diabetes and Alzheimer Disease cases only, the effect estimates attenuated although the results did not reach statistical significance. Baseline diabetes treatment, in particular in individuals on oral medication, was associated with higher risk of dementia (HR: 1.49, 95%CI: 1.05, 2.12) as compared to individuals without drug treatment (HR: 1.05, 95%CI: 0.8,1.38) (Supplementary Table 3).

Table 2. Association between prevalence of prediabetes and type 2 diabetes with incident of dementia in the Rotterdam Study.

		Model 1 (HR, 95% CI)	Model 2 (HR, 95% CI)
	Events/ No of participants		
Prediabetes	861/9352	1.12 (0.94, 1.30)	1.10 (0.95, 1.29)
Type 2 Diabetes	861/9352	1.28 (1.06, 1.54)	1.31 (1.08, 1.60)

Model 1: adjusted for age, sex, cohort

Model 2: adjusted for age, sex, cohort, body mass index, systolic and diastolic blood pressure, use of blood pressure lowering medication, statins use, cholesterol levels, high density lipoprotein, C-reactive protein, smoking, alcohol intake, physical activity, education , *APOE* genotype, history of heart failure, history of stroke, history of coronary heart disease.

Abbreviations: HR, hazard ratio; CI, confidence interval;

Diabetes related genetic variants and risk of dementia

Overall, we identified 70 LD-independent SNPs associated with diabetes in the recently published GWAS study among European from the DIAGRAM consortium and (11). While some of these SNPs were associated with diabetes within the Rotterdam study, none of the SNPs were found to be associated with odds of dementia (PBonferroni-adjusted for 70 SNPs=0.00071) (Supplementary Table 4). The genetic risk score of 70 SNPs was normally distributed among the study participants and was associated with diabetes (per log odds ratio of diabetes=0.10 (0.008), P value= $2 \times 10\text{-}16$). An unbiased estimate of the variance explained by the genetic risk score was 0.031 as tested through Nagelkerke R2. In the Rotterdam Study, we observed no association between diabetes genetic risk score and dementia (per log odds ratio of dementia: 0.008 (-0.02, 0.19)).

To boost our sample size, we then evaluated causal estimates in currently available GWAS of diabetes and dementia. However, not all of diabetes SNPs were present in the IGAP dataset. There were 8 non-overlapping SNPs between the diabetes-dementia GWAS results and we could only identify proxy SNPs for 4 of them (Supplementary Table 5). Moreover, 9 palindromic SNPs were identified with intermediate allele frequencies which were additionally removed. The total number of diabetes SNPs included in the final analysis was 57.

As shown in Table 3, we used several methods to calculate the causal estimate. IVW, which is an efficient method when all genetic variants are valid instruments, detected no evidence for a causal relation between diabetes and dementia (per log OR (95%CI): 0.02 (-0.05, 0.09)). MR-regression (-0.003 (-0.18, 0.17)) and weighted median estimator were consistent with these results (-0.02 (-0.12, 0.08)). We did not find evidence of deviation of the intercept from zero that would have point out towards the presence of pleiotropy (Figure 3).

Table 3. Results of genetically determined diabetes and dementia risk in the Rotterdam study and Mendelian randomization estimates in large genome-wide association studies.

	Method	Beta (95%CI)	Q (p value)
Rotterdam Study	Genetic risk score	0.008 (-0.02, 0.01)	
IGAP			
	Inverse-variance weighted	0.02 (-0.05, 0.09)	113.3 (9.15e-06)
	MR-Egger	-0.003 (-0.18, 0.17)	113.2 (6.47e-06)
	(intercept)	0.002 (-0.02, 0.02)	
	Weighted-Median	-0.02 (-0.12, 0.08)	113.3 (9.15e-06)

Data represented as beta coefficients with 95% confidence interval per log odds ratio of diabetes
Abbreviations: IGAP, International Genomics of Alzheimer's Project; Q, heterogeneity estimate; MR, Mendelian Randomization.

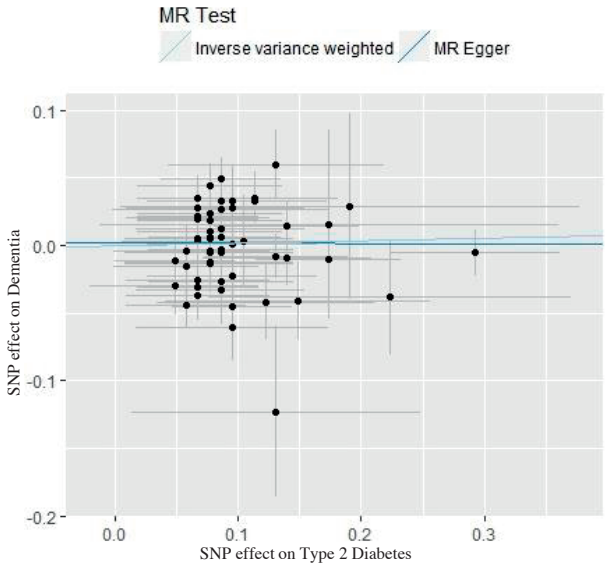

Figure 3. Inverse variance weighted and MR-Egger regression analysis. Results of the individual genetic instruments displayed as the causal effect on dementia in log odds and 95% confidence intervals.

In the sensitivity analysis, we were able to identify three subsets of diabetes-SNPs that were used as proxies for their biological mechanism of action: insulin secretion group (11 SNPs), insulin resistance group (4 SNPs) and body mass index/lipids group (4 SNPs) and were further investigated in relation to dementia risk. This analysis provided no evidence of an association between subsets of diabetes SNPs and dementia risk (Supplementary Table 6).

DISCUSSION

In this population-based study, we found a 31% higher risk of dementia among individuals with diabetes, whereas no association was shown among prediabetes individuals. Using an MR study design of diabetes-genetic variants of the most recent diabetes GWA study in Europeans as instrumental variables, did not support this association to be causal. Altogether, our results suggest that the association could be mainly due to reverse causation or possible confounding.

The direction of estimates in the observational analysis is in line with previous meta-analysis of 20 studies that reported a pooled relative risk of 1.73 among diabetics (3). However, the substantial heterogeneity (I2 >71.2%) observed between the studies among which >73% of dementia cases derived from two large Asian cohorts, together with the younger mean age might explain the difference in magnitude with our study. Several processes are thought to promote the onset of dementia in individuals with diabetes (25), however, the biological mechanism on the basis of this relationship is still uncertain. The pathogenesis shared between these two disease entities seems to be multifactorial involving insulin metabolism, hyperglycemic toxicity, inflammation and vascular changes. Insulin resistance may promote atherosclerosis and lead to vascular changes in the brain (26). On the other hand, oxidative stress due to chronic hyperglycemia and overall accompanying inflammation together with the accumulation of advanced glycation end products that are found in AD can worsen the vascular damage; the latter has been widely implicated in the pathogenesis of dementia (26, 27). Hypoglycemic states, often driven by diabetes treatment have also been associated with impaired cognitive function and dementia (26).

Although the genetic analysis in the Rotterdam Study was not well-powered to yield causal estimates, we were able to test some of the assumptions of the MR analysis. First, there should be a strong association between the genetic risk score and risk factor of interest- SNPs used in our study were associated with diabetes risk in RS and previous GWAS; second, the effect of genetic instrument on the outcome must be mediated exclusively by the exposure and there should be no direct effects (for example, a causal pathway between the genetic variants and outcome that does not involve the exposure and that can be introduced by horizontal pleiotropy or population stratification)- the

genetic risk score was not associated with any of the other diabetes-dementia confounders such as body mass index or triglycerides in the RS; third, the instrumental variables should affect the outcome only through the risk factor of interest- none of the diabetes SNPs have been implicated to play a role in dementia. Nevertheless, no-pleiotropy assumption is difficult to validate. New methods such as MR-Egger or Weighted median were applied as a sensitivity analysis to give consistent estimates when assuming 50% - 100% of genetic variants to be potentially invalid instrumental variables (23); however, no association was observed between the combined effect of the diabetes SNPs on dementia. A previous MR study investigating the effect of a myriad of cardiovascular risk factors including diabetes on dementia risk reported similar null results. Unlike our study, the analysis was based on strong assumption of a diabetes genetic risk score without considering any potential invalidity of the instruments (28). Similarly, another study by Walter et al (29), suggested no causal association between diabetes and dementia. The authors tried to validate the assumption of the MR analysis in a smaller longitudinal study with suboptimal ascertainment of both exposure (predicted diabetes in the population) and outcome (predicted probability of dementia), contrary to our study, in which both diabetes and dementia have been collected actively in the population. Moreover, our subsets of genetic variants used as proxy for potential biological mechanisms related to diabetes were identified by hierarchical clustering and further tested for validity of groups lowering the chance of misclassification between sets, as opposed to the priori literature search of SNP function performed by Walter et al. Taken together, the results of previous studies and ours, point out that the directionality of the association between diabetes and dementia is likely not causal, and (un)known confounding factors or disease processes might be influencing such relationship.

The major strength of this study is the large sample size for both diabetes and dementia in the Rotterdam Study, and the additional sample size utilized through GWAS data. By examining the association with prediabetes and dementia risk, we provided insights of early metabolic abnormalities and dementia. The lack of association hints toward more advanced features of diabetes to be involved in relation to dementia. Moreover, we used data from a well-characterized prospective population-based cohort study, which allowed us to have a comprehensive assessment of this association using both observational and genetic data. Some limitation warrants mention. First, we used multiple instruments selected from the GWA studies that included the current study sample. In case of weak genetic instruments, this might result in the direction of confounded associations. However, this is not the case here as out genetic instruments were strong (F-statistic >10). Although we were well-powered to run this analysis, the number of participants might still be too low for detecting small causal effects. Moreover, the genetic effect was much lower than the observational estimate; a possible true causal effect of diabetes on dementia would be close to zero and therefore have little meaningful clinical relevance.

In conclusion, we cannot verify any causal effect of the association between diabetes and higher risk of dementia. The observational findings are likely due to reverse causation or residual confounding.

Supplementary Table 1. Characteristics of diabetes SNP reported in the European ancestry GWAS from Scott et al. All the SNP were used to create a weighted genetic risk score of diabetes.

Chr	Gene	SNP	Effect allele	Other allele	Effect allele frequency	OR
14	*NRXN3*	rs10146997	G	A	0.2118	1.07
2	*BCL11A*	rs10193447	T	C	0.5979	1.07
7	*DGKB*	rs10238625	A	G	0.5397	1.07
7	*DGKB*	rs10276674	C	T	0.1981	1.09
11	*HSD17B12*	rs1061810	A	C	0.2785	1.08
11	*MTNR1B*	rs10830963	G	C	0.2661	1.08
7	*KLF14*	rs10954284	T	A	0.5017	1.06
9	*CDKN2A/B*	rs10965223	A	G	0.5918	1.08
9	*CDKN2A/B*	rs10965248	T	C	0.8191	1.15
12	*CCND2*	rs11063018	C	T	0.1902	1.09
11	*MAP3K11*	rs111669836	A	T	0.2485	1.07
10	*HHEX/IDE*	rs11187140	G	A	0.6222	1.14
10	*CDC123/CAMK1D*	rs11257659	T	C	0.2293	1.08
13	*SPRY2*	rs11616380	G	T	0.7148	1.09
3	*ADCY5*	rs11708067	A	G	0.7871	1.12
3	*PPARG*	rs11712037	C	G	0.8746	1.14
6	*CENPW*	rs11759026	G	A	0.2371	1.1
8	*TP53INP1*	rs11786613	C	A	0.0317	1.21
7	*MNX1*	rs1182436	C	T	0.7977	1.08
15	*PRC1*	rs12595616	C	T	0.3668	1.07
9	*TLE4*	rs13301067	G	A	0.9236	1.11
2	*GCKR*	rs145819220	G	C	0.0115	1.26
16	*FTO*	rs1558902	A	T	0.4155	1.13
7	*JAZF1*	rs1635852	T	C	0.5016	1.1
5	*ANKRD55*	rs173964	G	A	0.7436	1.06
22	*MTMR3/HORMAD2*	rs2023681	G	A	0.8878	1.13
11	*KCNQ1*	rs2237897	C	T	0.9456	1.25
12	*HMGA2*	rs2258238	T	A	0.1002	1.11
10	*PLEKHA1*	rs2292626	C	T	0.5038	1.09
2	*GRB14*	rs28584669	T	C	0.8333	1.05
16	*CMIP*	rs2925979	T	C	0.2977	1.08
2	*IRS1*	rs2972156	G	C	0.6143	1.08
1	*PROX1*	rs340874	C	T	0.5502	1.07

Supplementary Table 1. (continued)

Chr	Gene	SNP	Effect allele	Other allele	Effect allele frequency	OR
3	*UBE2E2*	rs35352848	T	C	0.7771	1.09
1	*MACF1*	rs3768321	T	G	0.1926	1.08
8	*SLC30A8*	rs3802177	G	A	0.6774	1.12
4	*WFS1*	rs3821943	T	C	0.5351	1.1
1	*NOTCH2*	rs406767	C	T	0.09	1.14
12	*CCND2*	rs4238013	C	T	0.2001	1.1
3	*IGF2BP2*	rs4402960	T	G	0.3056	1.15
8	*TP53INP1*	rs4734285	T	C	0.62	1.06
8	*ANK1*	rs516946	C	T	0.775	1.08
11	*KCNJ11*	rs5219	T	C	0.3828	1.07
19	*GIPR*	rs55864746	A	G	0.3085	1.07
12	*HNF1A (TCF1)*	rs56348580	G	C	0.6831	1.08
19	*CILP2*	rs58489806	T	C	0.0913	1.09
23	*DUSP9*	rs5945326	A	G	0.7455	1.2
4	*ACSL1*	rs60780116	T	C	0.8354	1.09
15	*ZFAND6*	rs62006309	A	G	0.5226	1.05
9	*ABO*	rs635634	T	C	0.18	1.08
5	*ZBED3*	rs6453287	C	A	0.3041	1.07
12	*TSPAN8/LGR5*	rs6581998	C	T	0.2707	1.06
2	*THADA*	rs6757251	C	T	0.9011	1.14
6	*SLC35D3*	rs6918311	A	G	0.5284	1.07
17	*ZZEF1*	rs7224685	T	G	0.3043	1.07
3	*ADAMTS9*	rs7428936	T	C	0.5905	1.07
6	*CDKAL1*	rs7451008	C	T	0.2606	1.19
17	*HNF1B (TCF2)*	rs757209	G	A	0.578	1.09
11	*ARAP1 (CENTD2)*	rs76550717	A	G	0.8299	1.1
17	*GLP2R*	rs78761021	G	A	0.3414	1.07
10	*TCF7L2*	rs7903146	T	C	0.2892	1.34
17	*GIP*	rs79349575	A	T	0.5061	1.07
12	*KLHDC5*	rs7953190	T	C	0.8031	1.08
18	*MC4R*	rs79851087	A	G	0.9721	1.19
16	*BCAR1*	rs8056814	G	A	0.9168	1.16
10	*ZMIZ1*	rs810517	C	T	0.5146	1.09
6	*HLA-DQA1*	rs9271774	C	A	0.7416	1.1
9	*TLE1*	rs9410573	T	C	0.5986	1.08
15	*HMG20A*	rs952471	G	C	0.6869	1.08
5	*ANKRD55*	rs9687833	A	G	0.1865	1.1

Abbreviations: Chr, chromosome; SNP, single nucleotide polymorphism; OR, odds ratio

Supplementary Table 2. Sensitivity analysis between the association of diabetes presence and the risk of dementia in the Rotterdam Study.

	Events/ No of participants	Model 2 (HR, 95% CI)
Censoring all dementia cases occurring in the first 4 years of the follow-up	729/8731	1.28 (1.03, 1.59)
Excluding co-morbidities such as history of cardiovascular disease (including coronary heart disease and stroke) and heart failure	754/8430	1.39 (1.12, 1.73)
Alzheimer Disease as outcome	571/9352	1.12 (0.87, 1.46)
Other than Alzheimer Disease as outcome	290/9352	1.67 (1.22, 2.28)

Abbreviations: HR, hazard ratio; CI, confidence interval;
Results are for Model 2: adjusted for age, sex, cohort, body mass index, systolic and diastolic blood pressure, use of blood pressure lowering medication, statins use, cholesterol levels, high density lipoprotein, C-reactive protein, smoking, alcohol intake, physical activity, education , APOE genotype, history of heart failure, history of stroke, history of coronary heart disease.

Supplementary Table 3. Hazard ratios for dementia of type 2 diabetes by baseline treatment category

	Events/ No of participants	Model 2 (HR, 95% CI)
No drug treatment	741/ 8492	1.05 (0.8, 1.38)
On treatment (oral medication or insulin)	861 / 8492	1.49 (1.05, 2.12)
On oral medication	861 / 8492	1.68 (1.29, 2.21)
On insulin treatment	861 / 8492	1.09 (0.58, 2.05)

Abbreviations: HR, hazard ratio; CI, confidence interval;
Results are for Model 2: adjusted for age, sex, cohort, body mass index, systolic and diastolic blood pressure, use of blood pressure lowering medication, statins use, cholesterol levels, high density lipoprotein, C-reactive protein, smoking, alcohol intake, physical activity, education , APOE genotype, history of heart failure, history of stroke, history of coronary heart disease.

Supplementary Table 4. Associations of individual SNPs with diabetes odds and dementia odds in the Rotterdam Study.

Chr	Gene	SNP	Type 2 Diabetes (1765/8837)			Dementia (950/8837)		
			Betas_ Diabetes	LCI_ Diabetes	UCI_ Diabetes	Betas_ Dementia	LCI_ Dementia	UCI_ Dementia
14	*NRXN3*	rs10146997	-0.065	-0.155	0.025	0.0287	-0.1003	0.1576
2	*BCL11A*	rs10193447	0.104	0.027	0.182	0.0098	-0.0971	0.1166
7	*DGKB*	rs10238625	-0.012	-0.087	0.063	0.0508	-0.0534	0.1551
7	*DGKB*	rs10276674	0	-0.101	0.101	0.0821	-0.0593	0.2236
11	*HSD17B12*	rs1061810	-0.031	-0.114	0.051	-0.0694	-0.1835	0.0448
11	*MTNR1B*	rs10830963	-0.187	-0.286	-0.089	-0.0868	-0.2247	0.0511
7	*KLF14*	rs10954284	0.039	-0.039	0.116	0.0241	-0.0841	0.1323
9	*CDKN2A/B*	rs10965223	0.045	-0.032	0.122	0.0239	-0.0837	0.1315
9	*CDKN2A/B*	rs10965248	0.058	-0.04	0.157	-0.0082	-0.1425	0.126
12	*CCND2*	rs11063018	-0.056	-0.161	0.049	-0.0984	-0.2449	0.0482
11	*MAP3K11*	rs111669836	0.008	-0.086	0.103	-0.1486	-0.2798	-0.0174

Supplementary Table 4. (continued)

Chr	Gene	SNP	Type 2 Diabetes (1765/8837)			Dementia (950/8837)		
			Betas_ Diabetes	LCI_ Diabetes	UCI_ Diabetes	Betas_ Dementia	LCI_ Dementia	UCI_ Dementia
10	*HHEX/IDE*	rs11187140	0.066	-0.014	0.145	0.1177	0.0059	0.2295
10	*CDC123/ CAMK1D*	rs11257659	-0.081	-0.174	0.012	-0.1075	-0.236	0.0211
13	*SPRY2*	rs11616380	0.023	-0.061	0.106	0.0011	-0.1152	0.1175
3	*ADCY5*	rs11708067	0.143	0.054	0.231	0.0045	-0.1157	0.1247
3	*PPARG*	rs11712037	0.066	-0.051	0.183	0.096	-0.0687	0.2607
6	*CENPW*	rs11759026	-0.068	-0.163	0.027	-0.0963	-0.2282	0.0356
8	*TP53INP1*	rs11786613	-0.023	-0.32	0.274	-0.139	-0.5411	0.2631
7	*MNX1*	rs1182436	0.139	0.036	0.241	0.0366	-0.104	0.1771
15	*PRC1*	rs12595616	0.009	-0.068	0.086	0.086	-0.0206	0.1925
9	*TLE4*	rs13301067	0.15	0.005	0.295	-0.0915	-0.2803	0.0973
2	*GCKR*	rs145819220	0.364	-0.321	1.048	0.4634	-0.4691	1.3958
16	*FTO*	rs1558902	-0.081	-0.157	-0.005	0.0399	-0.0667	0.1464
7	*JAZF1*	rs1635852	0.044	-0.031	0.119	-0.0246	-0.1289	0.0797
5	*ANKRD55*	rs173964	0.064	-0.026	0.154	0.0817	-0.0446	0.2079
22	*MTMR3/ HORMAD2*	rs2023681	0.183	0.04	0.327	-0.0665	-0.2574	0.1245
11	*KCNQ1*	rs2237897	0.12	-0.101	0.342	0.1074	-0.2125	0.4274
12	*HMGA2*	rs2258238	-0.103	-0.225	0.019	-0.1247	-0.2934	0.0439
10	*PLEKHA1*	rs2292626	0.005	-0.07	0.081	0.0843	-0.0214	0.1899
2	*GRB14*	rs28584669	-0.064	-0.161	0.033	-0.1131	-0.2473	0.0211
16	*CMIP*	rs2925979	0.003	-0.076	0.083	0.0709	-0.0406	0.1824
2	*IRS1*	rs2972156	0.128	0.049	0.207	-0.0216	-0.1305	0.0873
1	*PROX1*	rs340874	-0.034	-0.109	0.041	0.0069	-0.0966	0.1104
3	*UBE2E2*	rs35352848	0.062	-0.03	0.154	-0.087	-0.2119	0.038
1	*MACF1*	rs3768321	-0.007	-0.102	0.089	-0.0689	-0.2003	0.0625
8	*SLC30A8*	rs3802177	0.18	0.096	0.265	0.0458	-0.0705	0.162
4	*WFS1*	rs3821943	0.11	0.032	0.188	-0.0657	-0.1729	0.0414
1	*NOTCH2*	rs406767	-0.151	-0.441	0.139	-0.3958	-0.7824	-0.0092
12	*CCND2*	rs4238013	-0.104	-0.225	0.018	-0.1996	-0.3663	-0.0329
3	*IGF2BP2*	rs4402960	-0.068	-0.149	0.013	-0.067	-0.1789	0.045
8	*TP53INP1*	rs4734285	0.028	-0.053	0.109	-0.0454	-0.1574	0.0665
8	*ANK1*	rs516946	0.094	0.004	0.185	0.055	-0.0705	0.1804
11	*KCNJ11*	rs5219	-0.104	-0.18	-0.027	-0.0625	-0.1691	0.0441
19	*GIPR*	rs55864746	0.006	-0.078	0.09	-0.0061	-0.123	0.1108
12	*HNF1A (TCF1)*	rs56348580	0.139	0.055	0.223	0.0248	-0.0911	0.1407
19	*CILP2*	rs58489806	-0.148	-0.281	-0.015	-0.0944	-0.2802	0.0914

Supplementary Table 4. (continued)

Chr	Gene	SNP	Type 2 Diabetes (1765/8837)			Dementia (950/8837)		
			Betas_ Diabetes	LCI_ Diabetes	UCI_ Diabetes	Betas_ Dementia	LCI_ Dementia	UCI_ Dementia
23	*DUSP9*	rs5945326	-0.031	-0.132	0.07	-0.0434	-0.2446	0.1578
4	*ACSL1*	rs60780116	0.098	-0.01	0.205	0.0204	-0.1254	0.1661
15	*ZFAND6*	rs62006309	-0.022	-0.099	0.054	0.0845	-0.022	0.191
9	*ABO*	rs635634	-0.096	-0.188	-0.004	-0.0033	-0.135	0.1285
5	*ZBED3*	rs6453287	-0.009	-0.094	0.077	-0.0613	-0.1795	0.0569
12	*TSPAN8/LGR5*	rs6581998	-0.027	-0.109	0.056	0.104	-0.0129	0.221
2	*THADA*	rs6757251	0.112	-0.006	0.23	0.049	-0.1143	0.2124
6	*SLC35D3*	rs6918311	0.07	-0.007	0.147	0.0558	-0.0512	0.1628
17	*ZZEF1*	rs7224685	-0.1	-0.181	-0.019	0	-0.1136	0.1136
3	*ADAMTS9*	rs7428936	-0.092	-0.17	-0.015	-0.0163	-0.1236	0.0909
6	*CDKAL1*	rs7451008	-0.17	-0.254	-0.085	0.1869	0.0637	0.31
17	*HNF1B (TCF2)*	rs757209	0.072	-0.01	0.154	-0.0164	-0.1308	0.098
11	*ARAP1 (CENTD2)*	rs76550717	0.076	-0.027	0.179	0.0098	-0.131	0.1505
17	*GLP2R*	rs78761021	-0.052	-0.134	0.03	0.0815	-0.0346	0.1975
10	*TCF7L2*	rs7903146	-0.31	-0.391	-0.229	0.0785	-0.038	0.1949
17	*GIP*	rs79349575	-0.069	-0.148	0.009	-0.042	-0.1509	0.0668
12	*KLHDC5*	rs7953190	0.118	0.023	0.213	0.1122	-0.0198	0.2441
18	*MC4R*	rs79851087	0.091	-0.178	0.361	-0.1406	-0.4911	0.2099
16	*BCAR1*	rs8056814	0.091	-0.048	0.231	-0.0897	-0.2758	0.0963
10	*ZMIZ1*	rs810517	0.02	-0.055	0.096	-0.0562	-0.161	0.0486
6	*HLA-DQA1*	rs9271774	1.252	-1.495	3.999	-3.8788	-7.659	-0.0986
9	*TLE1*	rs9410573	0.048	-0.032	0.127	-0.1209	-0.2299	-0.0119
15	*HMG20A*	rs952471	0.113	0.03	0.196	0.0516	-0.0624	0.1656
5	*ANKRD55*	rs9687833	0.006	-0.09	0.102	-0.0798	-0.2113	0.0517

Abbreviations: Chr, chromosome; SNP, single nucleotide polymorphism; LCI/UCI (lower confidence interval/ upper confidence interval)

Supplementary Table 5. Overlapping diabetes SNPs in IGAP database. Missing SNPs in the IGAP database were substituted by proxy SNPs when available. Palindromic SNPs with intermediate allele frequencies were further removed.

Chr	Gene	Diabetes SNP	Presence in the IGAP database	Proxy for missing SNPs	Palindromic SNPs	Cluster
14	*NRXN3*	rs10146997	Yes			body-mass index + lipids
2	*BCL11A*	rs10193447	Yes			unclassified
7	*DGKB*	rs10238625	Yes			insulin secretion group
7	*DGKB*	rs10276674	Yes			unclassified

Supplementary Table 5. (continued)

Chr	Gene	Diabetes SNP	Presence in the IGAP database	Proxy for missing SNPs	Palindromic SNPs	Cluster
11	*HSD17B12*	rs1061810	Yes			unclassified
11	*MTNR1B*	rs10830963	Yes		Yes	hyperglycemic
7	*KLF14*	rs10954284	Yes		Yes	insulin resistance
9	*CDKN2A/B*	rs10965223	Yes			unclassified
9	*CDKN2A/B*	rs10965248	Yes			insulin secretion group
12	*CCND2*	rs11063018	Yes			unclassified
11	*MAP3K11*	rs111669836	Yes		Yes	body-mass index + lipids
10	*HHEX/IDE*	rs11187140	Yes			insulin secretion group
10	*CDC123/CAMK1D*	rs11257659	No	rs7077792		insulin secretion group
13	*SPRY2*	rs11616380	Yes			unclassified
3	*ADCY5*	rs11708067	Yes			insulin secretion group
3	*PPARG*	rs11712037	Yes		Yes	insulin resistance
6	*CENPW*	rs11759026	Yes			unclassified
8	*TP53INP1*	rs11786613	Yes			unclassified
7	*MNX1*	rs1182436	Yes			unclassified
15	*PRC1*	rs12595616	Yes			unclassified
9	*TLE4*	rs13301067	No	rs17791513		unclassified
2	*GCKR*	rs145819220	No			NA
16	*FTO*	rs1558902	Yes		Yes	FTO only
7	*JAZF1*	rs1635852	Yes			unclassified
5	*ANKRD55*	rs173964	Yes			insulin resistance
22	*MTMR3/HORMAD2*	rs2023681	No	rs6518681		unclassified
11	*KCNQ1*	rs2237897	Yes			unclassified
12	*HMGA2*	rs2258238	Yes		Yes	unclassified
10	*PLEKHA1*	rs2292626	Yes			unclassified
2	*GRB14*	rs28584669	Yes			NA
16	*CMIP*	rs2925979	Yes			body-mass index + lipids
2	*IRS1*	rs2972156	Yes		Yes	insulin resistance
1	*PROX1*	rs340874	Yes			insulin secretion group
3	*UBE2E2*	rs35352848	Yes			unclassified
1	*MACF1*	rs3768321	Yes			insulin resistance
8	*SLC30A8*	rs3802177	Yes			insulin secretion group

Supplementary Table 5. (continued)

Chr	Gene	Diabetes SNP	Presence in the IGAP database	Proxy for missing SNPs	Palindromic SNPs	Cluster
4	*WFS1*	rs3821943	Yes			unclassified
1	*NOTCH2*	rs406767	Yes			unclassified
12	*CCND2*	rs4238013	Yes			unclassified
3	*IGF2BP2*	rs4402960	Yes			insulin secretion group
8	*TP53INP1*	rs4734285	Yes			unclassified
8	*ANK1*	rs516946	Yes			unclassified
11	*KCNJ11*	rs5219	Yes			unclassified
19	*GIPR*	rs55864746	Yes			body-mass index + lipids
12	*HNF1A (TCF1)*	rs56348580	No	rs11065397	Yes	unclassified
19	*CILP2*	rs58489806	Yes			insulin resistance + lipids
23	*DUSP9*	rs5945326	No			NA
4	*ACSL1*	rs60780116	Yes			unclassified
15	*ZFAND6*	rs62006309	Yes			unclassified
9	*ABO*	rs635634	Yes			ABO only
5	*ZBED3*	rs6453287	No			unclassified
12	*TSPAN8/LGR5*	rs6581998	Yes			unclassified
2	*THADA*	rs6757251	Yes			insulin secretion group
6	*SLC35D3*	rs6918311	Yes			unclassified
17	*ZZEF1*	rs7224685	Yes			unclassified
3	*ADAMTS9*	rs7428936	Yes			unclassified
6	*CDKAL1*	rs7451008	Yes			insulin secretion group
17	*HNF1B (TCF2)*	rs757209	Yes			missing data (to delete)
11	*ARAP1 (CENTD2)*	rs76550717	Yes			proinsulin
17	*GLP2R*	rs78761021	Yes			unclassified
10	*TCF7L2*	rs7903146	Yes			insulin secretion group
17	*GIP*	rs79349575	No			unclassified
12	*KLHDC5*	rs7953190	Yes			unclassified
18	*MC4R*	rs79851087	Yes			NA
16	*BCAR1*	rs8056814	Yes			unclassified
10	*ZMIZ1*	rs810517	Yes			body-mass index + lipids
6	*HLA-DQA1*	rs9271774	Yes			unclassified
9	*TLE1*	rs9410573	Yes			unclassified

Supplementary Table 5. (continued)

Chr	Gene	Diabetes SNP	Presence in the IGAP database	Proxy for missing SNPs	Palindromic SNPs	Cluster
15	*HMG20A*	rs952471	Yes		Yes	unclassified
5	*ANKRD55*	rs9687833	Yes			insulin resistance

Abbreviations: Chr, chromosome; SNP, single nucleotide polymorphism; OR, odds ratio

Supplementary Table 6. Results of MR analyses on SNP subsets of the association between diabetes and dementia risk using DIAGRAM and IGAP consortia data.

Method	SNP set	Beta (95%CI)	Q (Q_pval)
Inverse-variance weighted method			
	Insulin secretion	0.08 (-0.07,0.23)	20.83 (0.02)
	Insulin resistance	-0.015 (-0.31,0.28)	1.077 (0.7)
	Lipids	0.08 (-0.13,0.29)	8.59 (0.03)
MR-Egger			
(intercept)	Insulin secretion	0.26 (-0.46,0.99)	20.11 (0.017)
	intercept	-0.01 (-0.08,0.04)	
	Insulin resistance	0.07 (-3.53,3.68)	0.21 (0.9)
	intercept	-0.008 (-0.30,0.28)	
	Lipids	2.7 (0.3,5.09)	2.46 (0.29)
	intercept	-0.20 (-0.02, -0.37)	
Weighted-Median			
	Insulin secretion	0.11 (-0.15,0.38)	20.83 (0.02)
	Insulin resistance	0.13 (-0.24,0.52)	1.07 (0.7)
	Lipids	0.002 (-1.50,1.50)	8.59 (0.03)

Abbreviations: DIAGRAM consortium, Diabetes Genetics Replication And Meta-analysis; IGAP, International Genomics of Alzheimer's Project; Q, heterogeneity; Q, heterogeneity estimate; MR, Mendelian Randomization.

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Chapter 5.2

A standard set of value-based patient-centered outcome for diabetes mellitus: an international effort for a unified approach.

Jana Nano[1,2*], Magdalena Walbaum[3*], Oluwakemi Okunade[4], Sarah Whittaker[4], Katharine Barnard[5], Daniel Barthelmes[6], Tim Benson[7], Paul Buchanan[8], Ronit Calderon-Margalit[9], Jihan Dennaoui[10], Rob Haig[7], Sergio Hernández-Jimenéz[11], Naomi Levitt[12], Jean Claude Mbanya[13], Saf Naqvi[14], Anne Peters[15], Mark Peyrot[16], William Polonsky[17], Andrew Pumerantz[18], João Raposo[19], Maria Santana[20], Andreas Schmitt[21], Søren Eik Skovlund[22], Cristina García Ulloa[11], Hwee-Lin Wee[23], Jelka Zaletel[24,25], Fabrizio Carinci[26*], Massimo Massi-Benedetti[27*] on behalf of Diabetes Working Group of the International Consortium for Health Outcomes Measurement (ICHOM)

[1] Department of Epidemiology, Erasmus Medical Center, Rotterdam, the Netherlands
[2] Institute of Epidemiology, Helmholtz Zentrum, Munich, Germany
[3] Institute of Epidemiology, University College London, United Kingdom
[4] International Consortium for Health Outcomes Measurement, Cambridge, United States
[5] Bournemouth University, United Kindom
[6] University Hospital Zürich, Switzerland
[7] Patient Representative, Australia
[8] GBDOC, Patient representative, United Kingdom
[9] The Hebrew University of Jerusalem, Israel
[10] National Health Insurance Company – Daman, United Arab Emirates
[11] Instituto Nacional de Ciencia Médicas y Nutrición, Mexico
[12] University of Cape Town, South Africa
[13] University of Yaounde I, Cameroon
[14] Imperial College London Diabetes Centre, United Arab Emirates
[15] The Keck School of Medicine of the University of Southern California, Los Angeles, CA, United States
[16] Loyola University Maryland, United States
[17] Behavioral Diabetes Institute, University of California, United States
[18] Western Diabetes Institute, Western University of Health Sciences, United States
[19] APDP/Nova Medical School Lisbon, Portugal
[20] O'Brien Institute for Public Health, Canada
[21] Diabetes Center Mergentheim, Germany
[22] Aalborg University and Aalborg University Hospital, Denmark
[23] Saw Swee Hock School of Public Health and Department of Pharmacy, Faculty of Science, National University of Singapore, Singapore
[24] National Institute of Public Health, Slovenia
[25] University Medical Centre Ljubljana Slovenia, Slovenia
[26] University of Bologna, Italy
[27] Hub for International Health Research (HIRS), Perugia, Italy

(In preparation)

ABSTRACT

Background

Value-based health care aims to bring together patients and health systems to maximize the ratio of quality in services to cost. To enable the assessment of value in diabetes mellitus care, a standard set of outcomes important to individuals with diabetes was defined for use in routine clinical practice across different settings worldwide.

Methods and Results

The International Consortium for Health Outcomes Measurement (ICHOM) brought together an international Working Group of health professionals and patient representatives from 6 continents in order to reach consensus on a minimum standard set of patient-centered outcomes and risk factors that would allow the tracking, comparison and improvement of diabetes care. Gathered in a series of teleconferences and using a modified Delphi method to reach consensus, the outcome domains and case mix variables considered by the group were generated from systematic literature reviews, input from patients, working group members and an online survey of patients and health professionals. The ICHOM Diabetes consensus measures were designed to be relevant for adult individuals (+18 years old) with type 1 or type 2 diabetes. Five domains were established including diabetes control (glycemic control and other management goals such as blood pressure, lipid profile and body mass index), acute events (related to diabetic ketoacidosis and hyperosmolar hyperglycemic syndrome, hypoglycemia and other acute complications such as stroke and myocardial infarction and lower limb amputation), chronic complications (related to vision, autonomic- and peripheral- neuropathy, Charcot's foot, lower limb ulcers, peripheral artery disease, ischemic heart disease, chronic heart failure, chronic kidney disease and dialysis, cerebrovascular disease, periodontal health, erectile dysfunction and lipodystrophy), health services outcomes (such as hospitalization, emergency room attendance and financial barrier to care) and survival. Baseline demographics, diagnosis profile, and other lifestyle, social and treatment related factors were included to improve the interpretability of comparisons.

Conclusions

This standardized minimum set of patient-centered outcomes is recommended to be collected in routine clinical settings to facilitate international comparisons of outcomes in diabetes care.

INTRODUCTION

The number of individuals with diabetes has more than doubled during the past 20 years with particular high numbers in low-income and middle-income countries resulting in large increases in the total burden of diabetes and diabetes-related deaths (1-4). In high income countries, the prevalence has stabilised due to improvements in early diagnosis and treatment, but the increasing burden of morbidity associated with diabetes has called for resolutions to improve treatment and public health approaches, bringing great strides in reducing the rate of complications (5-7). However, despite all these achievements, there is substantial variation in outcomes important to individuals with diabetes around the globe. This variation is in part due to differences in national diabetes programmes and strategies related to implementation, monitoring and evaluation (2, 8, 9). Seven-fold to 14-fold variation in avoidable hospital admissions and in particular admissions for major lower extremity amputation, respectively, is still evident across countries such as Italy, Colombia, Korea and Mexico (10). Historically, the definitions (and classification) of diabetes have varied by expert committees (11-13). Although both International Diabetes Federation and World Health Organization have recommended the use of diabetes registries as a key strategy in responding to the growing epidemic of diabetes (14, 15), application of such efforts has been localized in scope with limited international collaboration. A globally accepted standard for outcome data collection would help overcome this barrier.

There is an urgent need for strategies to monitor routine clinical practice and the quality of care efficiently and effectively, and enable outcome comparisons in a systematic and meaningful manner to reduce disparities between countries. With value defined as the best possible health outcomes important to patients achieved for the lowest cost, we are shifting to a new healthcare paradigm of value-based healthcare, a delivery model in which providers, including hospitals and physicians are paid based on these outcomes (16, 17).

To enable reliable national and international comparison so we could learn from best practices and improve healthcare globally, the International Consortium for Health Outcomes Measurement (ICHOM) (18), a non-profit organization founded in 2012, has initiated efforts to develop international consensus sets of outcomes that reflect patients' concerns and experiences. Until now, these sets have been successfully compiled for 24 medical conditions (19-36). The full list can be found here http://www.ichom.org/medical-conditions.

In accordance with the goals of ICHOM, an international multi-disciplinary diabetes working group aimed to define a minimum, consensus, standard set of outcomes with standard definitions that matter most to individuals with diabetes that can be tracked systematically across diverse health systems. A secondary aim was to identify a standard set of variables to enable case-mix adjustment, which would support comparison of diabetes outcomes among providers and health systems with different cases of people with diabetes.

METHODS

The Diabetes Expert Working Group

The development of the set was initiated by ICHOM in July 2017. The working group (WG) comprised internationally recognised experts, including clinicians, scientists in the field of diabetes, epidemiologists, and representatives of people living with diabetes. There were a total of 26 members from Africa, North America, Latin America, Asia, Australia, and Europe (Table S1). A smaller project team (J.N., M.W., O.O. (Project Leader), S.W., F.C. (Chair of the working group), M.B. (Chair of the working group) coordinated the efforts of the larger working group.

WG members were identified through their published work, active participation in patient groups or through recommendations from the chairs, existing working group members or advocacy groups.

Development of Diabetes Standard Set and Case-mix Variables

Between July 2017 and June 2018, the WG participated in 7 conference calls, all of which were followed by surveys on key decision points. Prior to each teleconference, the ICHOM project team developed the agenda, listed key proposals and summarized relevant evidence from the literature. Occasionally individual or small group calls with WG members with expertise on specific topics were conducted. A flowchart of the several phases (including definition of the scope, prioritization and definition of outcome domains, selection of outcome measures for clinical data and patient-reported outcome measurements (PROMs), prioritization and definition of case mix variables) for the development of the standard set is presented in Figure 1. Results of the decision of the WG on PROMs in diabetes are presented in a parallel publication.

A structured PubMed literature search was performed to identify relevant articles published from 1st January 2007 until July 12th 2017 with key terms relevant to diabetes clinical and patient-centred outcomes (Table S2). We found 3651 hits from our search strategy, 111 of which were excluded according to the exclusion criteria. Guidelines reports and other similar efforts published until the search date were added into the list and further evaluated in detail to extract diabetes outcomes (n = 3555 articles/reports). Moreover, diabetes registries were also identified based on previous articles of work members, manual search on google scholar and search links on registry website (Table S2, Table S3). The list of outcomes was collected and refined through consensus discussions to prioritize ease of interpretation and data collection across a variety of contexts. The working group selected outcomes based on 5 criteria: (1) importance to people with diabetes (2) clinical relevance (3) sensitive to changes in care (4) feasibility of capturing the outcome in clinical practice and (5) validity across cultures/internationally. Next, time points for data collection were selected for each outcome.

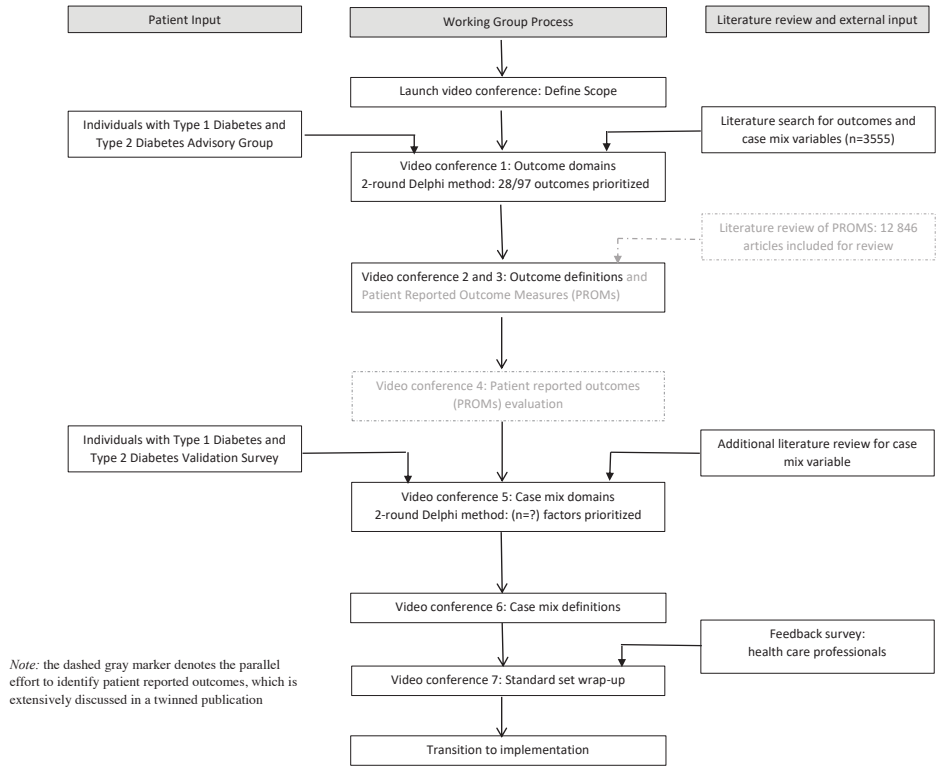

Figure 1. Flowchart of the project

To accommodate fair global comparisons, we aimed to identify additional variables that may impact the outcomes in the set. These case-mix variables would allow to the appropriate adjustment or stratification of the data. In order to identify these variables, we used the above described sources of articles and additionally, manually searched in clinical trials for matching factors (www.clinicaltrials.gov), and searched the literature for summary reviews on risk models for diabetes and its related outcomes. We applied the following criteria to prioritize the list of the variables: (1) feasibility of capture in routine clinical care, (2) validation as a case mix variable (whether it was a strong and well-accepted predictor of outcomes) and (3) validity across settings/regions/cultures.

To ensure patients' input in the outcomes and case-mix variable selection, small group calls with WG members living with diabetes (type 1 and type 2) were conducted to explore their perspective on the importance of different outcomes in their everyday life.

Modified 2-Round Delphi Method

After each videoconference, detailed minutes and an electronic survey to document voting on key decision points were shared with working group members. Through this

survey each member voted and provided feedback on the proposed minimum outcome set and the risk factors required as well as their definition.

A modified 2-round Delphi approach was used to reach consensus on which outcomes and case-mix variables were to be included in the standard set. In brief, the proposed outcomes or variables needed to be voted as very important (for example, score of 7-9 on a 9-point Likert scale) in either voting rounds by more than 80% of the working group members for inclusion in the set (Figure S1). Where consensus was not achieved, WG members were provided with the results of the first round, including the anonymous comments provided by the colleagues and additional research material from the project team before voting in a second round. The final standard set was approved unanimously by all members of the working group (Table S4, Table S5).

Review by persons with diabetes

The final list of outcomes was reviewed by 128 people with diabetes. Recruitment of respondents was done via ICHOM's website and social media channels, WG members' professional networks and the patient networks of JDRF and Imperial College London Diabetes Centre. Participants were asked to complete an anonymous survey, rating the importance of each outcome on a 9-point Likert Scale, with the option to include additional outcomes as free text. (Table S6, Figure S2). The survey was available in Arabic, English and Spanish.

Open review

Professionals with an interest in diabetes and/or outcome measurement were invited to provide feedback on the draft of the set through a survey distributed through ICHOM's websites, social media channels and professional network as well as WG member' professional networks (n= 176). They were asked to indicate whether or not they agreed with the inclusion of the outcomes and case-mix-variables in the set, the proposed definitions and tools and, if there was disagreement, what the reasons for this were.

RESULTS

Scope

The Working Group decided to limit the scope of the standard set to adults (aged over 18 years) with type 1 or type 2 diabetes. Children and young persons under 18 were excluded from the analysis. Further exclusion included (1) gestational diabetes as the aims of treatment are related to the success of pregnancy and childbirth and (2) secondary diabetes, is usually an underlying cause which is the treatment target.

The Standard Set: Outcome Domains

A total of 97 outcome measures were identified from a literature review, assessment of registries and input from the patient advisory group. The list was discussed with the WG and consensus vote was reached for 27 outcomes that were included in the standard set (Table 1, Figure 2). We categorized these outcomes as 1) diabetes control, 2) acute events, 3) chronic complications, 4) health services, and 5) survival. Moreover, the WG proposed specific outcome measurement time points that reflected the feasibility of data collection.

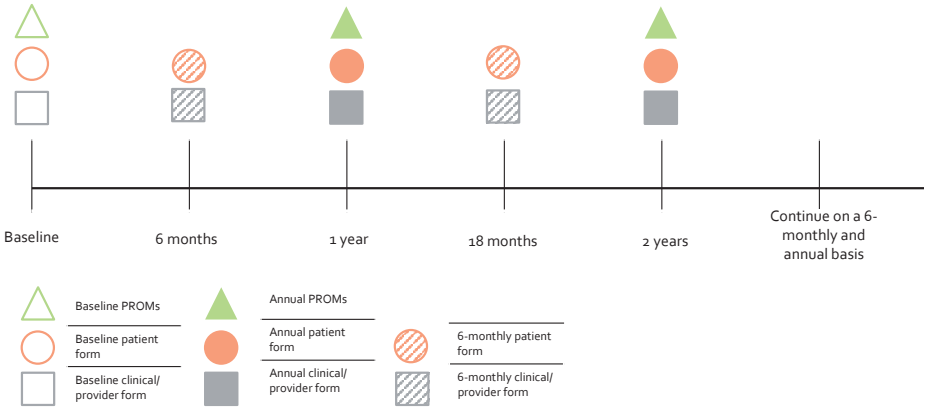

Figure 2. Timeline of data collection.

Diabetes control

There was an extended discussion by the working group and small break-out group for glycaemic control between calls 2 and 4. It was agreed unanimously that HbA1C should be included in the set as the measure for glycaemic control. As the international guidelines suggest HbA1C to be collected every 3-4 months, deciding on the frequency of data collection across worldwide healthcare settings seemed challenging. Although the standard set does not necessary represent treatment guidelines, the overall decision aligned with the idea that HbA1C should be used to assess care over the duration of the treatment cycle and 6-montly readings are an appropriate frequency to assess glycaemic control. For individuals having access to continuous glucose monitoring devices, in particular among type 1 diabetes individuals (37), the WG decided to collect data on the percentage of time in range when available. To date, time in range has been used to test the effectiveness of technologies designed to monitor blood glucose levels and maintain glucose control. There is an increase use of these devices, which hold the potential to improve substantially diabetes management in the future, for example, one of the most important ones, to capture the impact of acute interventions (38).

Table 1. Summary of the Standard Set of Outcomes for Diabetes

Patient Population	Measure	Supporting Information	Timing	Data Source
Diabetes Control				
All patients	Glycaemic Control	HbA1c and Time-in-range. Time in range is only measured for persons with diabetes who already have access to continuous glucose monitoring as part of their care.	Baseline and 6-monthly	Clinician / Healthcare provider
All patients	Intermediate outcomes	Includes disease management goals such as blood pressure, lipid profile and body mass index	Annually	Clinician / Healthcare provider
Acute Events				
All patients	Diabetic Ketoacidosis and Hyperosmolar Hyperglycemic Syndrome		Baseline and 6-monthly	Clinician / Healthcare provider
All patients	Hypoglycemia		Baseline and 6-monthly	Clinician / Healthcare provider or Patient
All patients	Acute Cardiovascular Events (Stroke and Myocardial Infarction)		Baseline and annually	Clinician / Healthcare provider or Patient
All patients	Lower Limb Amputation		Baseline and annually	Clinician / Healthcare provider or Patient
Chronic Complications				
All patients	Autonomic Neuropathy		Baseline and annually	Clinician / Healthcare provider
All patients	Peripheral Neuropathy		Baseline and annually	Clinician / Healthcare provider
All patients	Charcot's Foot		Baseline and annually	Clinician / Healthcare provider
All patients	Lower Limb Ulcers		Baseline and annually	Clinician / Healthcare provider
All patients	Peripheral Artery Disease		Baseline and annually	Clinician / Healthcare provider

Table 1. (continued)

Patient Population	Measure	Supporting Information	Timing	Data Source
All patients	Ischaemic Heart Disease		Baseline and annually	Clinician / Healthcare provider or Patient
All patients	Chronic Heart Failure		Baseline and annually	Clinician / Healthcare provider
All patients	Chronic Kidney Disease and Dialysis		Baseline and annually	Clinician / Healthcare provider
All patients	Cerebrovascular Disease		Baseline and annually	Clinician / Healthcare provider
All patients	Vision		Baseline and annually	Clinician / Healthcare provider or Patient
All patients	Periodontal health		Baseline and annually	Clinician / Healthcare provider
Only male patients	Erectile Dysfunction		Baseline and annually	Clinician / Healthcare provider or Patient
Persons on injectable insulin or non-insulin injectable therapies	Lipodystrophy		Baseline and annually	Clinician / Healthcare provider
Health Services				
All patients	Hospitalization		Annually	Clinician / Healthcare provider
All patients	Emergency Room Attendance		Annually	Clinician / Healthcare provider
All patients	Financial Barriers to Care		Annually	Clinician / Healthcare provider
Survival				
All patients	Vital Status		Annually	Clinician / Healthcare provider

In the category of diabetes control, the WG asserted that both blood pressure control and lipid profile control share the same importance in diabetes management. Together with measures of body mass index, they were uniformly voted for inclusion in the set.

Acute Events

The WG recognised that thresholds for hyper- and hypo-glycaemia are not internationally uniform and many studies or countries define individually. Based on a pragmatic perspective about the level of detail that could be collected, the WG decided to focus on the clinically relevant aspects of hyperglycaemia or hypoglycaemia. The WG focused on the number of episodes of Diabetic Ketoacidosis and Hyperosmolar Hyperglycaemic Syndrome, as the most clinically relevant conditions of hyperglycaemia for individuals living with type 1 or type 2 diabetes. Hypoglycaemia, is a potentially fatal complication of diabetes. It is associated with both barriers to achieving glycaemic goals, cardiovascular events, mortality and has negative consequences on quality of life and emotional status (38-41). Driven by the need to focus on clinically actionable data, the WG decided to measure the frequency of level two hypoglycaemia (glucose < 54 mg/dL) and level three hypoglycaemia (any severe hypoglycaemic episode associated with altered mental status requiring assistance) (38).

Acute cardiovascular events including (stroke myocardial infarction) and lower limb amputation were also voted to be measured in this category as they represent indicators of quality of care in diabetes.

Chronic Complications

Most of the diabetes burden is related to its long term complications including micro- and macro-vascular complication, nervous system complication and treatment related complications.

The WG highlighted the importance of identifying both objective clinical evidence of presence of *peripheral neuropathy* as well as subjective experience of people with diabetes when evaluating peripheral neuropathy. Moreover, long-term complications of peripheral neuropathy such as *Charcot's foot* were also included. Differentiating between specific modes of diagnosis (for example, pinprick vs tuning for test) was deemed as not important for the purpose of the set. Concerning *autonomic neuropathy*, extended discussion took place to find a single marker that could capture this entity, in particular cardiovascular autonomic neuropathy, as the latter is strongly associated with cardiovascular death (42). After considering the lack of standardisation for this outcome, the WG adopted a simple question on the presence of diabetic autonomic neuropathy as determined by the treating clinician.

From two classification systems of *lower limb ulcers*, Wagner-Meggitt Classification of Diabetic Foot or the University of Texas Diabetic Wound Classification System, the WG

selected the latter to assess both stage and grade of ulcers (43-45). The WG decided to include *lower limb amputation* to be collected. Upper limb amputations were deemed extremely rare and not necessary in a core set.

The cut-off of 0.9 for Ankle-Brachial-Pressure-Index (ABPI) was agreed as the accepted method of defining the presence of *peripheral artery diseases* (PAD) (46). In settings where ABPI may not be possible or feasible to measure, assessment of pedal pulses can be evaluated. In recognition of the fact that pain is a major symptom affecting individuals with PAD, the presence of pain as determined by patient report was also included.

Information on *ischaemic heart disease* was decided to be collected leaving open a variety of tests that can be used to reach the diagnosis. Concerning *heart failure*, the WG discussed the inclusion of ACC/AHA guidelines and the NYHA functional classification in the definition for heart failure (47). However, the ACC/AHA was chosen due to the opportunity that this classification system is able to identify patients are at risk of heart failure, even before they develop symptoms. Furthermore, there is clear progression through the ACC/AHA clinical stages of HF and, despite a lack of evidence-based therapies for heart failure with preserved ejection fraction in patients with type 2 diabetes, early detection of at-risk patients with stage B HF/PDD is likely to be beneficial and cost-effective (48-50).

For evaluation of *chronic kidney disease (CKD) and dialysis*, KDIGO classification system was accepted unanimously in the break-out group and in the WG, who highlighted the importance of including albuminuria measurements alongside with eGFR to determine the presence of CKD (51, 52). For the Diabetes Standard Set, the WG adopted the following threshold for CKD: <60mL/min/1.73m2 or ACR > 30mg/g. This recommendation is based on the International Society of Nephrology classification of risk in CKD.

The WG agreed that *cerebrovascular disease* is an important outcome to be assessed in people living with diabetes. However, they decided not to include any specific diagnostic criteria given the heterogeneous nature of this process across countries.

The WG agreed that measuring visual acuity was an important objective measure of *vision* related complications. For severe visual impairment, a visual acuity < 20/200 as defined by WHO was decided to be used. 20/200 is considered the threshold for legal blindness in many countries. For impaired vision, visual acuity was set at <20/40, a threshold at which social participation, such as the right to drive, is affected globally. Nevertheless, several sight-threating conditions in diabetes do not affect visual acuity until late in the course of the disease. Therefore, identification of the occurrence of these conditions as specific end-points of diabetes care was highlighted as important. The WG agreed that diabetic retinopathy and its subtypes, together with macular oedema, should be collected. Other oculopathies associated with aging such as cataracts and glaucoma are relatively common in individuals with diabetes as they are in the general population. Moreover, there is little evidence to suggest that tighter diabetes control

will change the course of these conditions. Therefore, they were both excluded from the set.

Periodontal health has been closely linked to glycaemic control in individuals living with diabetes (53, 54). However, there is under-awareness in the scientific community and this possible complication is often neglected by the multidisciplinary team caring for people with diabetes. Due to the lack of standard accepted classification for periodontal health worldwide, the WG proposed a simple classification that consisted of providing information on whether the individual had healthy gums or had evidence of gingivitis or periodontitis. There were still concerns from the WG that not all providers have expertise to assess periodontal health. However, incorporating this outcome in the set will, hopefully, raise awareness of its importance.

The project team initially proposed collecting data on *erectile dysfunction* in men using a Patient Reported Outcome Measure and included this in its separate literature search. Due to the difficulty to identify a concise tool focused on erectile dysfunction in persons with diabetes, a simple yes/no question adopted by the WG. Female sexual dysfunction was not deemed to be a core outcome in diabetes care and therefore, not included in the set.

Among treatment related complications, *lipodystrophy*, a skin lesion at the sites of injectable therapies, was voted for inclusion in the set by the WG. The project team had initially presented injection site complications, but the WG discussed that lipodystrophy, specifically, would be more appropriate. The condition slows down the absorption of the therapy, which in turn may jeopardise the progression of disease.

Health Service

Health Service was defined as the inclusion of three important outcomes hospitalisation and emergency room attendances and financial barriers to care. The data to be collected around hospitalisation includes date of admission and discharge, together with discharge diagnosis. The WG decided to collect specifically conditions of interest such as cardiovascular disease (myocardial infarction, acute coronary syndrome, unstable angina, stroke, decompensation of heart failure), acute kidney injury (AKI), foot- and lower limb-related complications and acute metabolic complications (including ketoacidosis, hyperosmolar syndrome, dehydration, failure to thrive, acute hypoglycaemia). The WG extensively discussed inclusion of acute kidney injury as defined based on KDIGO guidelines. People with diabetes are at increased risk for AKI as well as for CKD (55-57). AKI represents a major in-hospital complication, in particular when patients receive nephrotoxic agents (58, 59). Therefore, the inclusion of this category highlights the importance of evaluating the true global burden of this often misrepresented clinical entity in population of adults with type 1 and type 2 diabetes (55). Emergency room attendances were defined as the number of admissions for the same conditions as are

collected for hospitalisation in a one-year period. Financial barriers play a significant role in determining a patient's ability to access care, especially in countries without universal health coverage. Given the chronic nature of diabetes and the importance of continuous interaction with the health system to prevent the development of complications, the WG decided that tracking this outcome would give more insights to the dynamic of diabetes care in these countries.

Survival

The WG recommended gathering annual information on vital status cause of death. Given that obtaining data on the cause of death poses several difficulties both on accuracy (for example, information from death certification information in some settings) and logistics of data collection, the WG decided that they wanted to assess the quality of the data through identifying the source of the information. The WG discussed the possibility of collecting data on "diabetes-specific death", however, the lack of clarity around how specifically to define this led to a decision to incorporate the following response options: cardiovascular, acute metabolic complication of diabetes related to high or low blood glucose, renal and other. 'Cancer' was considered but later excluded due to current incomplete understanding of the relationship between these two disease entities.

In addition to the upper mentioned outcomes, the Working Group included in the standard set outcomes that will be collected through utilization of patient-reported outcome measures (PROMS). The main domains to be captured by PROMS voted in are: health-related quality of life, psychological well-being, diabetes distress and depression. The PROMs selection process is discussed in detail in a co-submitted paper.

Case-mix Variables for Risk Adjustment

The WG recommended a minimum set of baseline case-mix variables to enable meaningful comparisons across healthcare providers, institutions and treatment modalities. Following the voting, the final list comprised (n= 16) variables (Table 2). Demographic factors included year of birth, sex, ethnicity and education level. The WG decided to include ethnicity as an important determinant for health outcomes related to diabetes despite the difficulty to standardize among different countries. Given the close relation between diabetes and obesity, the IDF classification for ethnic specific values of waist circumference were adopted (60). Options for mixed ethnicities or other ethnicities not included in the list were added. Education level was defined as the highest level of schooling achieved and is being used as a surrogate for socioeconomic status due to the relative ease of international comparisons of education level and relatively little effort required to obtain this data (61).

Diagnosis profile category of case-mix variables included variables like type of diabetes, year of diagnosis and comorbidities status (including liver, lung disease, malignan-

Table 2. Summary of Case-mix Variables for Diabetes Standard Set

Patient Population	Measure	Supporting Information	Timing	Data Source
Demographic Factors				
All patients	Sex		Baseline	Clinician /Healthcare provider
All patients	Year of Birth	Used to calculate age	Baseline	Clinician /Healthcare provider
All patients	Ethnicity	This definition was based on categories in the IDF consensus Worldwide Definition of the Metabolic Syndrome	Baseline	Patient
All patients	Education Level	Education Level is based on the ISCED classification [International Standard Classification of Education]	Baseline and every 5 years	Patient
Diagnosis Profile				
All patients	Diabetes type		Baseline	Clinician/Healthcare provider
All patients	Year of Diagnosis	The estimated year of diagnosis based on person with diabetes' estimate or clinical records	Baseline	Clinician /Healthcare provider or Patient
All patients	Comorbidities		Baseline and Annually	Clinician /Healthcare provider
Lifestyle and Social Factors				
All patients	Smoking		Baseline	Patient
All patients	Alcohol Consumption		Baseline and Annually	Patient
All patients	Social Support		Baseline and Annually	Patient
Treatment factors				
All patients	Diabetes Treatment		Baseline and Annually	Clinician /Healthcare provider
All patients	Blood pressure lowering therapy		Baseline and Annually	Clinician /Healthcare provider

Table 2. (continued)

Patient Population	Measure	Supporting Information	Timing	Data Source
All patients	Statin/Lipid Lowering Therapy		Baseline and Annually	Clinician /Healthcare provider
All patients	Treatment Adherence	This scale was developed by the Diabetes Working Group and has not yet been validated	Baseline and Annually	Patient
All patients	Access to Healthcare		Baseline and Annually	Patient

cies, several infectious diseases, dementia, hemiplegia, mental-health related disease and thyroid disease). Given the strong relation between autoimmune disorder and type 1 diabetes, the WG group decided to collect annually thyroid-stimulating hormone levels in these people.

Lifestyle and social factors constituted of measuring smoking status, alcohol consumption, physical activity level, and social support captured by a simple question: who do you live with?

Treatment factors included information on diabetes treatment, blood pressure lowering therapy and statins or other lipid lowing therapy. Moreover, this category included two important determinants of diabetes outcomes: treatment adherence and access to care and medications. Due to the lack of global agreement on the methodology to capture these complex processes, the WG discussed their definition extensively. For treatment adherence, several tools were considered such as Adherence to Refills and Medications Scale and Morisky scale (62), but the WG decided to exclude these options due to the necessity to limit the burden of additional questions in the standard set or in the case of Morisky scale, the cost associated with licensing. Instead, the WG developed a treatment adherence scale (not yet validated) focusing on key components such as adherence to advice from the healthcare provider on diet, exercise, blood sugar monitoring, prescribed medication and/or insulin course. For access to healthcare, the WG highlighted the important impact that it has on diabetes outcomes in particular in developing countries. Due to the lack of standardized tools that would be able to capture the complexity of this process, the WG agreed to focus on two key important aspects of the difficulties of access: seeing a health care provider or obtaining the medicine needed.

Review by persons with diabetes

Of the 128 patients who participated in the online review survey between May and June 2018, 75% believed the proposed list of outcomes captured the most important outcomes. Respondents lived in Mexico, United Arab Emirates, United Kingdom and United States, the countries for which the group was able to obtain ethics approval within the narrow time frame of the project. Interestingly, the patients ranked the physical health outcomes such as visual outcomes, kidney health and lower limb amputations under the Complications domain as more relevant than the psychosocial outcomes. Some respondents provided free-text responses, in which access to treatment or equipment were highlighted as important issues. The WG addressed these concerns by suggesting simple questions that could capture these issues. Given the lack of validated or freely available measurement tools for these outcome domains, the WG recommends that the development of these tools be added to the research agenda. If such tools become available in future, these outcomes should be reconsidered for inclusion in the standard set.

Open review

Through an online survey, health professionals, care providers and other professionals within the diabetes community (n=176) responded positively to the comprehensive diabetes standard set, with the majority of outcomes receiving 80% or more agreement. They expressed confidence that the set included essential outcomes for individuals with diabetes and the data could be reasonably easy collected in routine clinical practice. Their main concerns were related to challenges around implementation of the standard set, availability of data, and the number of measures included in the set.

Data collection

The ultimate aim of this effort is to collect data that can be easily compared across different health care settings worldwide. Given the complexity of diabetes management affecting many areas of life, the WG made every effort to devise a timeline that the burden of data collection on health care providers and people living with diabetes. The WG agreed on a one-year care cycle, with the majority of data points being collected annually, while some are collected every 6 months. A reference guide has been developed by ICHOM (http://www.ichom.org/medical-conditions/diabetesmellitus/), which includes a data dictionary for all variables, potential data sources, and recommended timelines for data collection.

DISCUSSION

The ICHOM Diabetes Working Group identified a consensus standard set of outcomes for individuals with type 1 or type 2 diabetes providing a unique platform to allow outcome comparisons in routine clinical practice between countries and health systems to improve care quality. The set includes traditional outcomes related to glycaemic control and major complications as well as other less frequently reported outcomes that are important to patients such as periodontal health and erectile dysfunction and measures of psychological wellbeing and health related-quality of life. To allow for robust comparison, this set also includes baseline demographic information and other clinically relevant details to allow risk adjustment and stratification. To our knowledge, this is a first coordinated, multinational effort to recommend a standard set of diabetes outcome measures to improve the reliability and consistency of data collected by health care providers worldwide.

Moving trends of diabetes epidemiology and its complications, we are expecting more people to be diagnosed with diabetes earlier in life and to live with it for a longer period of time (63-65). These years are fuelling longer exposure to diabetes and diversification of its outcomes, which are placing a strain not only to patients but also on society and health care systems (66, 67). In this context, the framework of value based medicine has great potential to improve the quality of health care since it encompasses principles of medical ethics, prioritizing at first of patient's needs, and an emphasis of maximising utility gained per unit cost (68-70). We acknowledge the challenges of applying value-based medicine face due to the lack of standardization in outcome measurement in different geo-cultural settings. Therefore, data collected from routine clinical practice are of paramount importance in determining to what extent a care service, treatment or intervention is safe, effective, patient-centred, timely, efficient and equitable as underlined in the report for health care quality in the Institute of Medicine (71). To ensure the maximum rates of data collection, which is of critical importance when making comparisons across settings, we took a pragmatic approach to balancing the burden of collection to make meaningful comparisons at the population level while remaining useful for evaluating care at the individual patient level. More than 80% of the multinational survey respondents agreed with the set, providing support that the set captures the key outcomes relevant to people with diabetes.

There have been quite a few international comparisons of diabetes registries within selected populations, although the heterogeneity of definitions between registries make international comparisons very challenging (9). An OECD report shows that more than a 7-fold variation in hospital admission rates for diabetes is still evident across countries (10). Hospital admission for major lower extremity amputation reflecting long-term quality of diabetes care show an international variation rate over 14-fold (72). Some of these differences might reflect disease prevalence across countries, but not all of them.

Moreover, clear inconsistencies have been shown also in the data collection for proximal outcomes such as HbA1C control in clinical trials (73). The diabetes standard set can easily be implemented as a guide reference for randomized studies as well. Additional outcomes, based on trial requirements, could be added.

The strengths of this effort include the diversity of the Working Group representing experts in the diabetes field such as patient representatives, clinicians, researchers including low- and middle-income countries. Although the work summarized in this effort was heavily influenced by the professional opinion of a limited group of leaders in the field, the standard set is the result of current literature and best practice combined with a critical global expertise from the WG (in terms of own practices and efforts from the health systems of the country of origin) to compile a minimum set of outcomes important to diabetic individuals.

Initially, the standard set will be implemented in a number of pilot institutions. Results will be refined and the set will be reviewed and updated continuously by a steering committee comprising a subgroup of the current working group. The aim is to provide a well-rounded version ready for widespread adoption and to make sure that it remains relevant and up-to-date. We acknowledge that the implementation process of the standard set will be challenging due to reasons such as infrastructure costs for data collection, linkages with longitudinal administrative data sources, streamlining clinician data collection with electronic health records and existing disease registries. Further developments in health information technology would overcome the financial and logistic barriers in collecting and tracking these data.

The scope of this set includes type 1 and type 2 diabetes. The WG acknowledged that the challenges facing these two groups in the management of their condition vary significantly and as such, there may be differences in the relative importance of the outcomes selected between the two groups. They agreed that as a core minimum, the outcomes presented here do address the main issues faced by both groups, and there is room for providers of health systems to layer on additional outcomes if appropriate.

All the patient representatives in the WG were from high income or upper middle income countries. The same is true for the countries represented in the outcome validation survey. It is important that the relevance of the included domains is validated among lower income economies before wider adoption.

The Diabetes Working Group has defined a minimum recommended set of consensus patient-centred outcomes for collection, deemed to be most important to individuals with diabetes. The standard set is recommended to be used routinely in the clinical practice. This will facilitate and accelerate global improvements with an ultimate goal-international comparison of data on diabetes outcomes.

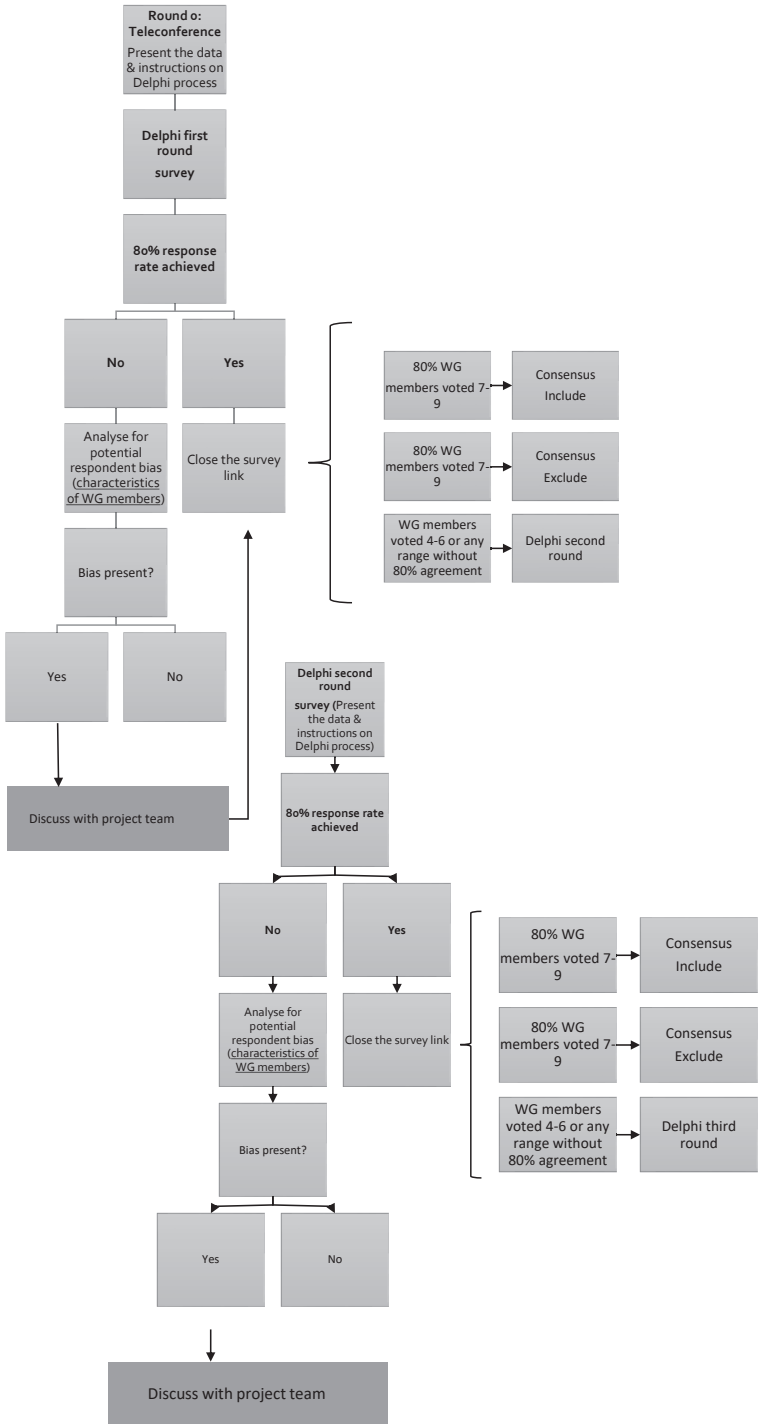

Figure S1. Delphi Method on Decision Process for the Outcome and Case-mix variable selection

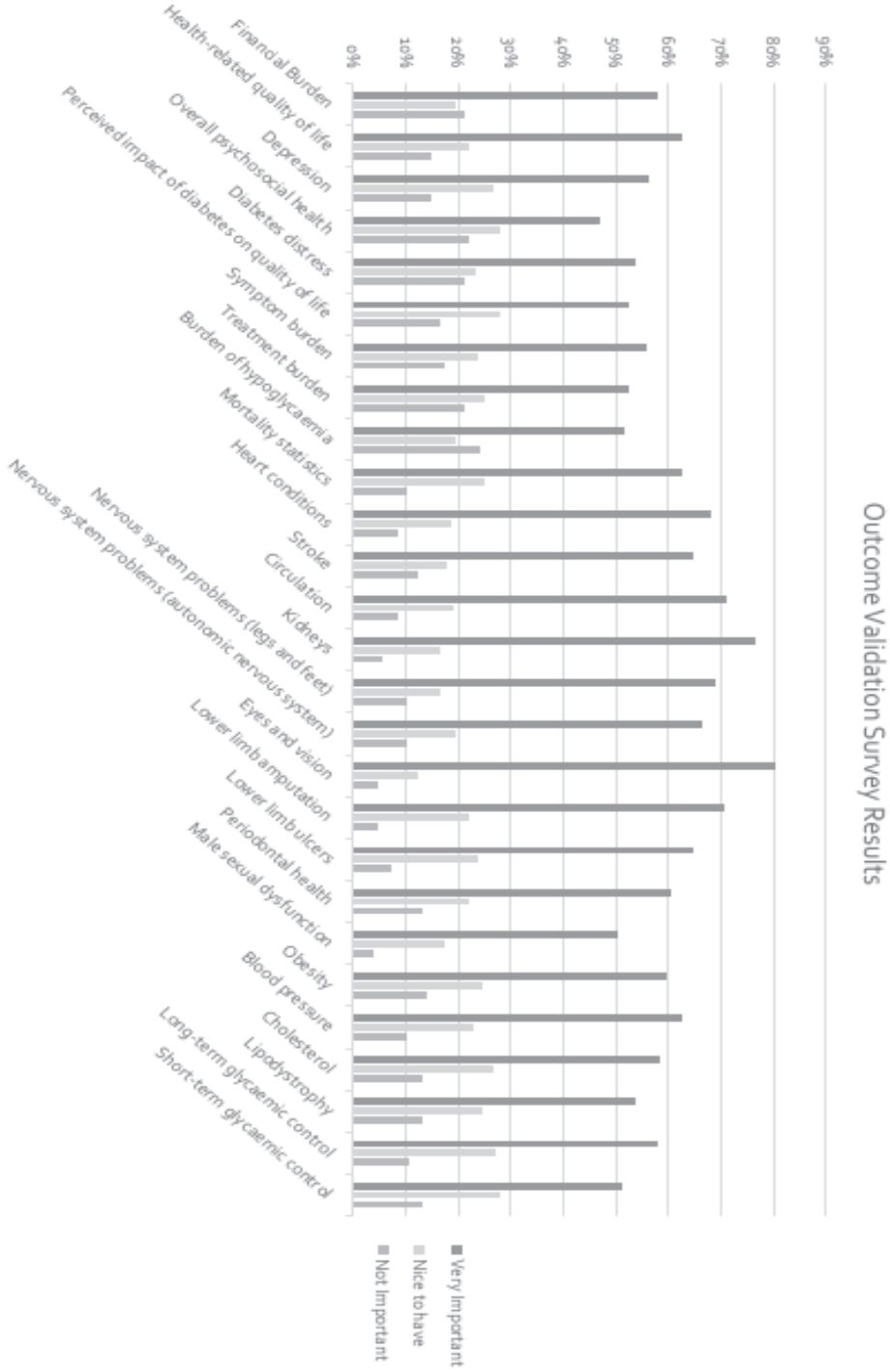

Figure S2. Outcome validation survey results from the people with diabetes

Table S1. Diabetes Standard Set Working group Members

Name	Primary affiliation	Country
Tim Benson	Patient Representative	Australia
Rob Haig	Patient Representative	Australia
Sharon Fraser	International Diabetes Federation	Belize
Jean Claude Mbanya	University of Yaounde I	Cameroon
Maria Santana	O'Brien Institute for Public Health	Canada
Søren Eik Skovlund	Aalborg University and Aalborg University Hospital	Danmark
Andreas Schmitt	Diabetes Center Mergentheim	Germany
Anil Bhansali	Postgraduate Institute of Medical Education and Research	India
Ronit Calderon-Margalit	The Hebrew University of Jerusalem	Israel
Mark Prabhaharan	Patient Representative	Malaysia
Sergio Hernández-Jimenéz	Instituto Nacional de Ciencia Médicas y Nutrición	Mexico
Cristina García Ulloa	Instituto Nacional de Ciencia Médicas y Nutrición	Mexico
João Raposo	APDP/NOVA Medical School Lisbon	Portugal
Hwee-Lin Wee	National University of Singapore	Singapore
Jana Klavs	University Medical Centre for Ljubljana	Slovenia
Jelka Zaletel	National Institute of Public Health Slovenia	Slovenia
Naomi Levitt	University of Cape Town	South Africa
Daniel Barthelmes	University of Zürich	Switzerland
Jihan Dennaoui	National Health Insurance Company - Daman	United Arab Emirates
Saf Naqvi	Imperial College London Diabetes Centre	United Arab Emirates
Katherine Barnard	Bournemouth University	United Kindom
Paul Buchanan	GBDOC, Patient representative	United Kindom
Anne Peters	USC Westside Center for Diabetes	United States
Mark Peyrot	Loyola University Maryland	United States
William Polonsky	Behavioral Diabetes Institute, University of California	United States
Andrew Pumerantz	Western Diabetes Institute, Western University of Health Sciences	United States

Table S2. Literature search strategy

Diabetes Outcomes		
Performed on July 12th, 2017		
Search terms		**Results**
#1	(("Diabetes Mellitus"[Mesh] OR "Diabetes Mellitus, Type 1"[Mesh] OR "Diabetes Mellitus, Type 2"[Mesh] OR diabet*[tiab] OR NIDDM[tiab] OR IDDM[tiab]) AND ("Quality of Life"[Mesh] OR "Quality of life"[tiab] OR QOL[tiab] OR "Quality Indicators, Health Care"[Mesh] OR "quality indicator*"[tiab] OR "Patient Outcome Assessment"[Mesh] OR "Patient Reported Outcome Measures"[Mesh] OR "patient reported outcome*"[tiab] OR "outcome report*"[tiab] OR "patient related outcome*"[tiab] OR "patient outcome*"[tiab] OR "patient assessment*"[tiab] OR "Treatment Outcome"[Mesh] OR "treatment outcome*"[tiab] OR outcome*[ti]) AND (index[tiab] OR indices[tiab] OR instrument[tiab] OR instruments[tiab] OR measure*[tiab] OR questionnaire*[tiab] OR profile*[tiab] OR scale*[tiab] OR scor*[tiab] OR status[tiab] OR survey*[tiab] OR rating*[tiab] OR tool[tiab] OR tools[tiab] OR metric*[tiab] OR reporting[tiab]) NOT (letter[pt] OR news[pt] OR comment[pt] OR editorial[pt] OR congresses[pt]) NOT (animals[Mesh] NOT humans[Mesh]))	3651
#2	Remove studies not meeting criteria (111 excluded in total), based on following in- and exclusion criteria: Inclusion criteria: Randomized controlled trials, systematic reviews, meta-analyses, cohort studies, registries, or guidelines assessing quality of life and clinical and patient-centered outcomes among individuals with type 1 and type 2 diabetes. Exclusion criteria: Studies on patients <18 years, unclear diagnosis, secondary diabetes diagnosis, gestational diabetes, study protocols, animal or basic science studies.	3540
#3	Guidelines from https://www.idf.org/e-library/guidelines.html (Categories: type 2 diabetes, diabetes complications, guideline development and diabetes management) and http://care.diabetesjournals.org/content/41/Supplement_1	12
#4	Other similar efforts (1-3)	3
#5	**#2 + #3 + #4**	**3555**
Diabetes Registries		
#6	Registries identified by Cunningham et al (1)	**14**
#7	Google search Search terms: diabetes registry First 50 references	**39**
#8	Search links on registry websites of #7 were accessible (for example from https://www.ncdr.com/WebNCDR/Diabetes/getstarted/datacollection)	**16**
#9	Remove registries not meeting criteria as follows (n= 8): Inclusion criteria: registries including type 1 and type 2 patients Exclusion criteria: no open access, pediatric registries (Final list of diabetes registries in Table S3)	**45**
	Case-mix variables	

Table S2. (continued)

Diabetes Outcomes		
#10	Manual search in www.clinicaltrials.gov Search terms: diabetes and individual complications outcomes First 10 trials per outcome	**NA**
#11	Google search Search terms on summary reviews on risk models for diabetes and its related outcomes First 50 references	**5**
#12	#5 + #10 + #11	

References

1. Cunningham SG, Carinci F, Brillante M, Leese GP, McAlpine RR, Azzopardi J, et al. Core Standards of the EUBIROD Project. Defining a European Diabetes Data Dictionary for Clinical Audit and Healthcare Delivery. Methods Inf Med. 2016;55(2):166-76.
2. Gandhi GY, Murad MH, Fujiyoshi A, Mullan RJ, Flynn DN, Elamin MB, et al. Patient-important outcomes in registered diabetes trials. JAMA. 2008;299(21):2543-9.
3. Murad MH, Shah ND, Van Houten HK, Ziegenfuss JY, Deming JR, Beebe TJ, et al. Individuals with diabetes preferred that future trials use patient-important outcomes and provide pragmatic inferences. J Clin Epidemiol. 2011;64(7):743-8.

Table S3. Additional search for outcomes in diabetes registries worldwide

	Name of the Registry	Country
1	The Northern California Kaiser Permanente Diabetes Registry	USA
2	Skaraborg Diabetes Registry	Sweden
3	Forum for Quality Systems in Diabetes Care, Styria	Austria
4	The Hong Kong diabetes registry	China
5	The Belgian Diabetes Registry.	Belgium
6	Tsukuba Kawai Diabetes Registry 2	Japan
7	The Wisconsin Diabetes Registry Study	USA
8	Thailand diabetes registry	Thailand
9	Karnataka Diabetes Registry	India
10	The Fukuoka Diabetes Registry	Japan
11	National Diabetes Register Croatia	Croatia
12	Saudi National Diabetes Registry	Saudi Arabia
13	Forum for Quality Systems in Diabetes Care, Rheinland-Pfalz	Germany
14	Chronic Disease Management Programme, Singapore	Singapore
15	The Diabetes Collaborative Registry	USA
16	Malaysian diabetes registry	Malaysia
17	National Institute of Diabetes and Digestive and Kidney Diseases	USA
18	Veterans Health Diabetes Registry	USA
19	Diabetes Distress and Care Registry at Tenri	Japan
20	The Pittsburgh Insulin-dependent Diabetes Mellitus (IDDM) Registry	USA
21	The Canterbury (New Zealand) insulin-treated Diabetic Registry population	New Zealand
22	New York City's Mandatory Diabetes Registry	USA

Table S3. (continued)

	Name of the Registry	Country
23	Swedish National Diabetes Registry	Sweden
24	Prospective documentation of diabetes patients	Germany
25	The LMC Diabetes Registry	Canada
26	Sardinian Conscript Type 1 Diabetes Registry	France
27	Bucharest Diabetes Database	Romania
28	Eritrea Diabetes Registry	Eritrea
29	National Diabetes Register	Norway
30	Colorado IDDM Registry	USA
31	Oguni diabetes registry	Japan
32	Diabetes Registry and Risk Stratification System	USA
33	The Diabetes Registry Outcomes Project for A1C Reduction	Canada
34	Qatar Diabetes Registry	Qatar
35	Canada Diabetes Registry	Canada
36	Israel National Registry	Israel
37	Scottish diabetes core dataset	UK
38	Penn State Hershey Diabetes Registry	USA
39	Larnaca Region	Cyprus
40	GPMSSP Database	Hungary
41	AMNCH, Tallaght	Ireland
42	Regione Umbria and the SID National Network	Italy
43	Mater Dei Hospital	Malta
44	Silesia University Hospital	Romania
45	DARTS, Tayside, Scotland	UK

Table S4. Voting results of 2-round Delphi method by working group on outcomes (Note Table S5 for the voting results of the case mix variables is still on the process of finalization)

	Outcome domain	Round 1*	Round 2*	Round 3**
Items included after round 1 (Inclusion threshold = 80% of WG ranking item 7-9 in importance)	Survival	95	N/A	N/A
	Mental health	95	N/A	N/A
	Financial burden	85	N/A	N/A
	Health-related quality of life	80	N/A	N/A
	Physical health status	95	N/A	N/A
	Peripheral neuropathy	90	N/A	N/A
	Lower limb ulcers	95	N/A	N/A
	Peripheral artery disease	80	N/A	N/A
	Limb amputation	95	N/A	N/A
	Ischaemic heart disease	95	N/A	N/A
	Heart failure	90	N/A	N/A
	Cerebrovascular disease	95	N/A	N/A
	Chronic kidney disease	95	N/A	N/A
	Erectile dysfunction	90	N/A	N/A
	Visual outcomes	95	N/A	N/A
	Glycaemic control	100	N/A	N/A
	Hospitalisation	85	N/A	N/A
	Cognitive function in context of hypoglycaemia	85	N/A	N/A
Items included after round 2	Satisfaction with care	67	85	N/A
Items included after round 3	Autonomy	57	63	61
	Feeling discriminated against	67	37	61
	Social relationships	71	68	67
	Obesity (Was initially termed "weight management"	76	79	83
	Injection site complication	48	47	67
	Cholesterol/ lipids*	76	79	72

*Inclusion threshold - >80% ranked item between 7 and 9

**(Inclusion threshold - >50% voted "yes"

Table S5. Voting results of 2-round Delphi method by working group on case mix variables.

	Case-Mix Variable	Round 1*	Round 2*	Round 3**
Items included after round 1 (Inclusion threshold = 80% of WG ranking item 7-9 in importance)				
Items included after round 2	N/A	N/A	N/A	N/A
Items included after round 3	Race/Ethnicity		67	90
	Education Level		54	95
	Alcohol Consumption		50	90
	Physical Activity		79	85
	Liver Disease		67	84
	Malignancy		52	79
	AIDS		38	53
	Chronic Obstructive Pulmonary Disease		48	63
	Peripheral Vascular Disease		71	95
	Dementia		63	84
	Hemiplegia		46	53
	Tuberculosis		33***	53
	Hepatitis B/Hepatitis C		33***	63
	HIV			
	Presence/history of anxiety disorders		58	85
	Presence/history of depression		63	95
	Presence/history of disordered eating behavior		67	95
	Presence of psychotic mental illnesses (e.g. schizophrenia)		58	75
	Cardiovascular risk score		54	58
	Family history of premature cardiovascular disease		50	58
	Thyroid disease (hyper/hypo)		50	79
	Thyroid hormones, Thyroid-stimulating hormone in patients with type 1 diabetes		52	53
	Functional Status		75	90
	Social Support	42	50	79
	Education and Behavioural Support History	48	55	53
	Treatment Adherence	55	62	55
	Access to healthcare		71	60

*Inclusion threshold - >80% ranked item between 7 and 9

**(Inclusion threshold - >50% voted "Include"

*** Round 1/Round 2 grouped Tuberculosis and Hepatitis B/Hepatitis C into 'Presence of Chronic Infections'

Table S6. Results of online review survey among 128 diabetes patients on the proposed outcomes

	%7-9	%4-6	%1-3	% Unable to score
Financial Burden	58%	20%	21%	2%
Health-related quality of life	63%	22%	15%	1%
Depression	56%	27%	15%	2%
Overall psychosocial health	47%	28%	22%	3%
Diabetes distress	54%	23%	21%	2%
Perceived impact of diabetes on quality of life -	52%	28%	16%	3%
Symptom burden	56%	24%	17%	3%
Treatment burden	52%	25%	21%	2%
Burden of hypoglycaemia	52%	20%	24%	5%
Mortality statistics - How long people with type 1 or type 2 diabetes live	63%	25%	10%	2%
Heart conditions- Whether or not people living with diabetes develop any complications with their heart; specifically, we are interested in heart failure, heart attacks and angina	68%	19%	9%	5%
Stroke - Whether or not people living with diabetes have had a Stroke or a Transient Ischemic Attack (also known as TIA or mini-stroke)	65%	18%	13%	5%
Circulation - Whether or not people living with diabetes have circulatory problems affecting blood flow to their legs or feet; circulatory problems can be painful and can cause long-term ulcers that are difficult to heal	71%	19%	9%	2%
Kidneys - How well kidneys of people with diabetes work and whether they are able to get rid of waste products from their body	77%	16%	5%	2%
Nervous system problems - (a) whether or not people living with diabetes have problems with the sensory nerves in their legs and feet (this can be in the form of pain, tingling or loss of feeling);	69%	16%	10%	5%
Nervous system problems - (b) whether or not the autonomic nervous system of people living with diabetes has been affected - this can affect various bodily systems including how your bowels work, abnormal sweating, and pulse and blood pressure abnormalities	66%	20%	10%	4%
Eyes and vision - How well people living with diabetes are able to see; we will also ask your healthcare provider about conditions that if treated early enough can prevent visual loss, such as diabetic retinopathy	80%	13%	5%	2%
Lower limb amputation - Whether or not a person living with diabetes has had to have part or all of the foot or leg removed due to a complication related to diabetes	71%	22%	5%	2%
Lower limb ulcers - Whether or not the person living with diabetes has a long-term ulcer that is not healing	65%	24%	7%	5%

Table S6. (continued)

	%7-9	%4-6	%1-3	% Unable to score
Periodontal health - Whether or not the person living with diabetes has healthy gums or is at risk of poor gum health	61%	22%	13%	4%
Male sexual dysfunction - Whether or not males living with diabetes suffer from impotence	50%	17%	4%	28%
Obesity - Whether or not the person living with diabetes is obese	60%	24%	14%	2%
Blood pressure - Whether or not the person living with diabetes has hypertension and if it is controlled or not	63%	23%	10%	4%
Cholesterol - Whether or not the person living with diabetes has high cholesterol	58%	27%	13%	2%
Skin changes at injection sites (Lipodystrophy) - Whether or not people who are on injectable therapies like insulin develop skin changes at the site of injection	54%	24%	13%	9%
Glycaemic control (long-term) How well your blood sugar is controlled and maintained within a normal range over the long term. This is often assessed with the HbA1c.	58%	27%	11%	4%
Glycaemic control – short-term. How well short-term fluctuations in your blood sugar levels are controlled to avoid hypo- or hyperglycemic episodes that might make you unwell.	51%	28%	13%	7%

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CHAPTER 6

General Discussion

The main objective of the work described in this thesis was to expand our knowledge on the role that traditional risk factors (e.g. obesity) and emerging biomarkers (e.g. hormones, bilirubin and liver-related markers such as gamma glutamyltransferase and fatty liver index) play in the etiology of T2D. The next step was to explore and unravel epigenetic determinants of type 2 diabetes and its risk factors by comprehensively investigating current literature and using the new approach of epigenome-wide association studies within the Rotterdam Study (1). We also extended our work by participating in a consortium of population based cohort studies, the CHARGE consortium and other collaborations. We further focused on diabetes related outcomes and investigated the role of diabetes on dementia risk. Moreover, upon the solid framework of value-based health care principles, we created a standard set of health outcomes important to diabetes patients and valuable to health care policies.

In this discussion the main findings are summarized. Furthermore, some methodological considerations are addressed, and potential clinical implications of the findings together with directions for future research are discussed.

REVIEW AND INTERPRETATIONS OF MAIN FINDINGS

Obesity, type 2 diabetes and mortality

In chapter 2.1, we evaluated the impact of overweight and obesity (as a function of body mass index (BMI)) on total life expectancy and the number of years lived with and without type 2 diabetes among individuals 55 years and older by obesity categories using multistate life tables. We constructed three different health states: free of diabetes, diabetes, and death and further evaluated how participants in our study moved from one state to another (incident diabetes, mortality among diabetics, and mortality among nondiabetics) to assess the difference in risk of mortality and diabetes by different categories of body mass index. The approach is discussed in details in the section of methodological considerations. There were no differences in total life expectancy by body weight status in both men and women. Nevertheless, men with obesity lived 2.8 years' fewer years free of diabetes than their normal weight counterparts, whereas, for women, the difference on years lived free of diabetes between obese and normal weight subjects was 4.7 years. Moreover, men and women with obesity lived 2.8 and 5.3 more years with diabetes compared to normal weight counterparts. Obesity increases the risk of developing diabetes earlier in life and the amount of years' individuals live with diabetes. As long as the obesity epidemic continues, we will observe more individuals living with diabetes and for a longer period of time. Our findings underscore the importance of preventing and treating obesity for clinicians, patients, and policy makers. Clare Garvey, PLOS Medicine Senior Research Editor, wrote a commentary on this evidence (special issue on Preventing Diabetes in July 19th, 2016) stating: "Clearly policy mak-

ers and governments need to support individuals and groups to educate and promote healthy choices [..]. The public health 'time-bomb' is just around the corner unless urgent action is taken [..]" (2).

In chapter 2.2, we tested the common assumption that people who experienced recent weight gain are more likely to be diagnosed with diabetes and explored more generally the patterns of body weight and composition in the years before developing diabetes. We used latent class trajectory analysis to identify several groups among all the people who eventually developed diabetes each with a distinct pattern of BMI development (with BMI measured based on a person's weight and height). Latent class trajectory analysis subdivides a number of people into groups that differ based on specified parameters. By using this method, in our population, we identified three distinct trajectories of change in BMI before the diagnosis of diabetes. Notably, the majority of individuals who developed diabetes were progressively gaining weight within the overweight range but their Framingham 8-year diabetes risk showed a decreasing trend throughout the period of follow-up suggesting that the diagnosis of diabetes might be biased towards enhanced screening efforts reserved to obese individuals rather than overweight.

Novel biomarkers for type 2 diabetes

The search for novel biomarkers in type 2 diabetes has been a priority of many researchers in the field. Significant resources continue to be devoted to unrevealing the complex pathophysiology of type 2 diabetes. In this thesis, we explored a few novel biomarkers.

Of note, conditions of hyperandrogenism in women, such as in polycystic ovarian syndrome (PCOS) or pregnancy, characterized of high endogenous estrogen levels have been linked to insulin resistance and increased risk of type 2 diabetes (3-6) in women. These data implicate endogenous sex hormone in the pathophysiology of type 2 diabetes. In chapter 3.1, we examined the role of endogenous sex hormones including (sex hormone-binding globulin (SHBG), free testosterone (FT), total testosterone (TT), free estradiol (FE) and total estradiol (TE)) in predicting the risk of type 2 diabetes in women. Combining original data analysis in the Rotterdam Study and performing a systematic review and meta-analysis, we showed that lower levels of SHBG and higher levels of TE are associated with higher risk of diabetes in postmenopausal women, independent of established risk factors such as glucose and inulin. Our study reported no association between TT and the risk of diabetes, though a suggestive positive association was observed between FT and diabetes. Our findings support the notion that estrogen and SHBG may play a role in the pathophysiology of diabetes in women, but it is not clear yet whether these measurements could be used as biomarkers to identify subjects at risk of developing type 2 diabetes.

Serum circulating total bilirubin, a breakdown product of normal heme catabolism, is useful for assessing liver function. Several epidemiological studies have suggested inverse associations between serum bilirubin levels and type 2 diabetes and its complications (7, 8). In chapter 3.2, we provide suggestive evidence from a systematic review and meta-analysis of the literature including 175,911 non-overlapping participants of an inverse association of serum levels of bilirubin with both metabolic syndrome and type 2 diabetes. However, the available evidence is mainly based on studies of cross-sectional design which makes the potential value of bilirubin as a biomarker and as a therapeutic target for prevention of type 2 diabetes is still an ongoing debate.

Studies described in chapters 3.3 and 3.4 provide further insights in the association of liver related phenotypes and risk of type 2 diabetes. In chapter 3.3, we showed that higher GGT levels were associated with risk of developing both prediabetes and type 2 diabetes. The association was strong and remained significant after adjustment for other risk factors for diabetes which was consistent with previous findings (9). To further elaborate whether the association was causal, we applied Mendelian Randomization analysis, a method that is widely used to examine causality in observational studies using genetic information. The approach is discussed in details in the section of methodological considerations. We found no evidence for a causal association between GGT levels, glycemic traits (glucose, insulin and HOMA-IR) and diabetes suggesting that the observed observational association might be due to confounding or reverse causation. Previous studies have shown that increased concentrations of compounds produced by GGT reaction generate reactive oxygen species that may trigger inflammatory responses (10, 11). Moreover, inflammatory cytokines such as tumour necrosis factor alfa has been reported to modulate expression of GGT which altogether, points toward an involvement of pro-inflammatory pathway in the link between GGT and diabetes that we were not able to control for in our analysis.

In chapter 3.4, fatty liver index (FLI), a proxy marker for fatty liver, which is calculated based on routine risk factors easily assessed in the clinical practice, was investigated in relation to risk of type 2 diabetes, cardiovascular disease, and mortality. FLI was significantly associated with diabetes incidence, independent of risk factors. Diabetes risk prediction models were improved significantly when FLI was added, but the latter did not perform better or beyond HOMA-IR, an established risk factor for diabetes. Therefore, our findings suggest that FLI has limitations on its utility in the clinical setting to predict cardio-metabolic events beyond HOMA-IR. This was further supported by our results on the associations of FLI with cardiovascular disease and mortality, for which null associations were found.

Epigenetics of type 2 diabetes and its risk factors

Following the advent of epigenetic investigations in populations-based studies, we systematically reviewed the current literature on two of the most studied epigenetic marks, DNA methylation and histone modifications, and their relation with type 2 diabetes and glycemic traits in chapter 4.1. In this review, we included 53 articles, based on 47 unique studies. No consistent associations were found between global DNA methylation and diabetes (including glycemic indexes). Most of the studies included were candidate driven and investigated genes with known importance in diabetes physiology in different tissues such as pancreatic islets, skeletal muscle, adipose tissue and blood cells. Several epigenome-wide association studies in these tissues were performed but the identified genes did not overlap with the candidate gene approaches or in-between tissues. Together, the studies reviewed in this chapter suggest a role of epigenetic modifications in the pathogenesis of diabetes and point out several pitfalls and recommendation on future research in the field.

Type 2 diabetes is characterized by chronic low-grade inflammation, a relevant factor contributing to the development of both diabetes and its complications (12). In chapter 4.2, we aimed to identify and synthetize all available evidence in humans and quantify the association of DNA methylation and histone modifications with circulatory inflammation markers. This review concluded an association between epigenetic marks and inflammation, suggesting an overall hypomethylation of the genome. Important gaps in the quality of studies were reported such as inadequate sample size, lack of adjustment for relevant confounders and failure to replicate the findings. In chapter 4.3, 4.4 and 4.5, we report results from three meta-analyses of EWA studies on diabetes risk factors including liver enzymes, fatty liver and obesity related traits. In chapter 4.3, we reported four new loci associated with GGT levels including cg06690548 (SLC7A11). As an amino acid transporter of cysteine and glutamate, SLC7A11 might be implicated in hepatic oxidative stress through GGT enzyme. Further, our knockdown experiment showed that reduced expression of SLC7A11 in liver cells resulted in alterations in lipid metabolism. Other important genes related to liver enzymes were PHGDH, SLC43A1 and SLC1A5, all of which have been previously shown to be related to adiposity. In chapter 4.4, we identified different levels of methylation in leukocytes DNA at 22 CpG sites to be associated with increased liver fat in 3,400 Caucasian participants in four cohort studies including some of the top genes ABCG1, SLC7A11, TXNIP. Gene ontology pathway analysis revealed a positive regulation of the cholesterol biosynthetic process. We further demonstrated that liver fat-associated DNA methylation at several loci are likely in causal pathways for dysglycemia, e.g., hypomethylation at cg08309687 (LINC00649) was associated with increased fasting glucose ($p = 0.04$) and hypermethylation of cg21429551 (GARS) was associated with increased fasting insulin ($p = 0.02$). In chapter 4.5, we investigated the association between DNA methylation and anthropological measures such as BMI and

waist circumference. The EWAS was conducted in the Rotterdam Study and replicated in the ARIC study with around 12 CpGs found for BMI and 13 CpG sites replicated for WC, most of which with known involvement to cardiometabolic outcomes such as ABCG1 and CPT1A. Moreover, we report novel epigenome-wide associations of methylation at MSI2 and LARS2 with body mass index and waist circumference. The genes have been associated with eating behaviors and bipolar disease, respectively. These results provide further insight in mechanisms underlying obesity-related traits, which can enable identification of new biomarkers in obesity-related chronic diseases.

Diabetes related outcomes

In chapter 5.1, we focused on the effect that type 2 diabetes poses on dementia risk and investigated whether this association might be causal. Diabetes, not prediabetes, was found to be a risk factor for dementia with a hazard ratio of 1.3 in the fully adjusted model for various confounders and mediators. Nevertheless, consistent with previous literature, we were unable to provide any causal estimate of genetically predisposing diabetes (including mechanism specific scores) on dementia risk. Using summary data from largest GWAS in the field did not provide additional information. The association between diabetes and dementia might be driven by any common unmeasured confounders or reverse causation.

In chapter 5.2, we developed a minimum Standard Set of patient-centred outcome measures for diabetes, for use in different healthcare settings. A consensus on the minimum set of standard outcomes measures and risk adjustment variables important in clinical management of diabetes was reached by integrating knowledge from expert's opinions, systematic review of existing literature, registry data and patient focus groups. A minimum Standard Set of outcomes was developed for individuals with diabetes [aged ≥ 18]. Outcome domains included survival and hospitalization, glycaemic control, hypoglycaemia, hyperglycaemia, visual outcomes, autonomic and peripheral neuropathy, lower limb ulcers, peripheral artery disease, ischemic heart disease and heart failure, chronic kidney disease, erectile dysfunction, periodontitis, lipodystrophy, obesity, cholesterol and blood pressure measured in 6 or 12 month intervals. Minimum risk adjustment data collected at baseline and annually compromised demographics, basic clinical information and treatment factors. This standard set provides an international template for meaningful, comparable and easy-to-interpret measures as a step towards achieving value-based healthcare in diabetes.

METHODOLOGICAL CONSIDERATIONS

Novel epidemiological methods

In this thesis, we applied a variety of methods used in epidemiological and clinical research. Through the past decades, the repertoire of analytical techniques in epidemiology has been growing fast following the need to answer complex research questions of population health. The new techniques seem necessary and generally they convey new information from the data. However, the clinical research and epidemiologist community might not be familiar with the methodology, thus, making the interpretation of the data a challenge. Careful and clear explanation of the methodology, analysis and results are critical.

In chapter 2.1, we used multistate life tables to examine the effect of obesity while people in our study were transitioning between 3 different states of health: "free of diabetes", history of diabetes" and "death". The multistate life table model is an important demographic method to document life cycle processes including change in marital status, migration to different places, living in socio-economically disadvantaged neighbourhoods (13-15), and in the last decades, change between healthy and unhealthy states ending in death by different causes (16-20). The setting of a cohort study with participants switching between different health status (entering, leaving or returning again to the same health status) through follow-up time, creates a good opportunity to transform epidemiological data into time-based health policy measures. While epidemiological data within a single homogenous cohort such as Rotterdam Study predict the number of disease events and deaths, the multistate life table enables estimation of the overall potential burden of diabetes in terms of years of life lost and lived with disease due to a risk factor of interest. However, a few limitations of the analysis warrant mentioning. All our analyses refer to membership of a disease state, which can only ever be an approximation of the disease burden without taking into account the severity of the health state, particularly for diabetes, which is derived from a heterogeneous group as we have shown in Chapter 2.2. The next step will be to add further layers of complexity such as for example, taking into account response to treatment or recovery, estimations of the severity of disease or disability associated with the specific health states.

In chapter 2.2, we used latent class trajectories analysis and mixed-effect models to examine the trajectories of change in body mass index and other cardiometabolic risk factors before diabetes development within a prospective population based study. This methodology has been increasingly recognized for their usefulness in identifying homogeneous subpopulations within the larger heterogeneous population and for the identification of meaningful groups or classes of individuals (21). Although latent class trajectory analysis has been widely used in criminology and behavioural research, it is new to health research (22-24). Traditional "strengths" of a good epidemiologic study (prospective design, repeated measurements for BMI, large sample size, long follow-up,

detailed data on cardio-metabolic risk factors and medication) facilitated the creation of the latent classes and analyses to estimate the trajectories of traditional cardio-metabolic risk factors. The study thus overcame some limitations of previous studies (e.g., classifying BMI into pre-defined categories). The approach allowed exploring heterogeneous growth patterns, which would not be identified using conventional methods.

Systematic reviews

In this thesis, some of the studies were systematic reviews and meta-analysis (chapter 3.1, 3.2 (partly), 4.1, 4.2, 5.2 (partly)). This study design is considered the best form of evidence (25) but if not well conducted, might provide unreliable results.

A systematic review will bring together different studies that are characterized by clinical heterogeneity (due to variation in participants, interventions and outcomes) or methodological heterogeneity (due to variation in study design and bias) or statistical heterogeneity (due to variation in methods used to assess the intervention/exposure effects) making it challenging to unify results. When heterogeneity is present, further exploration is necessary to understand factors contributing to this heterogeneity of the estimates. Although we observed some substantial heterogeneity between studies for example, in Chapter 3.2, we were unable to further explore the source of such heterogeneity in subgroup analysis due to small number of available studies. In this meta-analysis, we provided pooled effect estimates from studies mostly of a cross-sectional nature, which are prone to many epidemiological biases such as confounding and reverse causation and consequently. Nevertheless, the suggestive results from this meta-analysis point out a gap in the literature and the necessity of future prospective investigations between bilirubin and diabetes risk. Another important bias that could affect the results of a meta-analysis is publication bias. It occurs when the publication of results depends on the hypothesis tested and the significance and direction of effects detected (26). To investigate such presence, we used funnel plots, in which the estimates of the studies are plotted against a measure of precision or sample size. In the meta-analyses presented in this thesis, we did not find any evidence of publication bias, except for the results presented in chapter 3.1 where we observed a distorted funnel plot for the association of total testosterone levels and diabetes incidence, which was preserved with removal of the small studies with < 50 diabetes cases. In some other systematic reviews such as those in chapter 4.1 and 4.2, a meaningful quantitative pooling of the existing data was not feasible due to heterogeneity of definitions of exposure, measurement techniques, study design ect. Given the growing interest in the epigenetic field, we provided two comprehensive literature reviews of epigenetic marks and diabetes and glycaemic indexes and on inflammation (including evidence on several markers). Unlike narrative reviews, which often focused on a subset of studies in the chosen area based on availability of the author selection (25), systematic reviews apply

a standardised methodology for identification, selection and appraisal of the included studies which should allow an objective synthetisation of the literature without bias. Due to different epigenetic sites examined and heterogeneity in methodology and outcomes, it was not possible to perform a meta-analysis of epigenetics sited and outcomes investigated in these two papers.

Mendelian Randomization

Causal inference in traditional observational epidemiological studies is hampered by the possibility of confounding and reverse causation. Mendelian Randomization (MR) is a method that can be used to discover causal relationships between an exposure and outcome in the presence of such limitations using genetic variants as proxies for the exposure of interest. As Mendel's Laws of Inheritance dictates, alleles segregate randomly from parents to offspring. Thus, offspring genotypes are unlikely to be associated with confounders in the population. In addition, germ-line genotypes are fixed at conception, and therefore, temporally precede the variables under observation, avoiding issues of reverse causation. In understanding how MR works, it can be useful to think of a MR study as being analogous to a randomized controlled trial (RCT), except that genotypes are used to randomize participants into different levels of the exposure/treatment. However, it is important to realize that this analogy is not perfect e.g., RCTs typically involve treatments over a short duration, whereas an individual's genetics influences their biology from conception, meaning that many causal estimates from MR studies might reflect life-long exposures as well as developmental compensation that may arise from inheriting these mutations. Although initial applications of MR mostly focused on estimating the causal effect of environmental exposures on medically relevant outcomes, in recent years MR has found utility across a wide range of domains including the interpretation of high-dimensional omics studies.

Pitfalls in the interpretations of MR studies

Weak instrument bias. Typically, weak instruments are those that do not explain a large proportion of the variation in the exposure. If the instrument is weak, the chance difference in confounders may explain more of the difference in phenotype between subgroups than the instrument itself. Contrary, if the instrument is strong, the difference in phenotype between subgroups will be due to the genetic instrument and the difference in outcome will be due to this difference in phenotype. As common genetic variants frequently explain a small proportion of a phenotype's variance, use of multiple instruments increases this variance. Such an instrument can be chosen from genetic variants identified by GWAS. For example, in chapter 3.3, we provide investigation for both protein coding gene of gamma-glutamyl transferase GGT1 and also use a genetic risk score comprised of 26 SNPs genome-wide associated with GGT in the largest GWAS

so far to address the association with type 2 diabetes risk. Furthermore, increase sample size through utilization of large publically available GWAS dataset would be another possible solution. For example, in our project in chapter 3.3 and 4.1, we boosted the sample size utilizing diabetes and glycaemic indices GWAS datasets from DIAGRAM and MAGIC consortia and the current largest dementia GWAS from International Genomics of Alzheimer's Project (IGAP).

Population stratification. It refers to a particular form of confounding when the genetic variant-disease associations are distorted due to a third factor that might vary by population characteristics such as ethnicity. However, there are different statistical methods to adequately control for population substructures through for example, principal component analysis or linear mixed models that most of the summary results statistics from GWAS have taken into account. In chapter 5.1, to improve over previous literature on the associations between diabetes and dementia using trans-ethnic GWAS; we conducted our analysis utilizing the most recent diabetes GWAS results in Europeans and conducted the MR with dementia GWAS data from IGAP Consortia from the same ancestry.

Low power. Power is a function of sample size, variance explained in the exposure by the genetic variants, causal effect size, strength of confounding and type I error rate. In a low power setting, causal estimates are imprecise (wide confidence intervals) and it is difficult to detect a causal effect. There are several methods to approximate the power needed for a predefined hypothesis. For example, the online tool mRnd (http://cnsgenomics.com/shiny/mRnd/) calculates power to detect an effect using type 1 error (α) of 0.05 based on the genetic sample size and case/control ratios together with the proportion of variance of exposure explained by the instrument or genetic risk score. In this thesis, we used the limited sample of Rotterdam Study to verify the assumptions of the Mendelian Randomization approach and calculate the observational estimates controlled for a wide range of confounders. We further capitalized on summary results of publically available GWAS data and used genetic risk score of exposures to maximize the statistical power of the conducted studies.

Horizontal pleiotropy. This phenomenon happens when the genetic instrument is associated with the outcome via pathways that does not pass through the exposure of interest comprising one of the greatest threats of the validity of MR studies. New statistical methods have been developed to provide estimates robust to horizontal pleiotropy with different levels of relaxation of the instrumental variable assumptions: if the results from all these different models are largely consistent, this would reassure conclusions regarding causality. In both chapter 3.2 and 5.1, we used MR-Egger regression to account for horizontal pleiotropy. The slope of such regression in an estimate of the causal effect of the exposure on the outcome whereas the intercept in this regression is free to vary; the degree to which it departs from zero, is a function of the directional pleiotropy

present in the data. The MR-Egger approach relaxes the requirement of no horizontal pleiotropy among the SNPs. Instead it assumes that there is no correlation between the gene-exposure association and the direct effect of the genetic variants on the outcome. This is referred to as the InSIDE assumption (Instrument Strength Independent of Direct Effect) and is a weaker requirement than the stricter exclusion restriction criterion. A drawback of the MR-Egger method is that it tends to suffer from low statistical power and is particularly susceptible to bias from weak instruments. In Chapter 5.1, we further used a new method that permits up to 50% of the information in MR to come from SNPs that are invalid instruments called the weighted median estimator. Comparisons of such estimates was not possible in both studies included in this thesis as no suggestion of causal estimate was concluded. Moreover, as described above, we attempted to utilize GGT1 protein coding variant directly related to our exposure given our good understanding of the underlying biological function of the gene. However, we showed null causal association in this analysis as well.

Trait heterogeneity. Genetic instruments could be associated with multiple aspects or dimensions of a single trait. Such heterogeneity does not preclude causal inference but it does undermine the ability to infer causality for particular dimensions of heterogeneous exposure and makes interpretation of MR analysis more difficult. In chapter 5.1, we investigated genetically determined diabetes and dementia risk. Diabetes is a multifactorial disease with a complex pathophysiology. To account for such complexity, we used sets of variants with similar patterns of diabetes related quantitative trait associations such as insulin secretion, insulin resistance and body mass index/lipids in association with dementia risk. The sets of variants were identified through linkage hierarchical clustering methods of diabetes-related metabolic phenotypes. However, we were unable either to infer any causal association.

The above mentioned limitation and other are discussed at length elsewhere (27-36).

Applications of MR in epigenomics

In molecular epidemiology, many efforts have been directed to gain further insights into the mechanistic pathways between (1) genetic variants, (2) epigenomics and (3) causally associated traits (37-39). In this thesis (chapter 4.3 and 4.4), an analytical framework was applied in an attempt to integrate genetic predictors of DNA methylation levels with traits to evaluate bidirectional causal relationships and answer the following question: does DNA methylation resides along the causal pathways to traits or disease? (40).

Unbiased estimation and formal inference on the causal effect of methylation on phenotype (in our case liver enzyme GGT, chapter 4.3) heavily relies on strong genetic effects and typically requires large samples for adequate power (41). Since these sample sizes are currently not available for epigenomic datasets and Rotterdam Study had a finite sample, we instead explored consistency of the predicted effect of the genetic

variants (cis-mQTL) or genetic risk score versus the actually observed effect, thereby obtaining some indication on the plausibility of a causal effect of methylation on liver enzyme. This was performed in two directions, studying causality of the effect of DNA methylation on GGT and of GGT on DNA methylation (Figure S2A and S2B, Chapter 4.3). In chapter 4.4, a larger sample in the framework of a consortia investigation of epigenome wide association study of liver fat was used and two sample MR using summary statistics from instrument-exposure and instrument-outcome associations (IVW and MR-Egger method to explore pleiotropy effects) was implemented in a series of analysis to disentangle the potential causal association of epigenome-wide replicated CpGs with liver fat (1), gene expression and liver fat (2) and lastly, CpGs and gene expression (42). Although we used in the current projects the largest sample size available, the need for larger studies to investigate such hypotheses remains a paramount to ensure robust findings. To interpret the results of the causal investigations, tissue specificity is an important feature to take into account. Genetic proxies for methylation levels may be tissue specific. The need to assess the association between genetic variation and DNA methylation across tissues and validate the findings may be required. Although some databases exist to investigate such DNA methylation patterns, they are limited due to the fact that the tissues are extracted usually from diseased individuals and therefore, carry less information on environmental exposures.

Considerations and caveats about epigenome wide association studies

What and where to look for? Most appraisal of environmentally induced epigenetic alterations to date have either involved the assessment of global DNA methylation (methylation status is defined by measuring a representative sub-set of sites and regions such as LINE-1, Alu, Sat2, LUMA assays) or have adopted a genome-wide approach. Typically, epigenome-wide association studies (EWAS) with their hypothesis-free approach are a good example to investigate DNA methylation. Recent methylation arrays compromise a fast and cost-effective solution for profiling a relatively larger number of CpG sites in the EWAS. Some of the original work of this thesis is based on analysis performed with Illumina Infinium Human Methylation 450K Beadchip array which has high percentage of enrichment for CpG located in CpG islands only. Recent work suggests that other regions of the methylation outside these locations can be important for gene expression variations such as enhancers regions, inter-genic/intra-genic CpG island shores pointing out that investigations of a whole-some perspective of the genome might unravel important information for regulation phenotypic variation (43). In Chapter 4.4, we discovered and replicated 22 CpGs associated with liver fat. One of them, in particular, showed a potential causal association with the outcome and appeared to be located in an intergenic region of long non-coding RNA. lncRNA are a heterogeneous group of transcript with over 200 nt in length that exhibit no coding potential. Over the past few

years, it has become increasingly evident that these players together with histone modifications, another type of epigenetic marks, are important in the post transcriptional and translational coordination of gene expression. However, little is known about the general features and their molecular mechanism in human disease.

Before the era of EWAS, candidate gene approaches provided a suitable mean of identifying epigenetic loci related to different outcomes such as diabetes, cardiovascular disease, inflammation ect as we have summarized in chapter 4.1 and 4.2. Most of the studies were focused in exploring CpG-rich regions (or islands) in the regulatory regions of the genes, given that this is the primary mechanism that DNA methylation regulates gene expression. Other interesting regions investigated through candidate approaches were promoter regions and less often, intragenic regions. Although fruitful on occasions, the candidate gene approaches lack the statistical power required to identify replicable associations. We found no overlap between EWAS results and candidate gene approaches in our projects.

Choice of tissue. Interpreting EWAS results conducted in specific tissues has encountered several drawbacks in terms of generazibility of the results across cell types. The choice of the tissue type is limited by both accessibility and stability of epigenetic pattern. Most of EWAS investigated in relation to disease are often performed measuring DNA methylation in blood due to the ease of obtaining this tissue. But even in this case, other factors such as cell type composition (heterogeneity) influences DNA methylation patterns resulting in different methylation profiles (44, 45). To account for such effect, we corrected in our analysis for several counts of white blood cells when available or estimate these proportions by a well-calibrated formula from Housman et al who developed an algorithm to predict the blood cell type relative numbers (CD4+ T-cells, CD8+ T-cells, NK cells, monocytes and granulocytes) based on 100 CpG sites on the Illumina 450K methylation array (46, 47). Even after careful considerations to address the influence of cell composition, the outcome of the EWAS appeared to be very bimodal, with the majority of loci methylated (100%) or not methylated (0%), whereas intermediated levels indicate a cellular mosaicism that is difficult to interpret (48). Does this mosaicism of epigenetic profile differentiate the genetically similar cells of the same type in their functions and eventually lead to disease? With the development of single-cell techniques to study DNA methylation, these mosaic events will be able to be confirmed experimentally (49, 50).

Study design. Epigenetic patterns may change during the lifetime of an individual unlike the genome, which remains a static entity with the vast majority of genetic loci staying constant over time. Also, genetic variants are inherited randomly during birth and when this is not the case, "the non-randomness" across the genome is often identified as population stratification that can be corrected using statistical techniques. In contrast, epigenetics has all the same issues as any other measurement in classical epi-

demiology being susceptible to ascertainment bias, confounding or reverse causation. For example, the retrospective ascertainment introduces a risk of systematic difference between cases and controls in the handling or processing of samples, known as technical confounding including batch effects, for which we commonly adjust in our analysis. Other environmental factors can directly confound both DNA methylation and the trait into study, which can inflate type 1 error and exaggerate effect sizes estimates. We adjusted for confounders such as age and smoking that have been shown to influence DNA methylation (51, 52). Moreover, further adjustment for cardiometabolic factors in our EWAS were used in the models to allow a better delineation of the direct epigenetic effects. The issue of reverse causation, typical in EWAS, was addressed in our projects through various MR approaches as described above. On top of these factors, the choice of design remains crucial. Cross-sectional or case/control approach are a suboptimal choice, partly due to selection issues and possibility of reverse causation. However, they are more feasible and cost effective than other designs.

Replication. Like GWAS, the necessity to replicate hits in an independent study remains to be important also for EWAS to rule out any false positive findings. The hypothesis-free nature of an EWAS requires the need of replication to test the generated hypothesis in the discovery cohort. Moreover, the issues of correlation with genotype and reverse causation should both be addressed in replication analyses. However, epigenetics is a field which developed only the recent years and limited availability of tissue samples and sometimes study participants with a specific trait or disease make it difficult to achieve replication. In this thesis, we were able to apply two types of replication studies internal (within the same study, chapter 4.3) and external (across different studies, chapter 4.4 and 4.5). In the first case, we are able to avoid distorted associations due to differences in the study-specific characteristics, whereas in the second case, the opportunity to explore the potential replication of CpGs across different populations is of large benefit.

Genomic Inflation. Previous studies have shown a close relation between DNA methylation and DNA sequence polymorphism with an estimated variability of 22% to 80% (degree of change) in DNA methylation between individuals (53-55). Unlike genetic studies, where strategies exist to account for variability related to ancestry through population stratification and knowledge of linkage disequilibrium patterns helps further, for epigenomic studies this is more difficult. The influence of genomic variability, therefore, plays a role in the mosaic cellular response and might potentially affect the interpretability of EWAS results.

PUBLIC HEALTH/CLINICAL IMPLICATIONS AND FUTURE DIRECTIONS

Next phases of obesity and diabetes epidemics

Obesity and type 2 diabetes remain important public health concerns in our society. Life expectancy with and without disease provides simple concepts for patients and doctors to comprehend and these meaningful metrics are needed for education, counselling or health promotion. In our study, we expect more individuals with diabetes in the future suffering from the disease for a longer period of time. These years will be filled with different comorbidities, including T2D which will make response to interventions even more complex. Our findings have several implications in primary and secondary prevention strategies, which seem to have not kept pace with the incidence trends of diabetes and the increased lifespan of the population. In this thesis, we also found evidence that strategies tackling the obesity epidemics among the elderly might be beneficial when aiming at small weight reduction for the entire population, not only in high risk individuals. The profound impact of obesity would not only be reflected in the quality of life of people with comorbidities but also on the expected high toll on healthcare system in terms of costs. Future studies would need to calculate this burden in terms of additional financial consequences. Moreover, the use of multistate lifetable's approach among individuals of different age strata and other chronic diseases may raise an excellent opportunity to incorporate the findings into a more relevant public health policy framework.

Towards a value-based health care

In post 1990s era, evidence based medicine became the prevailing medical paradigm which is based on the fact that clinical decision-making is guided from well-designed and well-conducted research rather than only based on clinician's opinion, which may be limited by knowledge gaps or biases (56). However, a new perspective of medicine followed building upon the evidence based model by suggesting to take in consideration patient's values, with value defined as the best possible health outcomes achieved for the lowest cost (57, 58). Value-based healthcare is a healthcare delivery model in which providers, including hospitals and physicians are paid based on patient health outcomes (58). The proper unit for measuring value should encompass all services or activities that jointly determine success in meeting a set of patient's needs. This goal is what matters most to patients and what unites the interests of all actors in the medical system. Yet value in health care remains largely unmeasured and misunderstood. Within the ICHOM framework, we defined a standard set of outcomes and patient reported outcomes important for individuals with diabetes which hopefully will enable health care providers globally to compare, report and improve outcomes worldwide. Value based medicine has great potential to improve the quality of healthcare since it encompasses principles of medical ethics, reduces the uncertainties of clinical decisions and is in ac-

cordance with the goals of health economics (given its efficient allocation of resources by prioritizing the options by maximum utility per cost) (59-61). Achieving high value for patients is a paradigm change that is currently happening in healthcare.

The role of biomarkers in diabetes research

In this thesis, we investigated some current and promising biomarkers exploring their association with the risk of developing type 2 diabetes. Given the heterogeneous nature of diabetes, the use of biomarkers may help to better characterize diabetes risk and health care decision making (62, 63). Although the value of a biomarker lies in whether it adds to prediction above simple clinical information, in this thesis, we further investigated whether these biomarkers may be of value in identifying causal pathways to diabetes risk. This may in turn inform the development of new drug target for preventive or therapeutical interventions, as well as may be used to guide treatment choices. For example, GGT, a reliable, easy and inexpensive marker of liver health, is one of the most studied biomarkers in relation to diabetes (9), although its clinical predictive value remains minimal (64-68). Moreover, our findings suggest no causal evidence towards diabetes development. Other possible factors mediating such effect can be related to oxidative stress and inflammation. More research would be needed in this respect to establish the relative contribution of GGT in cardiometabolic health. Although GGT is an unspecific marker of diabetes and it is characterized as such with a limited value for clinical use, increased levels of GGT would be a 'red flag' for further clinical investigations of high risk individuals. The same implication applies for fatty liver index, which in our investigation did not perform better or beyond HOMA-IR, an established risk factor for diabetes. Its clinical value remains limited. Nevertheless, epidemiological evidence implicates a complex link between fatty liver and diabetes, direction of which has not yet been elucidated. On the other side, very little evidence from observational research has implicated bilirubin levels in diabetes development (69), although there is a reported potential causal relationship from a well conducted Mendelian Randomization study (7). This has opened new opportunities to test bilirubin as a drug target to prevent diabetes in clinical trials. Moreover, whether bilirubin improves diabetes prediction models still remains to be confirmed (70). In the same line, few investigations have been reporting on sex hormones and their association with diabetes development (71, 72); nevertheless, their clinical utility has never been investigated. In continuation to our meta-analysis of observational studies, hierarchical summary receiver operating characteristic curves and Fagan nomograms can be used to investigate the potential value of information on sex hormones for the prediction of type 2 diabetes and related outcomes (73). Given that early identification of diabetes risk provides an opportunity to introduce preventive interventions to stop or delay disease onset, future studies should focus on identifying

biomarkers that may help to better characterize the disease risk and healthcare decision making.

The dark road from association to function and clinical translation

The main aim of population-based molecular epidemiology is to identify new genes related to disease so we can discover and understand new pathways that drive the biological mechanism of disease and potentially, lead to novel therapies. Moreover, such "biomarkers" can improve diagnosis and prediction by explaining part of the uncertainly of who will develop or not the disease. In this thesis, we focused on exploring the epigenetic signatures of diabetes, inflammation, obesity-related traits such as anthropometric measures, fatty liver and liver enzymes. On the basis of the accumulating evidence, it is conceivable that epigenetic mechanism could provide significant contribution to the obesity and diabetes epidemics currently experiencing. The possibility to modulate the epigenetic machinery creates novel opportunities for curing and preventing disease on a population level. In an exploration of the randomized studies database https://clinicaltrials.gov/ with key terms 'epigenetics' and 'type 2 diabetes', it is noticeable that the ongoing efforts aim at elucidating whether modifiable risk factors such as obesity, diet, exercise and other intermediate factors such as insulin resistance and inflammation can epigenetically effect genes in diabetes people. Other studies focused on the epigenetic contribution to the pathophysiology of diabetes complications. On the other side, a handful of studies were investigating the role of epigenetics in fatty liver, mostly related to the effect of diet in this clinical entity. Overall, few of the clinical trials have been completed and results are not out yet. As opposed to the role of epigenetics in cancer research, current epigenetic studies have not been able to prove any validity in chronic disease prediction models. The complex relation between epigenetic regulation and disease development clearly demands further studies as the data until now seem to be merely provisional.

Unlike genome wide association studies, epigenome wide association studies are more prone to classical epidemiology caveats and therefore, clinical application seems distal limiting their value. The same applies for other types of 'omics' data (such as genomics, transcriptomics, metabolomics) which are of the same dynamic nature. Integrating together these data (such as in chapter 4.3, 4.4: genomics, expression and epigenetics) combined with functional work resulted in better understanding of the mechanism behind variation in traits or disease studied. Moreover, such integrative approach is likely to become increasingly popular in the forthcoming years as the technologies require to generate such data at scale are becoming always more feasible. Advancements in such technologies should allow a further detailed examination of the role of intermediate phenotypes in complex trait variation. For example, just recently, Illumina introduced a new human methylation array (Illumina Infinium MehtylationEPIC BeadChip Kit) that

builds upon the 450k array plus an additional of 350.000 CpGs in enhancer regions. In the near future, the generation of all these population 'big-omics data' will potentially unravel the 'hidden micro-universe' of molecular mechanisms through the application of machine learning algorithms that have foster success stories in the recent years in disease diagnosis (74, 75) To achieve this, large computational resources and data points are crucial. For the moment, multi-cohort projects with joint efforts to increase the sample size are currently ongoing in consortia such as CHARGE. Hopefully, we will be able to witness these discoveries that have the potential to lead to life-changing improvements for patients.

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CHAPTER 7

Appendices

SHORT SUMMARY (ENGLISH)

In the last years, the emerging threat of diabetes has called for resolution and intensified research efforts to analyse changing aspects of the epidemiology of traditional risk factors such as obesity and investigate the role of novel biomarkers in our ultimate aim to effectively prevent diabetes. The burgeoning interest in the field of epigenetics, an intersection between genetic determinism and environmental influences, has opened new opportunities to discover more about the molecular pathways involved in the regulation of diabetes risk factors from the etiological perspective, and possibly might give rise to new therapeutical strategies. Moreover, management and prevention of diabetes complications remain an exhaustive task not only for the diagnosed individual but also for the medical system. Patient-centered outcome measurements combined with efficient economical health systems, in what we now call value-based medicine, seem to be the new medical paradigm we are moving towards.

The upper mentioned aspects of diabetes have been studied in this thesis and are shortly summarized as follows:

Chapter 2 focuses in the association between obesity, type 2 diabetes and mortality by using some novel methodologies in epidemiological research to investigate the data: multistate lifetables and latent class trajectories. In chapter 2.1, we showed that obesity increased the risk of developing diabetes earlier in life, therefore, living with fewer number of years free of diabetes and extended the number of years with diabetes. Hypothesizing that individuals who experience recent weight gain are more likely to be diagnosed with diabetes in chapter 2.2, we explored patterns of body mass index changes before developing diabetes. We identified three distinct patterns of change in BMI and accompanying different trajectories of other cardiometabolic risk factors. The majority of individuals were categorized as progressive weight-gainers within the overweight range before the diagnosis of diabetes pointing out some possible failure of screening efforts reserved to obese individuals rather than those overweight.

Chapter 3 focuses on emerging risk factors for type 2 diabetes. In chapter 3.1, we studied the association between sex hormones with type 2 diabetes. Combining both original data analysis in the Rotterdam Study and performing a systematic review and meta-analysis of the current literature, we concluded that lower levels of sex hormone binding globulin and higher levels of total estradiol are associated with higher risk of diabetes in postmenopausal women, independent of established risk factors. These findings implicate endogenous sex hormones in the pathophysiology of type 2 diabetes. In chapter 3.2, we conducted a systematic review and meta-analysis on the role of bilirubin in the risk of metabolic syndrome and type 2 diabetes. Although current evidence came mostly from cross-sectional investigations, bilirubin levels increased the risk of these entities. Given that previous evidence has shown bilirubin as a potential causal biomarker of diabetes development, further studies with a longitudinal design or

clinical trials should explore more in depth the potential value of bilirubin in diabetes. In chapter 3.3, we confirmed previous evidence that higher GGT levels are associated with risk of diabetes in observational analysis. However, we found no evidence for a causal relationship between GGT levels, glycemic traits and diabetes suggesting that the corresponding observational association might be due to confounding or reverse causation. In chapter 3.4, fatty liver index, a proxy marker of fatty liver, which is calculated based on routine measurements in clinical practice such as GGT, waist circumference, body mass index and triglycerides, was investigated in relation to cardiometabolic diseases (type 2 diabetes and cardiovascular disease) and mortality. In relation to diabetes, increased levels of FLI were associated with diabetes risk. However, FLI did not improve diabetes risk prediction models better or beyond than HOMA-IR, a classical risk factor of diabetes. Altogether, FLI seem to have a limited clinical utility to predict cardio-metabolic events.

Chapter 4 describes several epigenome wide association studies. In chapter 4.1, we systematically reviewed the literature on global and site specific DNA methylation and histone modifications of type 2 diabetes and glycemic traits. A comprehensive review of such epigenetic signatures on circulatory inflammation markers was performed in chapter 4.2 as well. In both studies, we evidenced multiple caveats and challenges in the current investigations related mostly to study design, sample size, lack of proper adjustment for important confounders, failure of replication of findings disharmonized methodological assessment. In chapter 4.3, 4.4, 4.5, we report results from three meta-analyses of epigenome wide association studies on diabetes risk factors including liver enzymes, fatty liver disease and obesity related traits. In chapter 4.3, we reported four new loci associated with GGT levels including cg06690548 (SLC7A11), which we showed experimentally to be involved in lipid metabolism. In chapter 4.4, we identified differential methylation of leukocytes DNA at 22 CpG sites to be associated with increased liver fat in 3,400 Caucasian participants in four cohort studies. Interestingly, several liver fat associated CpGs are likely in the causal pathways to impaired levels of glycemic traits. In chapter 4.5, we report novel epigenome-wide associations of methylation at MSI2 and LARS2 with body mass index and waist circumference.

Chapter 5 focuses on diabetes related consequences and related outcomes. In chapter 5.1, we investigated the association between diabetes and dementia risk and observed that diabetes would pose a higher risk of dementia compared to individuals without diabetes. Within a Mendelian Randomization approach, we were not able to provide any evidence of a causal association between the two, highlighting the complexity of this relation. In chapter 5.2, we developed a minimum Standard Set of patient-centred outcome measures and risk adjustment factors for individuals with type 1 and type 2 diabetes, for routine clinical use in different healthcare settings. This standard set provides an international template for meaningful, comparable and easy-to-interpret measures as a step towards achieving value-based healthcare in diabetes.

In **chapter 6**, we reviewed our findings in the context of a general discussion. Methodological considerations with regard to the studies in this thesis and similar studies are discussed. Also, potential clinical implications and future direction of our findings are addressed.

NEDERLANDSE SAMENVATTING

In de afgelopen jaren heeft het opkomende probleem van diabetes het noodzakelijk gemaakt om oplossingen te vinden en geïntensiveerde inspanningen in wetenschappelijk onderzoek te gebruiken om de veranderende aspecten van de epidemiologie van traditionele risicofactoren, zoals obesitas, te analyseren en de rol van nieuwe biomarkers te onderzoeken, in ons uiteindelijke doel om diabetes effectief te voorkomen. De groeiende interesse in het vakgebied epigenetica, een kruising tussen genetische determinanten en omgevingsinvloeden, heeft nieuwe mogelijkheden geopend meer over de moleculaire routes, die bij de regulatie van risicofactoren voor diabetes betrokken zijn, vanuit het etiologische perspectief te ontdekken, wat mogelijk aanleiding kan geven tot nieuwe therapeutische strategieën. Daarnaast blijven het beheer en de preventie van complicaties bij diabetes een uitputtende taak, niet alleen voor de gediagnosticeerde persoon, maar ook voor het medische systeem. Patiëntgerichte uitkomstmaten in combinatie met efficiënte economische gezondheidsstelsels, in wat we nu *value-based medicine* noemen, lijken het nieuwe medische paradigma te zijn waar we naartoe bewegen.

De hierboven genoemde aspecten van diabetes hebben wij in dit proefschrift bestudeerd en kort samengevat als volgt:

Hoofdstuk 2 richt zich op de associatie tussen obesitas, diabetes type 2 en mortaliteit die door middel van nieuwe methodologieën in epidemiologisch onderzoek onderzocht wordt: *multistate life tables* en *latent class trajectories*. In hoofdstuk 2.1 toonden wij aan dat obesitas het risico op diabetes eerder in het leven verhoogt, wat betekent dat minder jaren zonder diabetes worden geleefd en meer jaren met diabetes. De hypothese volgend dat individuen die recent zijn aangekomen in gewicht een hogere kans hebben op de diagnose van diabetes komt in hoofdstuk 2.2 aan bod, waar wij patronen van veranderingen in de Body Mass Index onderzocht hebben vóór de ontwikkeling van diabetes. Wij identificeerden drie verschillende patronen van veranderingen in de BMI en bijbehorende verschillende trajecten van andere cardiometabole risicofactoren. De meerderheid van de personen werd gekarakteriseerd als personen met een progressieve gewichtstoename binnen de range van overgewicht vóór de diagnose van diabetes, dit wijst op een mogelijk falen van screening-inspanningen die zich richten op personen met obesitas in plaats van personen met overgewicht.

Hoofdstuk 3 richt zich op nieuwe risicofactoren van diabetes type 2. In hoofdstuk 3.1 hebben wij de associaties tussen geslachtshormonen en diabetes type 2 bestudeerd.

Door het combineren van zowel originele data analyses in de Rotterdam Studie en het uitvoeren van een systematische review en meta-analyse van de huidige literatuur concludeerden wij dat lagere niveaus van het geslachtshormoon bindende globuline en hogere niveaus van het totale oestradiol geassocieerd zijn met een hoger risico op diabetes bij postmenopauzale vrouwen, onafhankelijk van reeds bekende risicofactoren. Deze bevindingen wijzen op endogene geslachtshormonen in de pathofysiologie van diabetes type 2. In hoofdstuk 3.2 hebben wij een systematische review en meta-analyse over de rol van bilirubine bij het risico op metabolische syndromen en diabetes type 2 uitgevoerd. Hoewel de meest recente gegevens afkomstig waren uit cross-sectioneel onderzoek, verhoogden bilirubinespiegels het risico op deze entiteiten. Aangezien uit eerder bewijs is gebleken dat bilirubine een mogelijke causale biomarker voor de ontwikkeling van diabetes is, moeten verdere studies met een longitudinaal onderzoeksdesign of klinische onderzoeken de mogelijke waarde van bilirubine bij diabetes verder onderzoeken. In hoofdstuk 3.3 hebben wij eerder bewijs bevestigd dat verhoogde GGT-waarden geassocieerd zijn met het risico op diabetes bij een observationele analyse. We vonden echter geen bewijs voor een causaal verband tussen GGT-waarden, glycemische eigenschappen en diabetes, wat suggereert dat de observationele associatie het gevolg van verstorende of omgekeerde causaliteit zou kunnen zijn. In hoofdstuk 3.4 werden de index voor leververvetting, een proxy marker voor leververvetting die is berekend op basis van routinematige metingen in de klinische praktijk zoals GGT, middelomtrek, body mass index en triglyceriden, onderzocht in relatie tot cardiometabole aandoeningen (diabetes type 2 en hart- en vaatziekten) en sterfte. Met betrekking tot diabetes waren verhoogde waarden van FLI geassocieerd met het risico op diabetes. FLI verbeterede echter niet de voorspellingsmodellen voor diabetes beter of verder dan HOMA-IR, een klassieke risicofactor voor diabetes. Alles bij elkaar lijkt FLI een beperkte klinische bruikbaarheid te hebben om cardiometabole aandoeningen te voorspellen.

Hoofdstuk 4 beschrijft verschillende epigenoombrede associatiestudies. In hoofdstuk 4.1 hebben wij systematisch de literatuur over globale en plaatsspecifieke DNA-methylering en histone modificaties van diabetes type 2 en glycemische eigenschappen onderzocht. Een grondige review van dergelijke epigenetische handtekeningen op circulaire ontstekingsmarkers werd ook in hoofdstuk 4.2 uitgevoerd. In beide studies hebben wij meerdere voorbehouden en uitdagingen in huidig onderzoek aan het licht gebracht die voornamelijk betrekking hadden op het onderzoeksdesign, de steekproefomvang, gebrek aan juiste aanpassingen voor belangrijke *confounders*, mislukking van replicatie van bevindingen en problemen in de methodologische beoordeling. In hoofdstuk 4.3, 4.4, 4.5, rapporteren wij resultaten van drie meta-analyses van epigenoombrede associatiestudies over risicofactoren voor diabetes, waaronder leverenzymen, leververvetting en obesitas gerelateerde kenmerken. In hoofdstuk 4.3 rapporteerden wij vier nieuwe loci die geassocieerd zijn met GGT-niveaus, waaronder cg06690548

(SLC7A11), waarvan we experimenteel hebben aangetoond dat het betrokken is bij het lipide metabolisme. In hoofdstuk 4.4 identificeerden wij differentiële methylatie van leukocyten-DNA op 22 CpG-plekken die geassocieerd zijn met verhoogd levervet bij 3.400 Kaukasische deelnemers in vier cohortstudies. Interessant is dat verschillende CpGs die met levervet geassocieerd zijn waarschijnlijk in de causale routes naar verminderde niveaus van glycemische eigenschappen liggen. In hoofdstuk 4.5 rapporteren wij nieuwe epigenoombrede associaties tussen methylatie op MSI2 en LARS2 en Body Mass Index en middelomtrek.

Hoofdstuk 5 richt zich op de gevolgen die aan diabetes zijn gerelateerd en gerelateerde resultaten. In hoofdstuk 5.1 hebben wij het verband tussen diabetes en het risico op dementie onderzocht en vastgesteld dat diabetes een hoger risico op dementie oplevert in vergelijking met mensen zonder diabetes. Binnen een *Mendelian Randomization*-benadering konden we geen enkel bewijs leveren voor een causaal verband tussen deze beiden factoren, wat de complexiteit van deze relatie benadrukt. In hoofdstuk 5.2 hebben we een minimale standaard set van patiëntgerichte uitkomstmaten en risicoaanpassingsfactoren voor personen met diabetes type 1 en type 2 voor routinematig klinisch gebruik in verschillende instellingen binnen de gezondheidszorg ontwikkeld. Dit set biedt een internationaal model voor zinvolle, vergelijkbare en gemakkelijk te interpreteren metingen als een stap op weg naar het realiseren van op *value-based* gezondheidszorg bij diabetes.

In **hoofdstuk 6** hebben wij onze bevindingen in de context van een algemene discussie besproken. Methodologische overwegingen met betrekking tot de studies in dit proefschrift en vergelijkbare onderzoeken worden besproken. Daarnaast worden mogelijke klinische implicaties en toekomstige richting van onze bevindingen behandeld.

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During this experience I was lucky enough to have the best office mates that one could wish for: Magda, thank you for being a calming presence in office. Your dedication to cello inspired me to catch up with piano (although not for long). Maryam, your

scientific achievement is an inspirational model for a woman in science. Good luck as the new head of Cardiovascular Group. 2901 gang: Paul, you were a great supervisor of my master thesis and supported gently my transition to the PhD adventure. Both, a talented scientist and a great person, I am sure would make an inspiring Professor in the future! Layal, you are one of the toughest women I know and surely someone to look up to. It was great sharing my Boston experience with you; Symen, after you left, the office was a quieter place. We missed you and your genuine curiosity on the most random topics (of course related to medicine). Good luck with your PhD; Mohsen, I very much value our friendship, which grew even bigger with the liver project that we successfully brought to light! I always appreciated our coffee breaks and discussions on epidemiology, academia or life in general; the occasional visits of Sara in the department spiced it up! Thank you for all the support during this journey. Adela, we have shared a lot together of this journey especially 'hallet e kurbetit duke pergjysmuar cfare na peshonte rende ne zemer' and I am very thankful for this. From med school until now, you always remain 'me pikantja', Elsa! Carolina, Eliana, Irma and Silvana: thank you for being so cheerful and fun office mates (although shortly). I hope all that hard work and long hours would sum up to great PhD theses for each of you! Oscar, you always declined our invitations for after-work beers for NEJM, but I will make a reason out of it. Thank you for all the positive energy you brought to the office by your presence.

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Eralda, Fjorda, Tao: we enumerate many adventures together that remain my dearest life memories!

Enisa, If the world would have some more copies of you, it would definitely be a better place. You are a beautiful soul with a huge heart and I feel priviledged to have had you in my life and as a paranimf!! Thank you for the precious friendship and the great time together as roomies. I can not wait to have your book on my library shelf.

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Finally, my deepest debt is owed to my family, for their support and understanding. Although their dream was to see me become a doctor for patients, I hope they appreciate me as a doctor for populations! Nejva, one of the reasons I came back to Munich is to be close to my little sis'! I wished you could join me doing my defense and notice how high I have set the bar for you ☺ I am very proud of what you have become and I'm sure you will make a great doctor! Lulo, you have always been the coolest mom one would wish for; a great role model of work ethics and success! Thank you for always being my number one fan, fueling me with over-confidence! Jani, you always motivated me to give my best in everything I do. Our discussions have always been a source of guidance and very insightful for my next steps. I hope you are proud of what I have become today. Poliksenula, je gjyshja me e mire ne bote! Sa krenari qe jam mbesa jote! Kete liber ta dedikoj ty dhe gjyshit, i cili do te lumturohej shume po ta lexonte! Jeni mbreterit e zemres time! I take this chance to thank my other family members and in particular, my aunt Mira, the sublime human being on this earth, the strongest woman I know who is an inspiration to all around her and Suelino, for providing so much love and support, in particular with her happy voice behind the phone on my trips back and forth to work.

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Faleminderit nga zemra!

PHD PORTOFOLIO

Name PhD Student	Jana Nano
Department	Epidemiology, Erasmus Medical Center Rotterdam, Cardiovascular Epidemiology
Research School	Netherlands Institute for Health Sciences (NIHES), Rotterdam
PhD Period	September 2014 - February 2018
Promotor	Prof. dr. Oscar H. Franco
	Prof. dr. M. Arfan Ikram
Co-promotors	Dr. Taulant Muka
	Dr. Abbas Dehghan

	Year	Workload (*ECTS)
Training		
Doctor of Science in Public Health, NIHES, Rotterdam, the Netherlands	2014-2015	70
Erasmus Summer Program		
Health Economics	2015	0.7
Causal Inference	2015	0.7
History of Epidemiologic Ideas	2015	0.7
Causal Mediation Analysis	2015	0.7
Core Curriculum		
Linux for Scientists	2015	0.6
Advanced Short Courses		
Bayesian Statistics	2015	1.4
Women's Health	2015	0.9
Advanced Analysis of Prognosis Studies	2015	0.9
Advances in Genome-Wide Association Studies	2015	1.4
A First Encounter with Next-Generation Sequencing data	2015	1.4
From Problem to Solution in Public Health	2015	1.1
Public Health in Low and Middle Income Countries	2015	3.0
General Academic Courses		
Research Integrity, Erasmus MC	2016	2.0
Attended Seminars		
Seminars of the Department of Epidemiology	2014-2017	2.0
2020 Meetings	2014-2017	2.0
Cardiovascular Group Meetings	2014-2017	2.0
ErasmusAGE Group Meetings	2014-2015	2.0
Work report and discussion with promotors and copromotors	2014-2017	2.0
Molecular Epidemiology Meetings	2014-2017	2.0
Internal Medicine Meetings	2014-2015	1.0

International Conferences

Epigenomic of Chronic Diseases, Cambridge, UK	2017	
Clinical Epigenetic Society, Dusseldorf, Germany	2016	
European Diabetes Epidemiology Group (EDEG) Annual meeting, Ireland	2016	
International Congress of Biomedical Science, Tirana, Albania	2013	

Scholarship and grants

ERAWEB mobility scheme

3rd German Diabetes Center Research School, 2015

Albert Renold Fellowships, European Foundation for the Study of Diabetes, 2016

Netherland's Alzheimer Foundation, 2016

Erasmus University Rotterdam Trust Funds, 2016

Teaching Activities

Clinical Translation of Epidemiological Research Course (lecturer, NIHES, Erasmus MC, Rotterdam the Netherlands)	2017	2.0
Introduction to Epidemiology (assistant, Harvard School of Public Health – Boston, USA)	2017	2.0
Epigenetics Course (assistant, NIHES summer program, Erasmus MC, Rotterdam, the Netherlands)	2016, 2017	2.0
Study Design Course (assistant, NIHES summer program, Erasmus MC, Rotterdam, the Netherlands)	2016	2.0
Understanding Medical Research for Health Professionals Course (lecturer, International Congress of Biomedical Sciences, Tirana, Albania)	2014	2.0
An introduction to Epidemiology Course (assistant, Ludwig-Maximilian University, Munich, Germany)	2013	2.0

Participation in Consortia

DIAMANTE (DIAbetes Genetics Replication And Meta-analysis)	From 2015
CHARGE (Cohorts for Heart and Aging Research in Genomic Epidemiology)	From 2015
BBMRI (Biobanking and BioMolecular resources Research Infrastructure)	From 2016
ICHOM (International Consortium for Health Outcomes Measurement)	From 2017

Peer Review for Scientific Journals

Clinical Gastroenterology, Diabetes, European Journal of Epidemiology, British Medical Journal, Nutrition Metabolism and Cardiovascular Disease, Plos One ect.

Research Visit

Visiting Research Scientist, Harvard School of Public Health

Supervision

Valentina González-Jaramillo MD, MSc, PhD student

Erla Bare, Exchange Student

Other

Social Committee Member of Cardiovascular Group, Erasmus MC

Organizing Committee of Monday Scientific Seminars, Department of Epidemiology, Rotterdam

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

PUBLICATIONS AND MANUSCRIPTS

1. Mahajan A, Taliun D, Thurner M, Robertson N.R, Torres JM, Rayner NW, Steinthorsdottir V, Scott RA, Grarup N, Cook JP, Schmidt EM, Wuttke M, Sarnowski C, Mägi R, Nano J [et al] DIAMANTE Consortium. Fine-mapping of an expanded set of type 2 diabetes loci to single-variant resolution using high-density imputation and islet-specific epigenome maps. Nature Genetics 2018
2. Chen Z, Zuurmond MG, van der Schaft N, Nano J, Wijnhoven HAH, Ikram MA, Franco OH, Voortman T. Plant versus animal based diets and insulin resistance, prediabetes and type 2 diabetes: the Rotterdam Study. Eur J Epidemiol. 2018.
3. Dall'Aglio L, Muka T, Cecil CAM, Bramer WM, Verbiest M, Nano J, Hidalgo AC, Franco OH, Tiemeier H. The role of epigenetic modifications in neurodevelopmental disorders: A systematic review. Neurosci Biobehav Rev. 2018;94:17-30.
4. Dhana K, Braun KVE, Nano J, Voortman T, Demerath EW, Guan W, Fornage M, van Meurs JBJ, Uitterlinden AG, Hofman A, Franco OH, Dehghan A. An Epigenome-Wide Association Study (EWAS) of Obesity-Related Traits. Am J Epidemiol. 2018.
5. Dhana K, Nano J, Ligthart S, Peeters A, Hofman A, Nusselder W, Dehghan A, Franco OH. Obesity and Life Expectancy with and without Diabetes in Adults Aged 55 Years and Older in the Netherlands: A Prospective Cohort Study. PLoS Med. 2016;13(7):e1002086.
6. Glisic M, Kastrati N, Musa J, Milic J, Asllanaj E, Portilla Fernandez E, Nano J, Ochoa Rosales C, Amiri M, Kraja B, Bano A, Bramer WM, Roks AJM, Danser AHJ, Franco OH, Muka T. Phytoestrogen supplementation and body composition in postmenopausal women: A systematic review and meta-analysis of randomized controlled trials. Maturitas. 2018;115:74-83.
7. 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(3):577-86.
8. Muka T, Nano J, Voortman T, Braun KVE, Ligthart S, Stranges S, Bramer WM, Troup J, Chowdhury R, Dehghan A, Franco OH. The role of global and regional DNA methylation and histone modifications in glycemic traits and type 2 diabetes: A systematic review. Nutr Metab Cardiovasc Dis. 2016;26(7):553-66.
9. 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.
10. Nano J, Ghanbari M, Wang W, de Vries PS, Dhana K, Muka T, Uitterlinden AG, van Meurs JBJ, Hofman A, consortium B, Franco OH, Pan Q, Murad SD, Dehghan A. Epigenome-Wide Association Study Identifies Methylation Sites Associated With Liver Enzymes and Hepatic Steatosis. Gastroenterology. 2017;153(4):1096-106 e2.
11. 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(6):389-97.
12. Nano J, Muka T, Ligthart S, Hofman A, Darwish Murad S, Janssen HLA, Franco OH, Dehghan A. Gamma-glutamyltransferase levels, prediabetes and type 2 diabetes: a Mendelian randomization study. Int J Epidemiol. 2017;46(5):1400-9.
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PE, Stehouwer CDA, Schalkwijk CG, Arts ICW, Dekker JM, Dehghan A, Muka T, van der Kallen CJH, Nijpels G, van Greevenbroek M. Blood metabolomic measures associate with present and future glycemic control in type 2 diabetes. J Clin Endocrinol Metab. 2018.

14. Vargas KG, Milic J, Zaciragic A, Wen KX, Jaspers L, Nano J, Dhana K, Bramer WM, Kraja B, van Beeck E, Ikram MA, Muka T, Franco OH. The functions of estrogen receptor beta in the female brain: A systematic review. Maturitas. 2016;93:41-57.
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16. Meta-analysis of maternal smoking GFI1 loci and cardio-metabolic phenotypes in adults (Co-author, Appeceted October 2018 at The Lancet EBioMedicine)
17. A peripheral blood DNA methylation signature of hepatic fat reveals a potential causal pathway for non-alcoholic fatty liver disease (First shared author, submmited)
18. Fatty Liver Index and Risk of Type 2 Diabetes, Cardiovascular Disease and Mortality: The Rotterdam Study (First Author, submmited)
19. Epigenetics and Inflammatory Markers: a Systematic Review (Last Author, submmited)
20. Type 2 Diabetes and Dementia Risk: A Mendelian Randomization Study (First Author, in preparation)
21. Standardized Outcome Measurement for Patients With Diabetes Mellitus: Consensus From the International Consortium for Health Outcomes Measurement (ICHOM) (First shared author, in preparation)
22. Patient reported outcomes in Diabetes: Consensus From the International Consortium for Health Outcomes Measurement (ICHOM) (First shared author, in preparation)
23. Nutrients and DNA methylation across the life course: a systematic review (Co -author, submmited)
24. Sex difference of epigenetic mechanism in cardiovascular disease (Co -author, submmited)
25. Mendelian randomization provides evidence for a causal role of dehydroepiandrosterone sulfate in decreasing NT-proBNP levels in a Caucasian population (Co-author, submmited)
26. Epigenetic Links Between Statin Therapy and Type 2 Diabetes (Co-author, submmited)
27. Association between cutaneous manifestations of ageing with cardiovascular outcomes, risk factors and mortality: a systematic review and meta-analysis (Co-author, submitted)

ABOUT THE AUTHOR

Jana Nano (20th October 1987) was born and raised in Tirana, the vibrant capital city of Albania. In 2006, she graduated from 'Petro Nini Luarasi' High School and started her medical studies at the Faculty of Medicine, University of Tirana, Albania. During her studies (and later), she was engaged in youth activism supporting the reformation of the medical education in Albania, in particular promoting medical research, and improving medical literacy in the population. She spent the last year of her medical studies at the University of Medicine in Bologna, Italy. In October 2012, she was graduated cum laude from the medical school and immediately after, moved to Munich, Germany to pursue a Master in Clinical and Genetic Epidemiology. To expand her dazzling interest in epidemiology, she came at the Department of Epidemiology, Erasmus MC to work on her master thesis under the supervision of Prof. Oscar Franco and Dr. Abbas Dehghan. In October 2014, she was awarded EU funded grant for her Doctor of Science in Public Health degree and PhD studies in the same department. During the last three years and a half, she has worked on the research described in this thesis with an interest on diabetes epidemiology, liver related traits, obesity, (epi) genetics and lately, diabetes complications. In January 2017, she moved to Boston on a fellowship grant of the European Society of Diabetes and Netherlands Alzheimeir's Association as a Visiting Researcher at the Department of Epidemiology, Harvard School of Public Health (chair: Prof. Dr. Albert Hoffman). Upon return, she finalized her thesis and took over the leading and coordinating of the Metabolic Health and Diabetes group in the Rotterdam Study. Since March 2018, Jana accepted a post-doctoral position to continue her research work in diabetes complications at Helmholtz Zentrum, Deutsches Forschungszentrum für Gesundheit und Umwelt (Prof. Barbara Thorand; chair, Prof. Annete Peeters) in Munich, Germany, where she is happily living with her dog.

In the last years, the emerging threat of diabetes has called for resolution and intensified research efforts to analyse changing aspects of the epidemiology of traditional risk factors such as obesity and investigate the role of novel biomarkers in our ultimate aim to effectively prevent diabetes. The burgeoning interest in the field of epigenetics, an intersection between genetic determinism and environmental influences, has opened new opportunities to discover more about the molecular pathways involved in the regulation of diabetes risk factors from the etiological perspective, and possibly might give rise to new therapeutical strategies. Moreover, management and prevention of diabetes complications remain an exhaustive task not only for the diagnosed individual but also for the medical system. Patient-centered outcome measurements combined with efficient economical health systems, in what we now call value-based medicine, seem to be the new medical paradigm we are moving towards.