Novel insights into blood markers and cardiovascular disease: Results of the Netherlands Epidemiology of Obesity study

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CHAPTER 1

GENERAL INTRODUCTION

The research in this thesis is aimed at describing and understanding the role of factors related to body fat and body fat distribution in atherosclerosis and cardiovascular disease.

Atherosclerosis and cardiovascular disease

Cardiovascular disease (CVD) is a major cause of mortality, in 2015 17.7 million people died from CVD worldwide. [1] In the Netherlands alone, 38,647 people died from CVD in 2016. [2]

One of the most prominent markers of subclinical cardiovascular disease is atherosclerosis. Atherosclerosis is a progressive disease of the vessel wall, characterized by the accumulation of mainly low-density lipoprotein (LDL)- cholesterol and pro-inflammatory cells in the intima of the vessel. [3] Further development of atherosclerotic lesions is characterized by the proliferation of vascular smooth muscle cells in the media of the vascular wall [4], increased vascularization of the adventitia [5, 6], decreased responsiveness of the vascular smooth muscle cells to vasodilators [7], and endothelial wear. [8] Recent studies suggested that the pathology of atherosclerosis differs between women and men, in the sense that lesions typical for men are less dense and have a thin fibrous cap that is prone to rupture while lesions in women often have a more stable fibrous cap, but suffer from endothelial wear. [9]

Atherosclerosis may cause cardiovascular disease when it decreases the diameter lumen of a coronary artery and the restricted blood flow induces chest pain or dyspnoea with physical activity or cold-induced stress, i.e., angina pectoris, or when it causes total blockade of blood flow by its rupture and subsequent thrombus formation, i.e., myocardial infarction. In the brain, this may lead to ischaemic stroke. [10, 11]

Body fat distribution and atherosclerosis

One of the main risk factors for atherosclerosis is excess body fat, specifically body fat localised around the organs in the abdominal cavity, which is referred to as visceral fat. [12] Well-known underlying mechanisms in this association are low-density lipoprotein cholesterol (LDL-c), hypertension and diabetes, but there are several novel mechanisms that are under investigation for their potential role in this association, such as other blood lipids, inflammation and adipocytokines.

Traditional risk factors for atherosclerosis

Multiple factors contribute to the development of atherosclerosis. Genetic, lifestyle and environmental factors interact and therefore establish a complex causal structure that may eventually lead to cardiovascular disease. Since the conception of the famous Framingham Heart Study in 1948 [13], a plethora of studies have set out to investigate risk factors of atherosclerosis. Prominent and well-studied risk factors that resulted from these endeavours include smoking, high LDL-cholesterol concentrations, high blood pressure, and diabetes, which may in turn be caused by a lack of physical activity, stress, dietary habits, or obesity. [14, 15]

Overweight and obesity are increasingly prevalent risk factors for atherosclerosis. In 2017, almost half of the population in the Netherlands had overweight or obesity, defined as a body mass index of 25 or 30 kg/m^2 or higher. [16] Despite the ease of measurement of body mass index as a measure of overweight, in terms of cardiovascular risk visceral fat located in the abdominal cavity is regarded as a stronger cardiometabolic risk factor than the overall amount of body fat due to its higher metabolic activity and production of inflammatory markers and adipocytokines. [17] Visceral fat accumulation can be measured in research settings using computed tomography (CT) or magnetic resonance imaging (MRI), or approximated in clinical practice by measuring waist circumference.

While obesity approximately doubles the risk of cardiovascular disease, the mechanisms that explain this relation are not yet fully understood. As illustrated in Figure 1, the main cardiometabolic risk factors dyslipidaemia, hypertension, and diabetes are responsible for only half of the excess cardiovascular risk associated with obesity, acting as mediators of the relation between obesity and cardiovascular disease. [18] Consequently, half of the excess cardiovascular risk associated with obesity is yet unexplained. Quantifying the role of novel risk factors is essential to be able to improve identification of persons at risk for cardiovascular disease. Several other factors have been suggested as candidate intermediate mechanisms, such as HDL-cholesterol, postprandial triglyceride response [19], cholesteryl ester transfer protein (CETP) [20], adipokines [21], and inflammation [22]. The role of these factors in the development of subclinical atherosclerosis remains unclear.

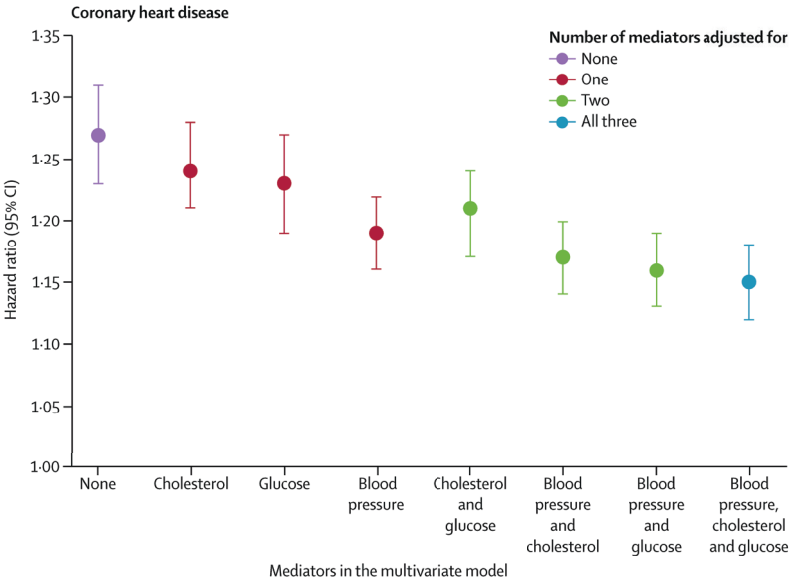

Figure 1 – Estimation of the relative contributions of metabolic mediators in the excess risk of coronary heart disease, separately and in combination. Adapted from The Global Burden of Metabolic Risk Factors for Chronic Diseases Collaboration. The Lancet 2014; 383:9921; 970-983.

Adipokines and inflammation

In the 1990s it became clear that adipose tissue does not merely store energy in the form of fat, but also secretes regulatory substances, such as inflammatory cytokines and adipose tissue specific peptide hormones. Adiponectin and leptin are hormones that are specifically secreted by adipose tissue, and therefore also referred to as adipokines or adipokine hormones. [23, 24]

Adiponectin (from Latin *adipo*, "fat"; *nectin*, "join, tie") is mainly produced by ectopic (visceral) fat, located in the abdominal cavity, and notably secretion of adiponectin is higher with lower amounts of visceral fat. [25, 26] The main physiological functions of adiponectin are yet unclear, but adiponectin has predominantly been described as a protective factor in the development of insulin resistance and type 2 diabetes. [27, 28]

In contrast, leptin (from Greek *leptos*, "small, slight, slender, delicate") is mainly produced by subcutaneous adipose tissue, and its primary function is to inhibit energy intake by signalling to the hypothalamus and activating the proopiomelanocortin (POMC) pathway. [29] Also, several studies suggested that leptin has off-target effects, which may be detrimental for cardiovascular health. [30-34]

However, these studies often have important limitations in design or available measurements. The main difficulty is the strong relation between body fat and serum leptin concentration, which may obstruct our view on potential underlying effects of leptin, because it is unknown to what extent leptin is a confounding factor, mediator, or merely a marker of body fat. Methods to circumvent this problem are statistical adjustment for body fat, or using genetic variants that predispose for higher serum leptin.

In the last three decades, the view of atherosclerosis as an inflammatory disease has emerged. [3] This is demonstrated by the higher risk of cardiovascular disease in the presence of several autoimmune diseases, such as rheumatoid arthritis or systemic lupus erythematous, which is even aggravated by the use of systemic corticosteroid treatment. [35, 36] The foremost factor suggesting a direct role for inflammation in the progression of atherosclerosis is the activation of macrophages in atherosclerotic lesions upon systemic inflammation. [37]

These macrophages incorporate LDL-cholesterol, which is subsequently oxidized causing the macrophages to form foam cells. [38] Abundance of pro-inflammatory immune cells and foam cell formation marks lesions at high risk for rupture, which motivated the development of anti-inflammatory therapies aimed at prevention of cardiovascular disease. The recent Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) indeed indicated a cardioprotective role of the anti-inflammatory drug canakinumab that targets interleukin-1β, but with a null effect on total mortality due to an increase in infection risk. [39] The potential role for inflammation is especially interesting in conditions of excess body fat, because body fat and specifically visceral fat depots have been associated with low-grade pro-inflammatory state. [40] The relative contribution of inflammation to atherosclerosis, as compared to other risk factors has not been studied in detail on a population level.

Lipids and lipoproteins

Overweight and obesity have a well-described and profound detrimental impact on the circulating lipoprotein profile, in particular an increase in atherogenic LDL-cholesterol. [41] LDL-cholesterol, together with its main structural component apolipoprotein B 100 (ApoB) is one of the most important constituents of atherosclerotic lesions. It contributes to lesion formation by its internalization in the vascular wall, and to lesion destabilization by being oxidized and being taken up by macrophages that subsequently form foam cells. Two main classes of LDL-cholesterol reducing and CVD-preventing drugs are currently being used: inhibition of the HMG-CoA reductase pathway using statin therapy, and advanced adjuvantia like PCSK9-inhibitors. [42]. However, as a side effect, both

LDL-cholesterol lowering treatments may lead to a small increased risk of type 2 diabetes. [43, 44]

Besides the well-studied effects of LDL-cholesterol on atherosclerosis, the interest has shifted towards other lipoprotein subclasses.

Specifically, the role of high-density lipoprotein (HDL)-cholesterol is highly debated. While low HDL-cholesterol concentrations are strongly associated with atherosclerosis and cardiovascular disease in observational studies, Mendelian randomisation studies have shown that on a population basis low levels of HDL-cholesterol have no causal effect on cardiovascular disease. [45] Consistently, clinical trials that aimed to increase HDL-cholesterol concentrations by inhibiting a key player in cholesterol metabolism, cholesteryl ester transfer protein (CETP) have all shown no effect of drug-induced HDL-cholesterol increase. [46-48] However, the failure of these trials is in contrast to previous reports of a cardioprotective role of genetic variants with a CETP-lowering effect. [20, 49, 50] Interestingly, the genetic studies that found a deleterious effect of CETP on cardiovascular disease were mainly performed in men and diabetic women. [20, 51] This suggests that the effect of CETP on the development of atherosclerosis and cardiovascular disease may be different in subgroups of the population.

Furthermore, fasting triglyceride concentrations have been suggested to be a risk factor for atherosclerosis. [52] However, many people are not in a fasting state for the majority of the day. Therefore non-fasting or postprandial triglyceride concentrations may be a better reflection of the total exposure of the vascular wall to triglycerides during the day. [53]

In all aforementioned mechanisms, there is a potential bias by confounding factors, i.e. common causes of the exposure and the outcome that may explain or distort the association of interest. [54] A factor that is often regarded as confounding factor is sex. In this thesis, sex is relevant as it is a determinant of adipose tissue accumulation and distribution [55], while it is also a determinant of increased cardiometabolic risk. [56] This causal structure is complicated further by the possibility that the relation between sex and atherosclerosis is partly mediated by visceral fat. There are several methods to mitigate the effects of confounding factors, of which adjusting using multivariable regression analysis is the method generally used in epidemiologic studies. Although adjustment in regression analysis is an efficient method to mitigate confounding effects while retaining statistical power, this method prohibits the interpretation of within-group effects in the case of effect modification. To facilitate interpretation of within-group effects, we aimed to stratify most analyses for sex. Other methods to reduce confounding effects include restriction, matching, and (Mendelian)

randomisation. [57] In this thesis, several manuscripts include Mendelian randomisation techniques, which are based on the random heritability of genetic variants that predispose for the exposure of interest. This implies that confounding factors would not be able to affect the genetically-determined part of the exposure phenotype. Therefore it is possible to use observational data to answer causal questions. [58]

Outline of the thesis

The aim of this thesis is to describe and improve understanding of the role of factors related to overweight and obesity in the development of cardiovascular disease.

This thesis can be divided into two main sections. The first section focuses on the role of inflammation and the adipokines leptin and adiponectin in the association between body fat and atherosclerosis. The second section includes studies that investigate determinants and effects of serum lipids and lipoproteins. Where possible, sex differences were investigated as well in the studies in this thesis. The main objectives of this thesis are visualised in the directed acyclic graph (DAG) in Figure 2.

In **chapter 2**, we studied to what extent the association between measures of total body fat and visceral fat, and subclinical atherosclerosis was mediated by measures of inflammation.

In **chapter 3** we investigated to what extent the sex difference in leptin and adiponectin concentrations were due to sex differences in body fat content and distribution. In **chapters 4 and 5**, potential cardiovascular effects of leptin and adiponectin are disentangled from cardiovascular effects of body fat and body fat distribution.

Chapter 6 builds on the long-standing controversy regarding the effects of CETP on cardiovascular disease. Considering previous genetic studies that showed marked effects of CETP in subgroups of the population we hypothesized that the effect of modulation HDL-cholesterol on atherosclerosis may be different in subgroups of the population (effect modification). Therefore we aimed to extensively investigate the relations of observational and genetically-determined CETP concentration with subclinical atherosclerosis in subgroups of the general population.

The role of the residual postprandial triglyceride response in the relation between HDL-cholesterol and coronary artery disease is investigated in **chapter**

7, and we studied the contribution of postprandial triglyceride excursions to subclinical atherosclerosis, in addition to the effects of fasting triglyceride concentrations in **chapter 8**.

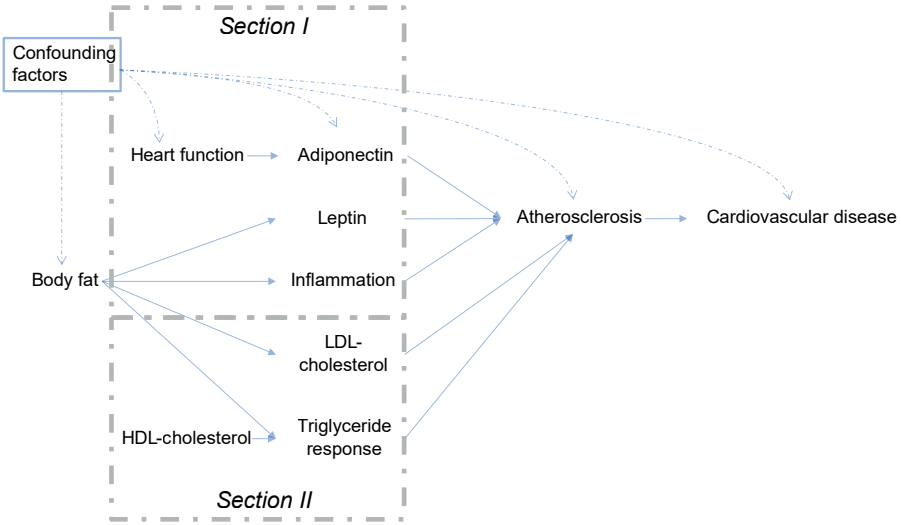

Figure 2 – Hypothesis diagram of the research presented in this thesis. In section I, mechanisms of inflammation and adipocytokines are described, while section II focuses on lipids.

Study populations

The observational analyses that are presented in this thesis were predominantly performed in the Netherlands Epidemiology of Obesity study (NEO study). The NEO study is a prospective cohort study including participants aged 45-65 years from the general area of Leiden, the Netherlands. Men and women with a self-reported body mass index (BMI) of 27 kg/m^2 or higher were eligible to participate. In addition, inhabitants of Leiderdorp (adjacent to Leiden) were invited to participate irrespective of their BMI. Unique aspects of the NEO study are the large population-based sample, detailed body fat measurements by MRI, extensive phenotyping including blood sample analyses and questionnaire data, metabolomic profiling, as well as a whole genome scan. A detailed description of the design and data collection of the NEO study can be found elsewhere. [59]

Participants with a BMI of 27 kg/m^2 were overrepresented in this study, which requires adjustment in order to be able to generalise results to a general population. All analyses presented in this thesis were performed weighted towards the BMI distribution of the participants of Leiderdorp, who had a BMI distribution that was similar to the BMI distribution of the Dutch population. [16, 60] Consequently, the results that are presented in this thesis apply to the general popula-

tion.

In addition, we performed several two-sample Mendelian randomization analyses in which we used external sources of genetic instruments and publicly-available summary statistics of genome-wide association studies. These Mendelian randomization analyses were performed on serum leptin concentrations, peak wave velocity and coronary heart disease (**chapter 4**), serum adiponectin and N-terminal pro-brain natriuretic peptide (NT-proBNP) concentrations, and measures of heart function (**chapter 5**), and serum lipid concentrations in **chapter 7**. Therefore data from several GWAS were used.

The CARDIoGRAMplusC4D consortium performed a GWAS on the risk of coronary heart disease in a case-control setting with 60,801 cases and 123,504 controls of European descent from 48 studies. [61] Coronary heart disease diagnoses included myocardial infarction, acute coronary syndrome, chronic stable angina or coronary stenosis of >50%.

The GWAS on pulse wave velocity as a measure of vascular function was performed within the UK Biobank project. [62] Pulse wave velocity was measured in 117,867 genotyped participants by using an infrared sensor at the fingertip.

The GWAS on serum leptin concentrations was performed in 32,161 participants of European descent from 23 studies. [63]

We used publicly-available summary statistics of the GWAS on adiponectin concentration that was performed by the ADIPOGen consortium in 39,883 individuals from European descent. [64]

The GWAS on circulating NT-proBNP concentrations was performed in 9,232 individuals with acute coronary syndrome, of whom 99% were of European descent, who participated in the PLATO trial. [65]

Genetic variants that predispose for heart structure and function were used from a GWAS by the EchoGen consortium, that was performed in 32,212 individuals of mostly European ancestry. [66]

The genetic variants that are associated with serum triglyceride, LDL-, and HDL-cholesterol concentrations were discovered in a GWAS that was performed by the Global Lipids Genetics Consortium, which was performed in 188,577 individuals of European, South Asian, and African ancestry [67]

SECTION I

INFLAMMATION AND ADIPOKINES

CHAPTER 2

THE ROLE OF INFLAMMATION IN THE ASSOCIATION BETWEEN OVERALL AND VISCERAL ADIPOSITY AND SUBCLINICAL ATHEROSCLEROSIS

T Christen, S Trompet, PCN Rensen, K Willems van Dijk, HJ Lamb, JW Jukema, FR Rosendaal, S le Cessie, R de Mutsert

Nutrition, Metabolism and Cardiovascular Disorders, 2019

Abstract

Background and aims

Inflammation may underlie the association between obesity, atherosclerosis and cardiovascular disease. We investigated to what extent markers of inflammation mediate associations between overall and visceral body fat and subclinical atherosclerosis.

Methods and Results

In this cross-sectional analysis of the Netherlands Epidemiology of Obesity study we estimated total body fat (TBF) by bio-impedance analysis, carotid artery intima media thickness (cIMT) by ultrasound, C-reactive protein (hs-CRP) and glycoprotein acetyls (GlycA) concentrations in fasting blood samples (n=5,627), and visceral adipose tissue (VAT) by magnetic resonance imaging (n=2,247). We examined associations between TBF and VAT, and cIMT using linear regression, adjusted for potential confounding factors, and for mediators: cardiometabolic risk factors (blood pressure, glucose and low-density lipoprotein cholesterol), and inflammation using CRP and GlycA as proxies.

Mean (SD) cIMT was 615 (90) µm. Per SD of TBF (8%), cIMT was 19 µm larger (95% confidence interval, CI: 10, 28). This association was 17 µm (95% CI: 8, 27) after adjustment for cardiometabolic risk factors, and did not change after adjustment for markers of inflammation. Per SD (56 cm^2) VAT, cIMT was 9 µm larger (95% CI: 2, 16) which changed to 5 µm (95% CI: -3, 12) after adjustment for cardiometabolic risk factors, and did not change after adjustment for inflammatory markers.

Conclusion

Our results suggest that associations between measures of overall and visceral body fat and subclinical atherosclerosis are not mediated by inflammation as measured by CRP and GlycA. Obesity may exert cardiovascular risk via other markers of systemic inflammation.

Introduction

Obesity, and in particular abdominal adiposity, is a well-established cause of coronary heart disease, stroke, and atherosclerosis [68, 69]. Between 40 and 50% of the association between obesity and cardiovascular diseases is mediated by three major cardiometabolic risk factors blood pressure, low-density lipoprotein cholesterol (LDL-c) and fasting glucose concentrations. [18] A low-grade systemic state of inflammation may partly mediate the relation between measures of body fat and cardiovascular disease (Figure 1).

Body fat, and in particular visceral fat, is infiltrated by pro-inflammatory (M1)-type macrophages, which contribute to a low-grade systemic inflammatory state by producing and secreting pro-inflammatory cytokines like IL-6, TGF-β, which is considered to have a detrimental effect on atherosclerosis [3, 70, 71]. Systemic inflammation is further marked by C-reactive protein (CRP) and glycoprotein acetyls (GlycA) [72]. CRP is produced by the liver upon stimulation by inflammatory cytokines, while GlycA is a composite marker of glycosylated acute phase protein concentrations [73]. Both markers are observationally associated with arterial stiffness and cardiovascular disease and can therefore be used as a proxy for low-grade systemic inflammation [74-78].

We aimed to investigate the role of low-grade systemic inflammation, in addition to the main known cardiometabolic risk factors, in the associations between overall and visceral body fat with subclinical atherosclerosis.

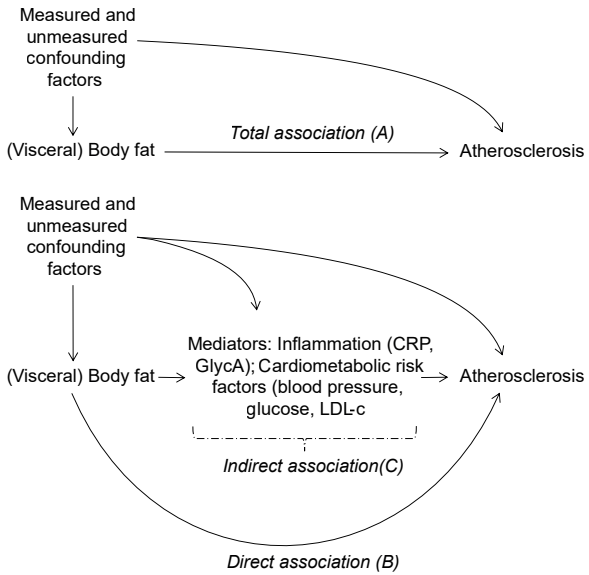

Figure 1 – Directed acyclic graph of the hypothesized relations between measures of body fat and (subclinical) atherosclerosis, the total association (A) may be separated in a direct association (B), and an indirect association (C) via inflammation

CRP, C-reactive protein; GlycA, glycoprotein acetyls; LDL-c, low density lipoprotein cholesterol

Methods

Study population

The present study is a cross-sectional analysis of baseline measurements of the Netherlands Epidemiology of Obesity (NEO) study, a population-based, prospective cohort study (n=6671). The study design and population are described in detail elsewhere [59].

Men and women aged 45-65 years with a BMI ≥27 kg/m^2 were eligible to participate in the NEO study. In addition, inhabitants of Leiderdorp were invited irrespective of their BMI to serve as a reference group with a BMI distribution of the general population. Prior to the study visit, participants completed questionnaires at home with respect to demographic, lifestyle, and clinical information. Research nurses recorded names and dosages of current medication.

The design of the NEO study was approved by the Medical Ethical Committee of the Leiden University Medical Center (LUMC). All participants gave their written informed consent.

Data collection

Self-reported ethnicity was regrouped into white and other. The highest completed level of education was reported in ten categories and regrouped into two categories: low education (no education, primary education or lower vocational education) and high education (other). Smoking was reported in three groups: never, former, and current smoker. The SQUASH questionnaire was used to self-report physical activity during leisure time in hours per week of metabolic equivalents of task [79]. Participants self-reported their medical history of diabetes and cardiovascular disease (defined as myocardial infarction, angina, congestive heart failure, stroke or peripheral vascular disease). Height was measured without shoes using a calibrated, vertically fixed tape measure. Blood pressure was measured seated on the right arm with a validated automatic oscillometric device (OMRON, Model M10-IT; Omron Health Care Inc, IL, USA). The mean of three measurements was used to calculate the mean systolic and diastolic blood pressure levels.

Measures of overall and visceral adiposity

Body weight and total body fat percentage (TBF) were assessed using a bio-impedance device (TBF-310, Tanita International Division, UK). BMI was calculated by dividing body weight in kilograms by body height in meters squared. Abdom-

inal visceral adipose tissue (VAT) was assessed by magnetic resonance imaging (MRI, 1.5 Tesla MR imaging, Philips Medical Systems, Best, the Netherlands) using a turbo spin echo imaging protocol in a random subgroup of 2580 individuals without contraindications for MRI (implanted metallic devices, a body circumference >1.70 m, or claustrophobia). At the level of the fifth lumbar vertebra, three 10 mm transverse images were obtained during a breath-hold. Visceral fat areas was quantified by converting the number of pixels to cm^2 for all three slices, of which the mean area was used in the analyses.

Carotid intima media thickness

To obtain cIMT, ultrasonography of the common carotid arteries (CCA) was performed. A 15 mm long section 10 mm proximal of the CCA bifurcation was measured while the subject was in supine position. IMT was measured using a 7.5–10 MHz linear-array probe and the Art.Lab system (ART.LAB version 2.1, Esaote, Maastricht, The Netherlands) in B-mode setting and using a wall-track system to detect boundaries between lumen and intima, and between media and adventitia. cIMT was reported as the mean of three measurements in angles of 180, 135 and 90 degrees (right CCA) and 180, 225 and 270 degrees (left CCA). In 183 randomly selected participants (n=183) we validated measurements of intima media thickness with an intra-observer coefficient of variation of 5.8%, and an inter-observer coefficient of variation of 9.0% [80].

Blood measurements

Plasma glucose, serum triglyceride, serum high-density lipoprotein-cholesterol (HDL-c), and serum total cholesterol concentrations were determined in fasting blood samples at the central clinical chemistry laboratory of the LUMC using standard assays. LDL-c concentrations were calculated using the Friedewald equation.

Serum concentrations of CRP were determined in a fasting blood sample using a high sensitivity CRP assay (hs-CRP, TINA-Quant CRP HS system, Roche, Germany and Modular P800, Roche, Germany). GlycA concentrations were measured in plasma that had undergone one previous freeze-thaw cycle, using a high-throughput proton nuclear magnetic resonance (NMR) spectroscopy (Nightingale Health Ltd., Helsinki, Finland) [81]. We calculated intraclass coefficients for 94 participants with repeated CRP and GlycA measurements during a second visit within 3-5 months of the first visit. For CRP, the intraclass coefficient was 0.31, and for GlycA 0.64. To examine multicollinearity, variance inflation factors (VIF) were calculated, which were all under 10 and therefore considered acceptable.

Statistical analysis

For the present analysis, we excluded participants with a medical history of cardiovascular disease, participants with hs-CRP concentrations ≥10 mg/L, or using medication that targets systemic inflammation (corticosteroids, lumiracoxib or naproxen [82, 83]), and participants with missing data.

Participants with a BMI ≥27 kg/m^2 were oversampled in the NEO study. To correct for this oversampling, analyses were weighted towards the BMI distribution of the NEO participants from Leiderdorp, whose BMI distribution was similar to the general Dutch population. [16, 60] All results were based on weighted analyses. Consequently, the results apply to a population-based study without oversampling of individuals with a BMI ≥ 27 kg/m^2.

Baseline characteristics were calculated for the total population, and for men and women separately, and expressed as mean (standard deviation, SD), median (25th-75th percentile) or percentage. The measures of adiposity were standardized to a mean of zero and standard deviation of one. Hs-CRP and GlycA concentrations were skewed, and therefore log-transformed to the natural logarithm.

Mediation analyses

To examine the role of inflammation in the associations between visceral and total body fat and atherosclerosis, we performed mediation analysis according to Baron and Kenny and evaluated the assumptions [84].

First, we estimated the total association between measures of body fat and intima media thickness with linear regression analyses, and adjusted crude analyses for potential confounding factors age, sex, tobacco smoking, physical activity, statin use, ethnicity, education, and the other measure of body fat and calculated regression coefficients with 95% confidence intervals.

Second, we examined the assumptions for the Baron and Kenny method of mediation analyses. One of the assumptions is the absence of interaction between the exposure and the mediator. To examine this assumption, we included an interaction term of the measures of body fat with the markers of inflammation in the model of the total association. We assessed linearity by plotting residuals, kernel density, P-P, and Q-normal distributions.

Furthermore we estimated the associations between the potential mediators and the exposure and the outcome variables by linear regression analysis. We reported the regression coefficients and 95% confidence intervals, which can be

interpreted as the difference in cIMT per standard deviation of measure of body fat. The regression coefficients from the models with log-transformed markers of inflammation as outcome were back-transformed, and can be interpreted as a relative change in inflammatory marker per standard deviation of body fat.

Subsequently, we assessed mediation of the total association between measures of body fat and intima media thickness by known cardiometabolic risk factors for cardiovascular disease: systolic blood pressure, LDL-c, and fasting glucose concentrations, and by the markers of inflammation separately and simultaneously. Therefore we additionally adjusted the regression models for the cardiometabolic risk factors and markers of inflammation. We reported regression coefficients with 95% confidence intervals, which can be interpreted as the direct effect [84].

To assess the robustness of the models, we repeated all analyses including previously excluded participants with a history of CVD. Initial exclusion of participants with hs-CRP concentrations ≥10 mg/L, may introduce selection bias [85]. Therefore, we performed sensitivity analyses including these participants. Furthermore, mediation analysis with a mediator that is measured with error may lead to strongly biased results [85]. Therefore, we performed a sensitivity analysis with an assumed random measurement error of 35% using the *eivreg* command in STATA (Statacorp, College Station, Texas, USA), version 14.0.

Results

Participants that used medication with effects on markers of inflammation (n=137) or had missing data (n=231), with a history of cardiovascular disease (n=437), or with hs-CRP concentrations ≥10 mg/L (n=267) were excluded. In total, 5627 participants with a mean age of 56 years (SD: 6), mean BMI was 26 kg/m^2 (SD: 4) and of whom 43% men were included in the analysis. Of these, 2247 underwent MRI and had assessment of VAT.

Total association

The analyses for the total association between body fat and intima media thickness showed that one standard deviation of total body fat (8%) was associated in the crude model with a 6 µm (95% CI: 2, 9) larger intima media thickness, which changed to 19 µm (95% CI: 10, 28) after adjustment for potential confounding factors. One SD of VAT (56 cm^2) was associated in the crude model with 24 µm (95% CI: 19, 29) larger cIMT, which changed to 9 µm (95% CI: 2, 16) per SD in the adjusted model.

Assessing the associations between exposure, mediators and outcome

We first examined associations between the measures of body fat and markers of inflammation. Adjusted linear regression analysis showed that per standard deviation of total body fat and visceral fat, hs-CRP concentrations were 71% (95% CI: 63, 79) and 33% (95% CI: 24, 42) higher. Per standard deviation of total body fat and visceral fat, GlycA concentrations were 9% (95% CI: 8, 9) and 6% (95% CI: 5, 7) higher. Measures of body fat were also associated with the cardiometabolic risk factors glucose, LDL-c, and blood pressure (data not shown). As for the association between the markers of inflammation and cIMT, the difference in cIMT was 1 µm (95% CI: -4, 7) per standard deviation of hs-CRP, and 2 µm (95% CI: -2, 5) per standard deviation of GlycA.

Table 1 – Baseline characteristics of participants of the NEO study aged between 45 and 65 years, without history of cardiovascular disease. (n = 5627).

	Total population	Men (43 %)	Women (57%)
Age (years)	56 (6)	56 (6)	55 (6)
BMI (kg/m^2)	26 (4)	27 (3)	25 (4)
Total body fat (%)	31 (8)	25 (5)	36 (7)
Visceral adipose tissue (cm^2) (n=2247)	90 (56)	115 (58)	67 (43)
Current smoking (%)	16	18	14
Alcohol intake (g/day)	10 (3-21)	16 (6-28)	8 (2-15)
Physical activity (MET h/week)	30 (16-50)	31 (15-51)	29 (16-49)
Education level (% high)	47	49	45
Diabetes (%)	5	7	3
Statin use (%)	8	11	6
Systolic blood pressure (mmHg)	131 (17)	135 (15)	127 (18)
Diastolic blood pressure (mmHg)	83 (10)	85 (10)	82 (10)
Triglycerides (mmol/L)	1.2 (0.8-1.7)	0.9 (0.7-1.3)	0.9 (0.7-1.3)
LDL cholesterol (mmol/L)	3.6 (0.9)	3.6 (1.0)	3.6 (1.0)
Glucose (mmol/L)	5.6 (1.2)	5.3 (0.8)	5.3 (0.7)
hs-CRP (mg/L)	1.1 (0.6-2.1)	1.2 (0.6-2.5)	1.1 (0.6-2.3)
Glycoprotein acetyls (mmol/L)	1.2 (1.1-1.3)	1.2 (1.1-1.3)	1.2 (1.1-1.3)
Carotid intima media thickness (µm)	615 (90)	625 (93)	605 (86)

Values are represented as mean (SD), median (25th-75th percentile) or percentage. Results were based on analyses weighted towards the BMI distribution of the general population (n = 5627).

hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein MET, metabolic equivalent; NEO, Netherlands Epidemiology of Obesity; SAT, abdominal adipose tissue; TBF, total body fat mass; SD, standard deviation; VAT, visceral adipose tissue

Table 2 – Linear regression analyses between measures of body fat and markers of inflammation, adjusted for potential confounding factors (total body fat: n = 5627; VAT: n = 2247).

		Relative change (%) in markers of inflammation per SD of measures of body fat	
		hs-CRP (%)	GlycA (%)
Total body fat (SD: 9%)	Crude	40 (35, 44)	3 (3,4)
	Adjusted	71 (63, 79)	9 (8, 9)
VAT (SD: 56 cm^2)	Crude	42 (35, 50)	9 (8, 10)
	Adjusted	33 (24, 42)	6 (5, 7)

Values represent relative change (%) in markers of inflammation per standard deviation of measures of body fat. Results were based on analyses weighted towards the BMI distribution of the general population (n= 5627; 2247 for VAT), and adjusted for potential confounding factors: age, sex, tobacco smoking, ethnicity, education and physical activity. Analyses with VAT were additionally adjusted for total body fat. GlycA, glycoprotein acetyls; hs-CRP, high-sensitivity C-reactive protein; SD, standard deviation; VAT, visceral adipose tissue

Table 3 – Linear regression analyses of associations between markers of inflammation and intima media thickness, adjusted for potential confounding factors (n = 5627).

	Model	
Difference in cIMT (μm) per SD of	Crude	Multivariate
hs-CRP	11 (5, 17)	1 (-4, 7)
GlycA	15 (9, 20)	2 (-2, 5)

Values represent difference in cIMT per standard deviation of markers of inflammation. Results were based on analyses weighted towards the BMI distribution of the general population (n= 5627), and adjusted for potential confounding factors: age, sex, tobacco smoking, ethnicity, education and physical activity, blood pressure, LDL-cholesterol, fasting glucose, and total body fat.

BMI, body mass index; cIMT, carotid intima media thickness; GlycA, glycoprotein acetyls; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; SD, standard deviation

No interaction was observed for associations between body fat and measures of inflammation in linear regression analyses between measures of body fat and intima media thickness.

Mediation analyses

The association between one SD of total body fat and intima media thickness decreased from 19 to 17 µm (95% CI: 8, 27) after adjustment for systolic blood pressure, LDL-c, and fasting glucose concentrations and to 16 µm (95% CI: 6, 25) after additional adjustment for hs-CRP and GlycA. The association between visceral fat and intima media thickness decreased from 9 to 5 µm (95% CI: -3, 12), and to 3 µm (95% CI: -5, 11) after additional adjustment for hs-CRP and GlycA.

Table 4 – Linear regression analyses of associations between measures of body fat and intima media thickness, adjusted for potential confounding factors (total association), hs-CRP and GlycA (direct association), and known cardiometabolic risk factors (systolic blood pressure, LDL-c, and glucose) (TBF: n = 5627; VAT: n = 2247).

	Difference in IMT (µm) per SD of:	
	Total body fat (SD: 9%)	VAT (SD: 55 cm^2)
Model 1 (crude)	6 (2, 9)	24 (19, 29)
Model 1 + age and sex	26 (22, 30)	19 (13, 24)
Model 2 (adjusted)	19 (10, 28)	9 (2, 16)
Model 3A (Model 2+ cardiometabolic risk factors)	17 (8, 27)	5 (-3, 12)
Model 3B (Model 2 + hs-CRP)	17 (8, 27)	7 (-0, 15)
Model 3C (Model 2 + GlycA)	17 (8, 26)	6 (-1, 14)
Model 3D (Model 2 + hs-CRP + GlycA)	16 (7, 26)	6 (-2, 13)
Model 4 (Model 2 + cardiometabolic risk factors + hs-CRP + GlycA)	16 (6, 25)	3 (-5, 11)

Results were based on analyses of the associations between measures of body fat and intima media thickness in participants of the Netherlands Epidemiology of Obesity study, weighted towards the BMI distribution of the general population (n=5627; 2247 for VAT). The adjusted analyses in Model 2 and further were adjusted for potential confounding factors: age, sex, tobacco smoking, ethnicity, education and physical activity. Model 3B-3D were additionally adjusted for hs-CRP, GlycA, and both. Model 4 was additionally adjusted for cardiometabolic mediating risk factors: systolic blood pressure, LDL-c and fasting glucose. Abbreviations: GlycA, glycoprotein acetyls; hs-CRP, high-sensitivity C-reactive protein; LDL-c, low-density lipoprotein cholesterol; SD, standard deviation; VAT, visceral adipose tissue

Sensitivity analyses

The observed associations did not change substantially when including participants with a history or with hs-CRP concentrations in excess of 10 mg/L (data not shown). Furthermore, the observed associations did not change after adjustment for measurement error in the mediators (Supplementary Table 1).

Discussion

In this population-based study, we confirmed that in particular total body fat and to a lesser extent visceral fat were strongly associated with markers of inflammation, and modestly associated with cIMT. However, we observed no association between markers of inflammation and cIMT, which violated one of the main assumptions of a mediation analysis: the presence of an association between mediator and outcome. Consequently, we did not observe mediation of the association between measures of body fat and cIMT by markers of inflammation.

We observed a stronger association with markers of inflammation for total body fat than visceral fat, which was discordant with previous literature that found stronger associations for visceral fat. [86] A potential explanation is that we used an accurate measures of visceral fat using MRI, and adjusted the associations for total body fat. Although visceral fat secretes cytokines at a higher rate than subcutaneous fat, visceral fat has a limited volume, and therefore contributes less to total inflammatory marker concentrations [25].

The role of inflammation in the development of atherosclerosis and cardiovascular disease has been debated for several years. While atherosclerosis is an inflammatory disease [22], CRP has been shown to be non-causal in the development of atherosclerosis and cardiovascular disease [87]. GlycA is a biological proxy of inflammation, as it is a measure of *N*-linked acute-phase proteins, which are secreted by the liver in response to stimulation by IL-6 [88]. Higher GlycA concentrations also predict an increased risk of CVD, independent of CRP [74]. Previous studies suggested that up to eight percent of the association between obesity and cardiovascular disease is mediated by inflammation [18, 89]. However, we did not observe an association between markers of inflammation and subclinical atherosclerosis, which precluded mediation.

This inconsistency between our and previous studies may be attributed to a difference in populations and markers of (subclinical) cardiovascular disease that were studied. Previous studies were performed in individuals at high cardiovascular risk, while the present study was performed in individuals from the general population. The main clinical trial that investigated the effects of inhibition of inflammation with the strong antiinflammatory drug canakinumab on cardiovascular disease incidence was also performed in patients at very high cardiovascular risk, and found only a small cardiovascular risk reduction but no net effect on all-cause mortality [39]. Second, atherosclerosis is considered an inflammatory disease [3]. Therefore it is possible that the inflammation of interest originates from atherosclerotic lesions, which may not be reflected by serum concentrations of hs-CRP and GlycA given the short half-lives of most cytokines.

Strengths of this study are that the analyses were performed in a large and well phenotyped population in which an objective measure of subclinical atherosclerosis and precise measures of overall and visceral adiposity were available. Two separate inflammation markers have been analysed with similar results. The possibility of measurement error was taken into account and our results showed that this error had very limited potential implications for our conclusions.

This study also has some limitations. First, cIMT measures subclinical atherosclerosis with a degree of uncertainty. However, our measurement of cIMT had a fair replicability [80]. Second, hs-CRP and GlycA are merely non-specific markers of low-grade systemic inflammation. Therefore, these concentrations may measure the true degree of systemic inflammation with error. This measurement error may severely bias the results of a mediation analysis [85]. However, as the markers of inflammation were robustly associated with total body fat and visceral fat, it is likely that concentrations of these markers truly reflected low-grade chronic inflammation associated with adipose tissue. Future studies could investigate more clinical measures of inflammation, such as leukocyte or monocyte counts. Also, the results did not essentially change upon correction for measurement error in all mediators. Furthermore, exclusion of participants with a history of cardiovascular disease to prevent reverse causation may have restricted our study population to individuals without clinically relevant atherosclerosis. However, our results did not change upon including these participants in sensitivity analyses. Finally, due to the observational nature of this study, unmeasured confounding or reverse causation may be present, that could specifically influence mediation analysis [90].

In conclusion, in this population-based study we observed no association between two markers of subclinical inflammation and cIMT, and no mediation by these markers of inflammation of the association between measures of body fat and intima media thickness. Further research may reveal other mediators of the relation between overweight and obesity and atherosclerosis.

CHAPTER 3

SEX DIFFERENCES IN BODY FAT DISTRIBUTION ARE RELATED TO SEX DIFFERENCES IN SERUM LEPTIN AND ADIPONECTIN CONCENTRATIONS

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Abstract

It is debated whether sex differences in fat-derived hormones adiponectin and leptin can be fully explained by sex differences in body fat distribution. In this analysis of the Netherlands Epidemiology of Obesity study, total body fat was assessed by bio-impedance (n=6,494), and visceral fat (VAT) by magnetic resonance imaging (n=2,516). Associations of measures of body fat and sex with serum adiponectin and leptin concentrations were examined using linear regression analysis. Sex differences were additionally adjusted for the measure of body fat that was most strongly associated with adiponectin or leptin concentrations. Median adiponectin concentrations in women and men were 10.5 mg/L (IQR, interquartile range: 7.7-13.9) and 6.1 mg/L (IQR: 4.5-8.2), the mean difference was 4.6 mg/L (95% CI: 4.3, 4.9). Median leptin concentrations in women and men were 19.2 µg/L (IQR: 11.5-30.0) and 7.1 (IQR: 4.6-11.1), the mean difference was 15.1 µg/L (95% CI: 14.4, 15.8). VAT was most strongly associated with adiponectin, while total body fat was most strongly associated with leptin. After adjustment for VAT, women had 3.8 mg/L (95% CI: 3.3, 4.3) higher adiponectin than men. After adjustment for total body fat, leptin concentrations in women were 0.4 µg/L lower than in men (95% CI: -1.2, 2.0). We observed that total body fat was strongly associated with leptin concentrations. Higher leptin concentrations in women than in men were completely explained by differences in total body fat. Visceral fat was modestly associated with adiponectin concentrations, and did not completely explain higher adiponectin concentrations in women than in men.

Introduction

The adipose tissue derived hormones adiponectin and leptin may mediate association between obesity and metabolic disease [32, 91-93]. Adiponectin is decreased in individuals with obesity, and may cause insulin resistance and diabetes [94, 95]. Leptin is associated with atherosclerosis via direct effects on endothelial function and inflammation [96].

Women have higher average leptin concentrations than men [97]. Individuals with overweight or obesity have elevated blood concentrations of leptin compared with individuals at normal weight, mainly due to increased depots of subcutaneous fat [93, 98]. Women also have higher adiponectin concentrations than men [97, 99]. Adiponectin is decreased in the presence of excess visceral fat [98]. It is unclear whether sex differences in fat distribution explain the differences in concentrations of leptin and adiponectin between sexes, as previous studies investigated non-specific measures of body fat, or did not take into account skewed distributions of adiponectin and leptin in the analyses [97, 100-103]. In the present study, we aimed to extensively investigate to what extent sex differences in adiponectin and leptin concentrations are explained by sex differences in body fat. Therefore, we investigated the associations of measures of body fat with adiponectin and leptin concentrations.

Materials and Methods

Study design and population

The Netherlands Epidemiology of Obesity (NEO) study is a population-based, prospective cohort study of 6,671 men and women aged between 45 and 65 years. The study design and population are described in detail elsewhere [59]. Men and women with a self-reported body mass index (BMI) of 27 kg/m^2 or higher and living in the greater area of Leiden, the Netherlands were eligible to participate in the NEO study. In addition, all inhabitants aged between 45 and 65 years from one municipality adjacent to Leiden (Leiderdorp, the Netherlands) were invited to participate irrespective of their BMI. Prior to the study visit, participants completed questionnaires at home with respect to demographic, lifestyle, and clinical information. Participants visited the NEO study centre after an overnight fast for an extensive physical examination including blood sampling. In a random subgroup of participants without contraindications (body circumference ≥ 170 cm, implanted metallic devices, or claustrophobia) magnetic resonance imaging (MRI) of abdominal fat was performed. Research nurses recorded current medication use by means of a medication inventory.

The Medical Ethical Committee of the Leiden University Medical Center (LUMC) approved the protocol. All participants gave their written informed consent.

Data collection

Measures of body fat

Height was measured without shoes using a calibrated, vertically fixed tape measure. Body weight and percent total body fat were measured by the Tanita bio-impedance balance (TBF-310, Tanita International Division, UK) without shoes, one kilogram was subtracted to correct for the weight of clothing. Body mass index (BMI) was calculated by dividing body mass in kilograms by body height in meters squared. Total fat mass was calculated by multiplying total body fat with body weight. Waist circumference (WC) was measured halfway between the iliac crest and the lowest rib using a flexible steel tape measure.

Abdominal subcutaneous adipose tissue (aSAT) and visceral adipose tissue (VAT) were quantified by MRI (1.5 Tesla MR imaging, Philips Medical Systems) using a turbo spin echo imaging protocol in a random subgroup. At the level of the fifth lumbar vertebra, three transverse images with a slice thickness of 10 mm were obtained during a breath-hold. The fat areas were quantified by converting the number of pixels to centimetres squared for all three slices. The mean of the three slices was used in the analyses.

Blood sampling and analysis

Glucose, high-density lipoprotein cholesterol, and total cholesterol concentrations were determined in the central clinical chemistry laboratory of the LUMC by using standard methods. Low-density lipoprotein cholesterol was calculated using the Friedewald equation.

Serum adiponectin concentrations were measured using a latex particle-enhanced turbidimetric immunoassay (Cat Nr A0299, Randox Laboratories Limited) on an automated analyzer (Roche Modular P800).

The concentration of leptin was measured in serum with a human leptin competitive RadioImmunoAssay (RIA) (Cat Nr HL-81HK, Merck Millipore, Darmstadt, Germany) . The concentration was counted using a gamma counter (Wizard 2 3470, Perkin Elmer, StatLia software). Coefficients of variation for leptin as determined with internal control materials were calculated based on 22 runs over 105 days and were 12-14% at concentrations between 19 and 55 µg/l.

DNA was extracted from blood and genotyping was performed by the Centre National de Génotypage (Paris, France), using the Illumina HumanCoreExome-24 BeadChip (Illumina Inc., San Diego, CA, USA). Subsequently, genotypes were imputed to the 1000 Genome Project reference panel (v3 2011) using IMPUTE (v2.2) software. [104, 105]

Population characteristics and other variables

Ethnicity was self-identified in the questionnaire and was regrouped into white (reference) and other. Highest completed level of education was reported in ten categories according to the Dutch education system and regrouped in two categories: low education (no education, primary education or lower vocational education) and high education (other). Participants reported the frequency and duration of their physical activity during leisure time using the Short Questionnaire to Assess Health-enhancing physical activity questionnaire and this was expressed in metabolic equivalents hours per week . Smoking status was self-reported. Menopausal state was categorized in pre-, and postmenopausal state according to information on ovariectomy, hysterectomy and self-reported state of menopause in the questionnaire. Carotid intima media thickness (cIMT) was used as a measure of subclinical atherosclerosis. cIMT was assessed by ultrasonography of the common carotid arteries, using a 7.5–10 MHz linear-array probe and the Art.Lab system in B-mode setting and using a wall-track system (ART.LAB version 2.1, Esaote, Maastricht, The Netherlands) [80].

Statistical analysis

In the NEO study individuals with a BMI of 27 kg/m^2 or higher were oversampled. To correctly represent baseline associations in the general population adjustments for the oversampling of individuals with a BMI ≥ 27 kg/m^2 were made [60]. This was done by weighting all participants towards the BMI distribution of participants from the Leiderdorp municipality , whose BMI distribution was similar to the BMI distribution of the general Dutch population [16]. All results were based on weighted analyses. Consequently, the results apply to a population-based study without oversampling of individuals with a BMI ≥ 27 kg/m^2.

In the present analyses, we excluded participants with missing blood samples, as well as participants who used hormone replacement therapy. Analyses with MRI measures as exposure variable were restricted to the participants who underwent MRI.

Descriptive characteristics were summarized as mean (SD), median (25^{th}, 75^{th} percentiles), or as percentage, and stratified by sex. We made scatterplots of the different measures of body fat and adiponectin and leptin concentrations. We observed extreme high leptin concentrations in a small group of women, and we decided to investigate the background of these extreme concentrations further in post-hoc analyses which are described below.

For straightforward comparison, we standardised the values of BMI, total body fat (mass), waist circumference, VAT, and aSAT and calculated z-scores with a mean of zero with a standard deviation of one. Visual inspection of histograms of adiponectin and leptin concentrations indicated that adiponectin and leptin concentration distributions were skewed. Furthermore, scatterplots of adiponectin and leptin concentrations with measures of body fat showed non-linear relations with body fat. To be able to perform linear regression analysis, we transformed adiponectin and leptin concentrations to the natural logarithm.

First, linear regression analyses were performed to examine associations between the standardized measures of body fat and transformed concentrations of adiponectin and leptin. The results were back-transformed and can be interpreted as the relative change per standard deviation of the measure of body fat. We performed all analyses separately for men and women.

Second, we performed linear regression analyses between sex and non-transformed adiponectin and leptin concentrations to assess the absolute difference in adiponectin and leptin concentrations between men and women. Subsequently, to investigate to what extent these absolute sex differences in leptin and

adiponectin concentrations are explained by differences in body fat, we adjusted these absolute differences for the measure of body fat that was most strongly associated with either adiponectin or leptin concentrations.

All crude analyses were adjusted for age, ethnicity, education, smoking status, physical activity, menopausal status, and serum C-reactive protein concentrations. Because abdominal fat is strongly related to total body fat, for the study of specific effects of abdominal fat we additionally adjusted models of VAT for total body fat, and vice versa [54]. Analyses were performed with STATA Statistical Software (Statacorp, College Station, TX, USA), version 14.

Post-hoc analyses in women with high leptin concentrations

Several women were found to have leptin concentrations exceeding 100 µg/l, which have seldom been observed in previous studies [106]. These women all had a body fat percentage in excess of 44%. In an attempt to uncover why these women had such high leptin concentrations, without complaints or clinical symptoms, we performed various post-hoc analyses on this specific group of women. These analyses were not weighted towards a normal BMI distribution. First, we compared demographic and clinical characteristics between women with and without extreme leptin concentrations, further stratified for total body fat. Second, genetic variants may explain extreme leptin concentrations [63, 107]. Therefore, we performed a candidate gene study of the genes coding for leptin (*LEP*), and leptin receptor (*LEPR*), and leptin-associated genes in a recent genome wide association study (*GCKR, CCNL1, SLC32A1, COBLL,* and *FTO*). Because the phenotype was only observed in women, and sex hormones may play a role in leptin expression, we additionally targeted the estrogen and androgen receptor genes *ESR1* and *AR1* [100]. Single nucleotide polymorphisms (SNPs) in the target genes, within 50 000 base pairs up- or downstream of the target genes or in a quantitative trait locus (QTL) according to the GTEx V6p database were indexed [108]. We calculated odds ratios with 95 percent confidence intervals for SNPs in high leptin (≥100 µg/l) compared with normal leptin concentrations (<60 µg/l). *P*-values lower than $5*10^{-5}$ were considered indicative for a suggestive signal. Genetic analyses were performed in white women to avoid admixture, and we additionally excluded women with intermediate leptin concentrations (60-100 µg/l), women with less than 44.5% total body fat, and women with insufficient genotyping quality, or indications of relatedness [109].

Results

After exclusion of 177 participants that did not meet inclusion criteria, 6,494 participants (56% women, of whom 60% were postmenopausal) were included in the present study, with a mean age of 56 (SD: 6) years, and a mean BMI of 26.3 (SD: 4.4) kg/m^2 (Table 1). Of the participants that underwent MRI examination, 52% were women. Women had more total body fat than men, while men had more visceral adipose tissue than women. The median adiponectin concentration in women was 10.5 (IQR: 7.7-13.9) mg/l, in men this was 6.1 (IQR: 4.5-8.2) mg/l. Women had a median leptin concentration of 19.2 (IQR: 11.5-30.0) µg/l, while in men this was 7.1 (IQR: 4.6-11.1) µg/l.

Table 1 – Characteristics of participants in the Netherlands Epidemiology of Obesity (NEO) study (n=6,494), stratified by sex.

	Total population	Men (44 %)	Women (56 %)
Age (y)	56 (6)	56 (6)	55 (6)
BMI (kg/m^2)	26 (4)	27 (4)	26 (5)
Total body fat (%)	32 (9)	25 (6)	37 (7)
Total body fat (kg)	25 (10)	23 (9)	28 (10)
Waist circumference (cm)	92 (13)	98 (11)	87 (13)
Abdominal subcutaneous adipose tissue (cm^2)	235 (97)	209 (81)	259 (104)
Visceral adipose tissue (cm2)	90 (56)	115 (58)	67 (43)
Menopausal status (%)	n.a.	n.a.	60
Diabetes (%)	6	7	4
Cardiovascular disease (%)	6	8	4
Fasting blood concentrations			
LDL cholesterol (mmol/L)	3.5 (1.0)	3.5 (1.0)	3.5 (1.0)
Glucose (mmol/L)	5.5 (1.0)	5.7 (1.1)	5.3 (0.8)
Leptin (µg/L)	12.1 (6.7-22.6)	7.1 (4.6-11.1)	19.2 (11.5-30.0)
Adiponectin (mg/L)	8.3 (5.6-11.9)	6.1 (4.5-8.2)	10.5 (7.7-13.9)

Values are represented as mean (SD), median (25th -75th percentile) or percentage. Results were based on analyses weighted towards a normal BMI distribution (n = 6,494).

BMI, Body mass index; LDL, Low density lipoprotein; SD, standard deviation

Measures of body fat with adiponectin concentrations in men and women

High waist circumference and visceral adipose tissue were associated with reduced adiponectin concentrations, while we observed no association for measures of overall body fat and adiponectin concentrations (Table 2). The strongest association was observed between VAT and adiponectin concentrations, one SD of VAT (56 cm^2) was associated with 0.77-fold reduced adiponectin concentrations (95% CI: 0.75, 0.79). In women, one SD increased VAT was associated with 0.80-fold (95% CI: 0.75, 0.85) reduced adiponectin concentrations, while in men, one SD increased VAT was associated with 0.94-fold (95% CI: 0.90, 0.98) reduced adiponectin concentrations.

Women had 6.1 mg/l (95% CI: 5.6, 6.6) higher serum concentrations of adiponectin than men. After additional adjustment for VAT, the association attenuated but remained 4.4 mg/l (95% CI: 3.5, 5.4) higher adiponectin concentrations than in men (Table 3).

Measures of body fat with leptin concentrations in men and women

All measures of body fat were associated in linear regression analysis with higher leptin concentrations (Table 2). Total body fat was most strongly associated with leptin: per SD increased total body fat (9%), leptin concentrations were 1.89-fold increased (95% CI: 1.79, 1.99). The associations between total body fat and leptin concentrations were similar in men and women.

Mean leptin concentrations in women were 18.6 µg/l (95% CI: 17.6, 19.7) higher than in men, while after adjustment for total body fat this attenuated (0.4 µg/l; 95% CI: -1.2, 2.0) (Table 3).

Table 2 – Regression coefficients of linear regression analysis between measures of body fat and adiponectin and leptin concentrations in the total population (n=6,494), between MRI-determined aSAT and VAT, and adiponectin and leptin concentrations (n=2,516), and for men and women separately.

		Adiponectin (%)			Leptin (%)		
		Relative change	Men (44%)	Women (56%)	Relative change	Men (44%)	Women (56%)
BMI (kg/m2)	Crude	0.86 (0.84, 0.87)	0.88 (0.85, 0.90)	0.89 (0.87, 0.90)	1.65 (1.61, 1.70)	1.92 (1.84, 2.01)	1.69 (1.65, 1.73)
SD: 4 kg/m2	Adjusted	0.97 (0.94, 1.00)	0.98 (0.92, 1.05)	0.98 (0.94, 1.01)	1.17 (1.12, 1.22)	1.24 (1.12, 1.38)	1.16 (1.11, 1.21)
Waist circumference (cm)	Crude	0.80 (0.79, 0.81)	0.89 (0.86, 0.91)	0.86 (0.85, 0.88)	1.31 (1.28, 1.34)	1.87 (1.80, 1.94)	1.68 (1.64, 1.72)
SD: 13 cm	Adjusted	0.91 (0.88, 0.94)	0.98 (0.93, 1.04)	0.88 (0.85, 0.91)	1.17 (1.12, 1.21)	1.34 (1.24, 1.44)	1.08 (1.03, 1.13)
Total body fat (%)	Crude	1.12 (1.10, 1.14)	0.86 (0.83, 0.89)	0.86 (0.83, 0.88)	2.12 (2.07, 2.16)	2.22 (2.09, 2.35)	2.14 (2.07, 2.22)
SD: 9%	Adjusted	0.96 (0.92, 1.00)	0.95 (0.90, 1.01)	1.01 (0.95, 1.07)	1.89 (1.79, 1.99)	1.94 (1.76, 2.14)	1.85 (1.73, 1.98)
Total fat mass (kg)	Crude	0.96 (0.94, 0.98)	0.88 (0.86, 0.91)	0.88 (0.86, 0.90)	2.04 (1.98, 2.09)	1.94 (1.85, 2.03)	1.80 (1.75, 1.85)
SD: 10 kg	Adjusted	0.99 (0.95, 1.02)	0.97 (0.92, 1.02)	1.04 (0.99, 1.10)	1.67 (1.60, 1.74)	1.76 (1.64, 1.89)	1.61 (1.51, 1.71)
aSAT (cm2)	Crude	0.99 (0.96, 1.02)	0.92 (0.88, 0.97)	0.90 (0.87, 0.93)	2.06 (1.98, 2.15)	2.02 (1.88, 2.17)	1.80 (1.73, 1.87)
SD: 97 cm2	Adjusted	1.01 (0.98, 1.04)	1.03 (0.98, 1.08)	1.04 (1.00, 1.08)	1.61 (1.56, 1.66)	1.70 (1.61, 1.80)	1.57 (1.51, 1.64)
VAT (cm2)	Crude	0.77 (0.75, 0.79)	0.89 (0.86, 0.92)	0.79 (0.76, 0.82)	1.24 (1.19, 1.30)	1.61 (1.52, 1.71)	1.90 (1.79, 2.02)
56 cm2	Adjusted	0.89 (0.86, 0.93)	0.94 (0.90, 0.98)	0.80 (0.75, 0.85)	1.18 (1.13, 1.23)	1.18 (1.12, 1.24)	1.17 (1.09, 1.25)

Adjusted: Adjusted for age, sex, total body fat, smoking status, physical activity, type II diabetes, fasting glucose, C-reactive protein concentrations, use of glucose lowering medication

Results were based on weighted analyses (n=6,494 for BMI, waist circumference, total body fat, and total fat mass; n=2,516 for aSAT and VAT)

aSAT, abdominal subcutaneous adipose tissue; BMI, body mass index; VAT, visceral adipose tissue; WC, waist circumference

Table 3. Absolute difference (95% confidence interval) in leptin and adiponectin concentrations between men and women, and adjusted for visceral fat area (adiponectin) or total body fat (leptin).

	Difference in adiponectin concentration (mg/l)		Difference in leptin concentration (µg/l)	
	Adjusted	+ VAT	Adjusted	+ TBF
Men versus women (ref)	6.1 (5.6, 6.6)	4.4 (3.5, 5.4)	18.6 (17.6, 19.7)	0.4 (-1.2, 2.0)

Adjusted: age, total body fat, smoking status, physical activity, type II diabetes, fasting glucose, C-reactive protein concentrations, use of glucose lowering medication and VAT (adiponectin) or TBF (leptin)

Results were based on weighted analyses (n=6,494 for BMI, waist circumference, total body fat, and total fat mass; n=2,516 for aSAT and VAT)

SD, standard deviation; TBF, total body fat

Post-hoc analysis of women with high leptin concentrations

Descriptive characteristics

Forty-four women had leptin concentrations of ≥100 µg/l, combined with total body fat was over 44.5 % (Figure 1). Table 4 shows the characteristics of women stratified by leptin concentrations and total body fat.

Women in the extreme leptin group used more thyroid hormone medication, glucose-lowering drugs, and lipid-lowering medication, and had higher fasting concentrations of glucose, insulin, LDL-cholesterol, and CRP than women in both other groups (Table 4).

Table 4 – Characteristics of 44 female participants in the NEO study with leptin concentrations ≥ 100 µg/l, compared with 3,319 female participants with lower leptin concentrations, stratified by total body fat. A, B, and C correspond with groups plotted in Figure 1.

	A	B	C
	TBF <44.5%	TBF ≥44.5%	TBF ≥44.5%
	Leptin <100 µg/l	Leptin <100 µg/l	Leptin ≥100 µg/l
	n=2,122	n=1,197	n=44
Age (years)	56 (6)	56 (6)	56 (6)
BMI (kg/m2)	28 (4)	35 (5)	42 (7)
Height (cm)	166 (6)	168 (6)	165 (6)
Thyroid hormone use (%)	5	9	16
Glucose lowering drug use (%)	3	7	9
Lipid lowering drug use (%)	9	15	20
Weight at age 20 (kg)	60 (55-65)	65 (60-74)	67 (60-79)
Total body fat (%)	39 (5)	48 (2)	51 (3)
Total fat mass (kg)	30 (7)	47 (9)	59 (15)
VAT (cm2)	78 (41)	136 (52)	136 (25)
Fasting glucose (mmol/l)	5.4 (0.9)	5.9 (1.1)	6.2 (1.5)
Fasting insulin (mmol/l)	8.1 (5.4-11.8)	13.1 (9.4-19.2)	21.8 (14.2-30.3)
Adiponectin (mg/l)	10.0 (7.3-13.3)	8.6 (6.2-11.5)	8.3 (6.2-10.2)
CRP (mg/l)	1.5 (0.8-2.8)	3.3 (1.8-5.9)	4.8 (3.0-8.7)
Leptin (µg/l)	25.2 (16.2-34.9)	45.8 (35.0-58.9)	136.5 (116.5-171.4)
cIMT (µm)	612 (86)	632 (84)	645 (90)

CRP, C-reactive protein; cIMT, carotid intima media thickness; IQR, interquartile range; SD, standard deviation; TBF, total body fat; VAT, visceral adipose tissue.

Values are represented as mean (standard deviation), or median (interquartile range)

Candidate gene study

After exclusion of men (n=3,131), women with leptin concentrations 60-100 µg/l, less than 44.5% total body fat, or who did not meet genotyping criteria (n=2,631), a total of 830 women were analysed in the candidate gene study, of whom 41 had leptin concentrations ≥100 µg/l and 789 had leptin concentrations <60 µg/l. In total, 23,076 SNPs in and in close proximity to the *LEP*, *LEPR*, *GCKR*, *CCNL1*, *SLC32A1*, *COBLL*, *FTO*, *ER* and *AR* genes and 6 cis- and trans-QTL genes

of leptin were indexed. Of the women with leptin concentrations ≥100 µg/l, 45% were heterozygous, and 10% were homozygous for the risk allele of a single genetic variant (rs4731420-G), while in the women with leptin concentrations <100 µg/l, 27% were heterozygous, and 2% were homozygous for the risk allele. This corresponded to an odds ratio of 2.8 (95% CI: 1.7, 4.6, p= $1.70*10^{-5}$) for high leptin concentrations in women (Table 5). The rs4731420 SNP, with a minor allele frequency of 0.16, is located upstream of the *LEP* gene, annotated as *LOC101928423* and is in close linkage with a known SNP that increases the risk of type 2 diabetes (rs791595; D'=1.0). In a further linear regression analysis including all men and women, one risk allele of this SNP was associated with 3 µg/l higher leptin concentrations in women (95% CI: 1, 4), but not in men (-0 µg/l per allele, 95% CI: -1, 1). No other SNPs in the *LEP*, *LEPR*, *ER*, and *AR* genes were associated with leptin concentrations.

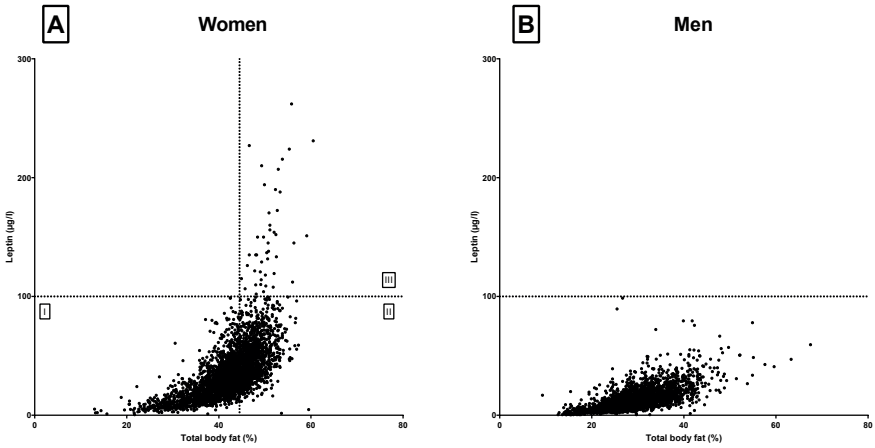

Figure 1 – Scatter plot of total body fat percentage and leptin concentrations in 3379 women (panel A) and 3115 men (panel B) in the NEO study, area III indicates a group of 44 women with leptin concentrations in excess of 100 µg/L, who were further compared with women in areas I and II in Table 4.

Table 5 – Odds ratio of the risk of leptin concentrations ≥100 µg/l in women as compared with leptin concentrations <60 µg/l (reference), related to a genetic variant in proximity to the LEP gene (n=830).

SNP	location	location relative to LEP	p-value	MAF	OR (95% CI)
rs4731420:G	7:127,863,295	-377,906 bp	1.70×10-5	0.161	2.8 (1.7, 4.7)

Analysis performed in 41 women with leptin concentrations ≥100 µg/l and 789 with leptin concentrations <60 µg/l.
GAS, genetic association study; MAF, minor allele frequency; OR, odds ratio; SNP, single nucleotide polymorphism

Discussion

In the present study, we confirmed that total body fat was strongly associated with leptin concentrations. We also confirmed that women had higher leptin concentrations than men, and showed that this sex difference was fully explained by differences in total body fat. Furthermore, we showed that visceral fat was most strongly associated with adiponectin concentrations. We confirmed that women had higher adiponectin concentrations than men, and we also found that this sex difference was not fully explained by differences in visceral adipose tissue. Finally, we observed remarkably high leptin concentrations in 44 women (1.3%) without clinical symptoms, but with high total body fat. A genetic variant in proximity to the *LEP* gene was associated with this phenotype only in women.

Our findings that VAT was more strongly negatively related to adiponectin concentration in women than in men were in line with findings of previous studies showing stronger correlation coefficients between visceral fat and adiponectin in women than in men [97, 99]. This sex difference in adiponectin concentrations may be due to a higher adiponectin mRNA expression in ectopic fat in women than in men [110]. However, this study also found that subcutaneous adipose tissue transcribed more mRNA than ectopic fat tissue, which seems in contrast to existing evidence that the main producer of adiponectin is visceral fat. A potential explanation could be that posttranscriptional regulation plays a major role in the secretion of adiponectin. This posttranscriptional regulation may be affected by androgens or inflammatory cytokines [111-113]. Previous studies also suggested that subcutaneous fat may modulate production of adiponectin by visceral fat [26, 102, 114], but the sex difference in our study remained after adjustment for total body fat. Further research could focus on inflammatory cytokines as a regulatory mechanism for adiponectin production in visceral fat.

In contrast with previous reports, we did not observe a sex difference in the association between total body fat and leptin concentrations [97, 101, 115]. This may in part be due to the inclusion of younger participants in previous studies than in ours, in which a sex difference in the association between total body fat and leptin concentrations may be more notable. Otherwise, the difference may be due to different methods to analyse the sex difference. Most notably, previous studies did not transform leptin concentrations in order to achieve a normal distribution, or used correlation analyses instead of multivariate regression analyses. It remains unclear which method would fit the natural relations most optimally. Sex hormones may affect leptin concentrations, which has been suggested in studies on exogenous sex hormone administration in transgender persons [116, 117].

Our result suggest that a genetic variant is associated with leptin concentrations only in women. To our knowledge, this sex-specific effect has not been described previously. This may indicate that the regulation of leptin expression is to some extent different in men and women. The SNP is located upstream of the *LEP* gene, which may have a regulatory function. A linked SNP, rs791595, has previously been linked to an increased risk of type 2 diabetes [118], suggesting a role for leptin in the development of type 2 diabetes. However, other studies suggest that leptin has a protective effect on the development of type 2 diabetes [119]. Further research is needed to unravel the interrelations between body fat, leptin concentrations, and type 2 diabetes.

The major strength of this study is the direct assessment of visceral fat using MRI, as previous literature related adiponectin specifically with visceral adipose tissue. Further strengths of the present study are the large number of participants with extensive phenotyping of potential confounding factors and leptin and adiponectin concentrations, as well as genotyping.

The present study also has several limitations that need to be considered. First, inherent to the observational cross-sectional design, we are not able to draw conclusions regarding the directionality or causality of the relations between body composition and adiponectin and leptin concentrations. Second, the present study included mainly participants of European ancestry. The associations may be different in people with other ethnic backgrounds. Last, due to the non-normal distribution of adiponectin and leptin concentrations in the study population, concentrations were log-transformed. Interactions between sex and measures of body fat may depend on appropriate transformation. Other studies have used log-transformation [120], quadratic transformation [106], or no transformation [121] in their statistical models, which may explain the difference in conclusions between different studies. However, log-transformation of biomarker data is often appropriate [122].

Conclusion

This study shows that higher concentrations of adiponectin in women than in men may not be completely explained by differences in visceral fat, while the sex dimorphism in leptin was completely explained by the difference in total body fat between women and men. Furthermore, we showed that within a sample of the general population, there are middle-aged women with high total body fat who have apparently asymptomatic extreme leptin concentrations. Our results suggest a role for other factors related to sex, most likely sex hormones, in the regulation of adiponectin and leptin concentrations.

CHAPTER 4

THE RELATION BETWEEN LEPTIN AND (SUB)CLINICAL CARDIOVASCULAR DISEASE

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Submitted

Abstract

Context Leptin has been associated with adverse effects on cardiovascular disease, but the effect of confounding by body fat in these associations remains unclear.

Objective To investigate associations between leptin and heart function and subclinical cardiovascular disease adjusted for total body fat, and to investigate the causal relation between leptin and cardiovascular disease using Mendelian randomisation.

Design and setting Leptin concentrations, total body fat and diverse measures of subclinical cardiovascular disease were determined in participants of the Netherlands Epidemiology of Obesity study. Linear regression between leptin concentration and measures of heart function, ECG measures, and carotid intima media thickness as a measure of subclinical atherosclerosis was adjusted for potential confounding factors, and additionally including total body fat. We analysed the combined effects of genetic variants from a GWAS on leptin concentrations in publicly-available summary statistics of coronary heart disease GWAS (CARDIoGRAMplusC4D, n=184,305).

Participants 6,107 men and women, mean (SD) age 56 (6) years, BMI 26 (4) kg/m^2, and median leptin concentration 12.1 µg (IQR: 6.7-22.6).

Results In observational analyses, leptin was weakly associated with heart function and subclinical cardiovascular disease, but these associations attenuated when adjusting for total body fat. A doubling of genetically-determined leptin concentration was associated with an odds ratio of cardiovascular disease of 0.69 (0.37, 1.27).

Conclusion Observational associations between leptin and subclinical measures of cardiovascular function and disease were largely explained by differences in total body fat. Results of analyses of genetically-determined leptin and coronary heart disease risk were inconclusive due to a large confidence interval.

Introduction

Leptin is a satiety hormone that is derived from adipose tissue, and is important in the feedback loop of central regulation of body weight [123]. There are indications that leptin has a regulatory function in immunity, and vasoconstriction [124-126]. Furthermore, in a meta-analysis on the association between leptin and clinical cardiovascular disease, individuals in the top tertile of leptin concentrations had a 36-50% higher risk of developing cardiovascular disease than those in the lowest tertile, which persisted after adjustment for BMI [91]. While leptin seems detrimental for cardiovascular health in population studies, studies on subclinical markers of cardiovascular disease show conflicting results. Leptin has been associated with detrimental effects on some risk factors for cardiovascular disease, such as decreased muscle mass, hypertension, kidney damage, and increased arterial stiffness with consequent cardiac morphological changes, but also beneficial effects on atherosclerosis and cardiac repolarisation [30-34, 126, 127]. Small human studies have also suggested that an increased sympathetic nervous system activity is a mechanism in the relation between leptin and detrimental outcomes like hypertension, kidney damage, and increased arterial stiffness [31]. However, since leptin is mainly produced by white adipose tissue, a problem in many previous studies is the lack of adequate adjustment for total body fat. Some studies instead adjusted for body mass index (BMI) which is subject to misclassification of body fat due to its relation to height and muscle mass [93]. Therefore, residual confounding may have distorted the results of several previous studies. For this reason, we aimed to study the association between leptin and three domains of subclinical cardiovascular measurements (heart function, electrocardiogram parameters, and subclinical atherosclerosis) while taking the influence of total body fat into account. Furthermore, we aimed to elucidate the causal effect of leptin on clinical cardiovascular disease by a Mendelian randomisation approach.

Methods

Study design and study population

The Netherlands Epidemiology of Obesity (NEO) study is a population-based, prospective cohort study including 6,671 individuals aged 45 to 65 years, in which individuals with overweight or obesity were oversampled. The study design and population have been described in detail elsewhere [59].

Men and women living in the greater area of Leiden (in the West of the Netherlands) were invited by letters and by local advertisements. They were invited to respond if they were aged between 45 and 65 years and had a self-reported body mass index (BMI) of 27 kg/m^2 or higher. In addition, all inhabitants aged between 45 and 65 years from one municipality (Leiderdorp) were invited to participate irrespective of their BMI, allowing for a reference BMI distribution. The present analysis is a cross-sectional analysis using baseline measurements. We excluded participants with a medical history of cardiovascular disease (defined as myocardial infarction, angina, congestive heart failure, stroke, or peripheral vascular disease).

The Medical Ethical Committee of the Leiden University Medical Center (LUMC) approved the design of the study. All participants gave their written informed consent.

Data collection

Participants were invited to a baseline visit at the NEO study centre of the LUMC after an overnight fast. Prior to this study visit, participants completed a general questionnaire at home to report demographic, lifestyle and clinical information. The participants were asked to bring all medication they were using to the study visit. A research nurse registered all medication and dosing. At the baseline visit an extensive physical examination was performed, including anthropometry and blood pressure, and blood samples were drawn from the antecubital vein. At the study centre, participants completed a screening form, asking about anything that might create a health risk or interfere with magnetic resonance imaging (MRI)), most notably metallic devices, claustrophobia or a body circumference of more than 1.70 meter. Of the eligible participants, 2,580 were randomly selected to undergo MRI of the abdomen, of whom a random subset underwent additional cardiac imaging (n=1,207).

Venous blood samples from the antecubital vein were obtained after an overnight fast of >10 hour. Fasting serum total cholesterol, HDL-cholesterol, tri-

glycerides, C-reactive protein, and plasma glucose and insulin were determined in the fasting blood samples at the central clinical chemistry laboratory of the LUMC using standard assays. LDL cholesterol concentrations were calculated using the Friedewald equation. [128] The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as the product of plasma glucose and insulin, divided by 22.5. [129] Leptin concentrations were determined in fasting plasma samples using enzymatic colorimetric reactions with a human leptin competitive RadioImmunoAssay (RIA) [130] (Cat Nr HL-81HK, Merck Millipore, Darmstadt, Germany) and the Roche P800 automated analyser (Roche Diagnostics, Almere, The Netherlands), according to standard laboratory protocols. Analytical total CV's as determined with internal control materials were calculated based on 22 runs over 105 days and was 12-14% at levels between 19 and 55 µg/L. Samples with a concentration >100 µg/L were diluted 1:5 with buffer from the kit.

Furthermore, aliquots of plasma and serum were stored after centrifugation at -80°C. DNA was extracted and genotyping was performed by the Centre National de Génotypage (Evry Cedex, France), using the Illumina HumanCoreExome24 BeadChip (Illumina Inc., San Diego, California, United States of America). Subsequently, genotypes were imputed to the 1000 Genome Project reference panel (v3 2011) using IMPUTE (v2.2) software [61, 131]. Five known genetic variants for leptin were extracted, that were discovered in a BMI-adjusted genome-wide association study for leptin concentrations [63]. We assumed all genetic effects to be additive.

Subclinical cardiovascular outcome measures

Electrocardiographic measurements

A 12-lead electrocardiogram (ECG) was obtained in all participants using a Mortara Eli-350 electrocardiograph (Mortara Instrument Inc., Best, the Netherlands) after a resting period of at least 10 minutes. Standard 10-second ECGs were stored in an 8-lead (I, II, II, V1-V6), 5,000 sample comma-separated-value file. The Kors matrix was used to calculate a vector cardiograms (VCG) from the eight independent ECG leads [14]. ECGs and VCGs were analysed using the automatic MATLAB-based (The MathWorks, Natick, MA) program BEATS and the semiautomatic program LEADS [132, 133]. BEATS was used to detect the timings of all QRS complexes and calculated R-R intervals (ms) and coefficient of variation. All ECGs were checked for falsely identified QRS complexes or nonsinus beats, and the timings were manually adjusted. Also, complexes surrounding the nonsinus beat were removed from the mean R-R interval calculations. Mean HR in beats/min was calculated as 60 divided by the mean R-R interval in

seconds. LEADS was used to calculate QT time (ms), QTc (corrected according to the Bazett formula), and Tpeak-end duration (ms). The QRS and T integral vectors were approximated by calculating the numerical sum of x-y-z deflections (amplitudes of positive deflections are added and those of negative deflections subtracted). The spatial QRS-T angle was defined as the angle (°) between the integral QRS vector and the integral T vector. The spatial ventricular gradient (mV*ms) was calculated as the vectorial sum of these vectors.

Intima media thickness

Carotid intima media thickness (cIMT) was used as a measure of subclinical atherosclerosis. cIMT was assessed by ultrasonography of the common carotid arteries in all participants. A 15 mm long section 10 mm proximal of the carotid artery bifurcation was measured while the subject was in supine position. cIMT was measured using a 7.5–10 MHz linear-array probe and the Art.Lab system in B-mode setting and using a wall-track system (ART.LAB version 2.1, Esaote, Maastricht, The Netherlands) to detect boundaries between lumen and intima, and adventitia. cIMT was measured during six heart beats in angles of 180, 135 and 90 degrees (right CCA) and 180, 225 and 270 degrees (left CCA).

Cardiac imaging

In a random subset of the MRI population (n=1,207), through-plane flow measurements of the ascending, proximal descending, mid-descending, and distal descending aorta were acquired in participants with MRI imaging. Aortic peak wave velocity (PWV) was calculated by dividing the aortic path length between the measurement sites by the transit time between the arrival of the systolic wave front at these sites, and it is expressed in meters per second. The heart was imaged in the short-axis orientation by using electrocardiographically gated breath-hold balanced steady-state free precession imaging to assess ventricular dimensions and mass. An electrocardiographically gated gradient echo sequence was performed with velocity encoding to measure blood flow across the mitral valve to determine diastolic function. Systolic parameters included ejection fraction (EF). Diastolic parameters included peak filling rates of the early filling phase (E) and atrial contraction (A) and their ratio (E/A ratio). Image postprocessing was performed with in-house–developed software packages (MASS and FLOW; Leiden University Medical Center, Leiden, the Netherlands).

UK Biobank

In the UK Biobank, a population-based cohort study of approximately 500,000 individuals from the United Kingdom, who were genotyped, and phenotypic data was collected in subgroups of the study population. Pulse wave velocity was measured in 117,867 genotyped participants using an infra-red sensor at the tip of a finger of the warm hand while the participant was sitting. The sensor detected the blood flow velocity, and recorded the velocity over time. The time between the two peaks of flow velocity was divided by the participant's height to obtain the pulse wave velocity index (PWVi)

Other variables

Blood pressure was measured seated on the right arm with a validated automatic oscillometric device (OMRON, Model M10-IT; Omron Health Care Inc, IL, USA). Three measurements were performed with five-minute rests in between measurements, and the mean systolic and diastolic blood pressure levels were calculated.

Height was measured without shoes using a calibrated, vertically fixed tape measure. Body weight and percent total body fat (TBF) were estimated by the Tanita bio-impedance balance (TBF-310, Tanita International Division, UK) without shoes and one kilogram was subtracted to correct for the weight of clothing. Body mass index (BMI) was calculated by dividing body mass in kilograms by body height in meters squared.

Statistical analyses

Baseline characteristics were presented as means (standard deviation), medians (25th-75th percentile) or percentages. As the NEO study population included an oversampling of participants with BMI of 27 kg/m^2 or higher, we weighted all analyses towards the BMI distribution of participants from the Leiderdorp municipality, who had a similar BMI distribution to the general Dutch population. [16] Consequently, the results apply to a population-based study without oversampling of individuals with a BMI≥27 kg/m^2. For comparison of the results in the figures, all measures of subclinical cardiovascular disease were standardized to a mean of zero and standard deviation of one.

We performed linear regression analyses between leptin and EF and E/A ratio, heart rate and heart rate variability, QRS and P duration, PR and QT interval, P, QRS, and T axis, and carotid intima-media thickness as outcomes. These analyses were adjusted for the potential confounding factors age, sex, smoking sta-

tus, HOMA-IR, triglyceride, glucose, HDL-, and LDL-cholesterol concentrations, blood pressure, use of antihypertensive and glucose and lipid lowering medication. We additionally adjusted the association between leptin and cardiovascular risk factors for total body fat. The regression coefficients can be interpreted as SD difference in the outcome measure per 10 µg/ml difference in leptin concentration. We repeated all analyses stratified by sex.

For the Mendelian randomisation analysis with genetically-determined leptin concentrations, we calculated a weighted genetic risk score including five known genetic variants from a recent genome-wide association study (GWAS) on leptin: rs10487505 (LEP), rs780093 (GCKR), rs900400 (CCNL1), rs6071166 (SLC32A1), and rs6738627 (COBLL1) [63]. The genetic risk score was calculated by multiplying the number of leptin concentration-increasing alleles per variant with the BMI-adjusted per-allele effect of the variant from the genome-wide association study. We estimated the effect of the genetically-determined leptin on the cardiovascular measurements using two-stage least squares regression analysis. The regression coefficients can be interpreted as SD difference in the outcome measure per 10 µg/ml difference in genetically-determined leptin concentration. As for pulse wave velocity, the sample size in the NEO study was limited, we performed additional analyses on publicly-available data on the pulse wave velocity index, as recorded in the UK Biobank. [62] Finally, we estimated the effect of genetically-determined leptin on clinically overt coronary artery disease by combining data on our genetic instruments for leptin with a publicly available database of summary statistics of genome-wide associations for coronary artery disease. Data on coronary artery disease in 60,801 cases and 123,504 controls of European descent have been contributed by CARDIoGRAMplusC4D investigators and have been downloaded from www.CARDIoGRAMplusC4D.org. [134] Of these individuals, 6,688 had also been included in the GWAS of leptin concentrations. [63, 134, 135] For the analyses in the UK Biobank and CARDIoGRAMplusC4D data we used the MR-base application to estimate the inverse-variance weighted effect of our set of BMI-adjusted genetic variants for leptin. [63, 136] To perform this estimation, we performed a weighted regression of the association (beta) between the gene and leptin, and the betas between the gene and the cardiovascular outcome. The regression was weighted towards the inverse of the standard error of the betas, and the regression line was constrained to intersect the origin. The resulting regression coefficient can be interpreted as the change in the outcome per doubling of genetically-determined leptin concentration. One genetic variant (rs10487505, LEP) was not indexed in the CARDIoGRAMplusC4D dataset, therefore we used a proxy in perfect linkage (rs6979832; LD 1.0). [137]

Results

Baseline characteristics

A total of 6,671 participants were included in the NEO study. After exclusion of participants with missing blood samples (n=44), missing values for leptin (n=35), and a history of cardiovascular disease (n=485), data of 6,107 participants of whom 2,844 men and 3,263 women were used for the observational analyses. For cardiac MRI, analyses were performed in 1,060 participants. Further exclusion of related participants, or participants with genetic data of insufficient quality yielded 5,639 participants for the analyses using genetic data. Analyses of the CARDIoGRAMplusC4D dataset were performed in 60,801 cases and 123,504 controls.

Table 1 presents baseline characteristics of the NEO study, stratified by leptin concentration above or below the median (12.1 µg/L). Mean (standard deviation, SD) age was 55 (6) years, and 43% were men. Median (25th -75th percentile) leptin concentrations in men were 6.9 µg/L (4.5- 10.7), and in women 19.0 µg/L (11.4-29.7).

Subclinical cardiovascular parameters

The results of the observational and Mendelian randomisation analyses of the association between leptin and MRI measures of heart function are presented in Figure 1, between leptin and ECG parameters in Figure 2, and between leptin and measures of subclinical atherosclerosis presented in Figure 3. In adjusted observational models, 10 µg/l increased leptin concentration was associated with 0.18 SD (95% confidence interval: 0.13, 0.23) increased right ventricular ejection fraction, and 0.04 SD (95% CI: 0.00, 0.08) increased left ventricular ejection fraction, and weak associations with ECG measures of cardiac conduction with confidence intervals including the null. Additional adjustment for total body fat reduced these and other associations towards the null: 10 µg/L increased leptin concentration became associated with 0.05 SD (95% CI: -0.01, 0.12) of RVEF, and 0.04 SD (95% CI: -0.02, 0.1) of LVEF. All associations were similar in women and men (data not shown). Mendelian randomisation analyses showed no associations between genetically raised leptin and the subclinical markers of cardiovascular disease, and wide confidence intervals.

Table 1 – Characteristics of participants in the Netherlands Epidemiology of Obesity (NEO) study (n = 6,107), stratified by leptin concentration below or above median (12.1 µg/L).

	Total population	Leptin <12.1 µg/L	Leptin ≥12.1 µg/L
Age (y)	55 (6)	56 (6)	55 (6)
Sex (men, %)	43	68	17
BMI (kg/m2)	26 (4)	25 (3)	28 (4)
Total body fat (%)	31 (8)	25 (6)	37 (6)
Smoking (current, %)	16	18	14
Diabetes (%)	5	4	6
Antihypertensive use (%)	20	16	25
Glucose lowering medication use (%)	2	2	3
Lipid lowering medication use (%)	8	7	10
Glucose (mmol/l)	5.4 (1.0)	5.4 (1.1)	5.5 (0.8)
HOMA-IR	1.8 (1.2, 2.9)	1.5 (1.0, 2.3)	2.1 (1.4, 3.4)
HDL cholesterol (mmol/l)	1.6 (0.5)	1.6 (0.5)	1.6 (0.4)
LDL cholesterol (mmol/l)	3.6 (1.0)	3.6 (0.9)	3.6 (1.0)
Systolic blood pressure (mmHg)	130 (17)	131 (17)	130 (17)
Diastolic blood pressure (mmHg)	83 (10)	83 (11)	84 (10)
Carotid intima media thickness (µm)	615 (90)	611 (91)	619 (89)
Genetic risk score leptin	4.51 (1.43)	4.43 (1.44)	4.62 (1.41)
Leptin (µg/l)	12.1 (6.7-22.6)	6.7 (4.5, 9.0)	22.6 (16.0, 32.7)

Values are represented as mean (SD), median (25th -75th percentile) or percentage. Results were based on analyses weighted towards a normal BMI distribution (n = 6,107).

Abbreviations: BMI, Body mass index; HDL, high density lipoprotein HOMA-IR, homeostatic model of insulin resistance; LDL, low density lipoprotein; SD, standard deviation

Publicly-available summary statistics

Analyses using the UK Biobank data indicated that leptin did not causally affect pulse wave velocity: per doubling of genetically-determined leptin concentration, the pulse wave velocity index was 0.02 lower (95% CI: -0.23, 0.18)

Inverse variance weighted regression in publicly-available summary statistics on coronary artery disease (CARDIoGRAMplusC4D) showed that a doubling of ge-

netically determined leptin concentration was associated with an odds ratio of 0.69 (95% CI: 0.37, 1.27) for coronary artery disease.

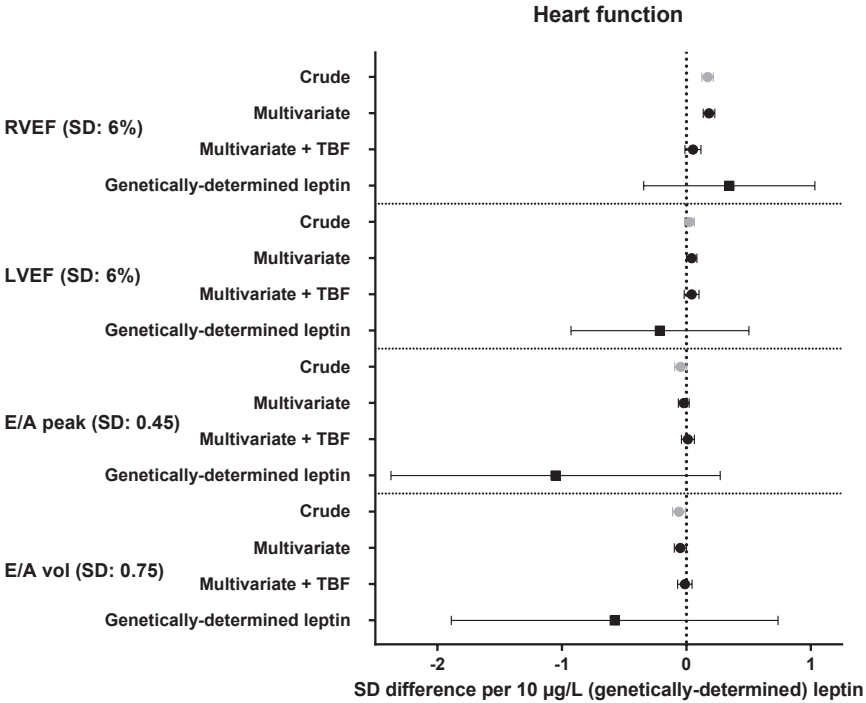

Figure 1 – Standardized differences in MRI measures of heart function with 95 % confidence intervals as associated with observational (circles) and genetically-determined (squares) leptin concentration. Multivariate analyses were adjusted for age, sex, smoking status, HOMA-IR, triglyceride, glucose, HDL- and LDL-cholesterol concentrations, blood pressure, use of antihypertensive and glucose and lipid lowering medication. Multivariate + TBF analyses were additionally adjusted for total body fat. Results were based on weighted analyses (n=1,060). Abbreviations: CI, confidence interval; E/A, early/atrial (filling phase) HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin response; LDL, low-density lipoprotein; LVEF, left ventricle ejection fraction; RVEF, right ventricle ejection fraction; SD, standard deviation; TBF, total body fat.

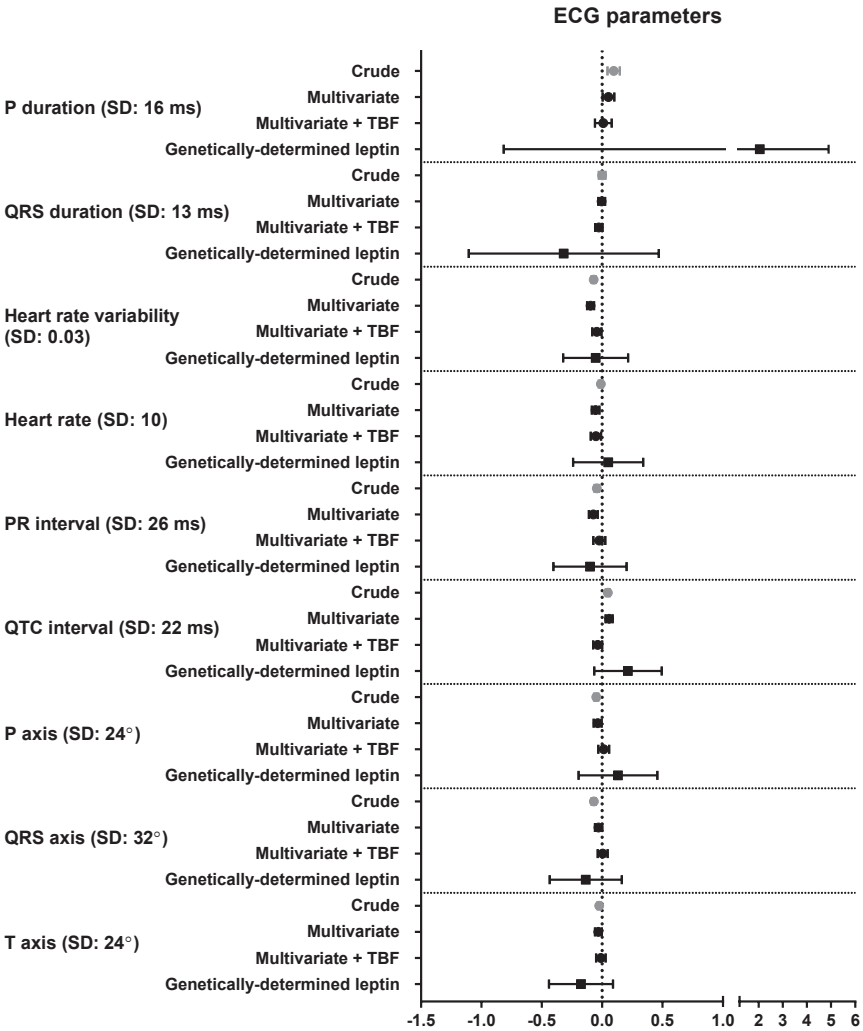

Figure 2 – Standardized differences in ECG parameters with 95 % confidence intervals as associated with observational (circles) and genetically-determined (squares) leptin concentration. Multivariate analyses were adjusted for age, sex, smoking status, HOMA-IR, triglyceride, glucose, HDL- and LDL-cholesterol concentrations, blood pressure, use of antihypertensive and glucose and lipid lowering medication. Multivariate + TBF analyses were additionally adjusted for total body fat. Results were based on weighted analyses (n=6,107).

Abbreviations: CI, confidence interval; ECG, electrocardiogram; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin response; LDL, low-density lipoprotein; SD, standard deviation; TBF, total body fat.

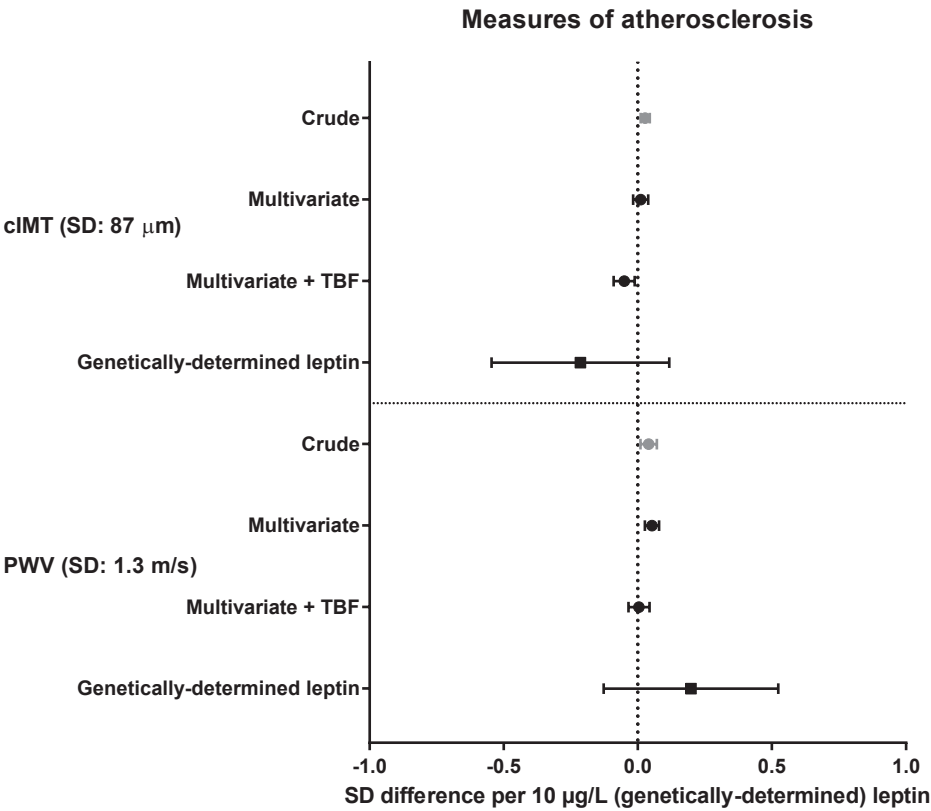

Figure 3 – Standardized differences in measures of atherosclerosis with 95 % confidence intervals as associated with observational (circles) and genetically-determined (squares) leptin concentration. Multivariate analyses were adjusted for age, sex, smoking status, HOMA-IR, triglyceride, glucose, HDL- and LDL-cholesterol concentrations, blood pressure, use of antihypertensive and glucose and lipid lowering medication. Multivariate + TBF analyses were additionally adjusted for total body fat. Results were based on weighted analyses (n=6,107 for cIMT, n=2,320 for PWV). Abbreviations: CI, confidence interval; cIMT, carotid intima media thickness; ECG, electrocardiogram; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin response; LDL, low-density lipoprotein; PWV, peak wave velocity; SD, standard deviation; TBF, total body fat.

Discussion

Our results show that in a well-phenotyped population-based cohort, observational and genetically-determined leptin concentration was not associated with various measures of heart function and subclinical cardiovascular disease. Furthermore, Mendelian randomisation analysis in a large publicly-available database of coronary heart disease indicated no effect in either direction, with large uncertainty, but a strong detrimental effect seems unlikely.

The results of this study extend the current knowledge on the relation between leptin and cardiovascular disease, since we investigated these associations with adjustment for total body fat, which has been done in only a few studies. [91] We have shown that multivariable observational associations between leptin and subclinical measures of cardiovascular disease disappeared after adjustment for the main producer of leptin, total body fat. In the light of literature on leptin and cardiovascular disease, there are two potential explanations for the discrepancy between observational studies that consistently show an association between leptin concentration and several cardiovascular parameters. [30-34]

First, adipose tissue may act as a confounding factor in the study of the association between leptin and cardiovascular parameters, and was previously not adequately adjusted for. In this case, leptin is produced by adipose tissue, which is a cause of cardiovascular risk via other mechanisms. [138] Based on previous research, adipose tissue predominantly affects cardiovascular parameters through mechanisms that do not involve leptin, such as insulin resistance and diabetes [18] Also, previous studies often adjusted for body mass index instead of total body fat. [139] Body mass index is partly determined by lean mass, which is regarded as a cardioprotective factor, in part because of insulin sensitizing properties of muscle mass. Adjusting for body mass index therefore does not completely solve the confounding by total body fat, and leaves residual confounding. The role of high leptin concentrations that are due to leptin resistance is unclear, but by adjustment for total body fat we attempted to correct for this as well. Also, the possibility exists that leptin is a mediator of the effect of body fat on cardiovascular disease. However, if this were true, any remaining causal effect of leptin on cardiovascular disease parameters would not disappear by adjustment for total body fat. Therefore our analyses would still have been able to identify such effects.

Second, the previously observed associations between leptin and cardiovascular parameters might be due to reverse causation, i.e., worsening of these cardiovascular parameters may affect leptin concentrations when subclinical disease leads to weight change. Therefore, in addition to observational analyses adjust-

ed for total body fat, we used a Mendelian randomisation approach specifically to mitigate problems due to unmeasured confounding or reverse causation. In our Mendelian randomisation analyses we observed estimates of the effect of leptin on measures of subclinical cardiovascular disease close to the null, some of which with broad confidence intervals. This leaves the possibility of mostly small effects that may be observed in larger populations, but for most outcome measures this indicates the absence of large clinically relevant effects. The results of the CARDIoGRAMplusC4D GWAS suggested that if there is an effect of leptin on clinical cardiovascular disease, it is likely to be beneficial. However, this study showed large uncertainty despite a large population and reasonably strong genetic instruments. Therefore, this study should be repeated when stronger genetic instruments become available. It is unlikely that this study could be repeated in a larger cardiovascular disease cohort in the near future.

Potential beneficial effects of leptin may arise downstream of its receptor, which further signals via the JAK-STAT3 pathway. [140] This pathway is pivotal in regulation of gene expression and apoptosis, and small alterations in activation of this pathway may lead to diverse effects on homeostasis. [141] The main effect of leptin remains on the regulation of energy expenditure and body weight, but leptin may also have off-target effects that could be beneficial with regard to cardiovascular disease. [142]. Previous studies have related leptin to inhibition of the angiotensin receptor in vascular smooth muscle cells [126], myocardial de- and repolarisation [127], and decreased atherosclerosis [143] in tissue culture and rodents, and to slightly improved heart function in a population-based study [144]. This is partly reflected in our results, indicating small effects on subclinical measures of cardiovascular disease in both adverse and beneficial directions. However, these effects were small and therefore may have a negligible impact on clinical cardiovascular risk.

The major strength of this study is the large number of well-phenotyped participants from the general population. Furthermore, the use of genotyping allowed us to study causal associations as well. However, this study also has some limitations. First, the cross-sectional observational analyses may be subject to residual confounding and reverse causation. Since our population was genotyped, we were able to perform additional genetic analyses to validate our initial findings without the risk of unmeasured confounding. However, in genetic studies the risk of pleiotropy remains, i.e. the genetic variants may affect the outcome via other mechanisms than the exposure. A second potential limitation of genetic studies on leptin may be the possible introduction of collider stratification bias, that might have been caused by the adjustment for BMI of the genome-wide association study on leptin. [63] By correcting for BMI, the remaining significant in-

struments may have become associated with BMI. Therefore these instruments may affect measures of subclinical cardiovascular disease through BMI instead of through leptin. However, the loss of genome-wide significance of the variant in the *FTO* gene after adjustment for BMI indicates that at least a part of the BMI-mediated effects may be adjusted away. In addition, we presented mostly null findings. An effect of BMI would potentially be away from the null. Third, a disadvantage of genetic studies is the large number of participants needed in the presence of a weak genetic instrument, such as in the present study. Even the analyses using publicly-available summary data may be inadequately powered, as the broad confidence intervals suggest. Also, these data do not provide sex-stratified statistics, therefore analyses separate for women and men are not yet possible. However, a larger dataset on cardiovascular disease is not yet publicly available, therefore the current analysis is the most precise analysis we could have performed. A potential limitation of the analyses of publicly-available data is that the populations in which the GWAS on leptin was performed and CARDIoGRAMplusC4D partly overlap. Overlap is mainly a problem when weak instruments are used in strongly overlapping studies. However, the present study used strong genome-wide significant genetic instruments with a small overlap of 3.6 %, therefore this sort of bias is unlikely to affect our results. [135]

In summary, our results show that observationally, leptin concentration was associated with various measures of heart function, but appropriate adjustment for total body fat reduced these associations towards the null. These results are supported by Mendelian randomisation analyses in the same study. However, Mendelian randomisation analysis of the causal relation between leptin and coronary heart disease in a large publicly-available dataset suggested large uncertainty in the effect of leptin on coronary heart disease.

CHAPTER 5

MENDELIAN RANDOMIZATION STUDY OF THE RELATION BETWEEN ADIPONECTIN AND HEART FUNCTION, UNRAVELLING THE PARADOX

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In preparation

Abstract

High adiponectin concentrations are generally regarded as beneficial with regard to cardiometabolic health, but have been paradoxically associated with increased cardiovascular disease risk, specifically heart failure, in individuals at high cardiovascular risk. We aimed to investigate the association between adiponectin and heart function parameters, and inversely, we estimated the effect of genetically-determined heart function and NT-proBNP as the main marker of heart failure on adiponectin using Mendelian randomisation.

Observational analyses between adiponectin and measures of heart function, i.e. E/A ratio, left, and right ventricular ejection fraction, were performed in participants of the Netherlands Epidemiology of Obesity (NEO) study, assessed by MRI of the heart (n=1,138). Two-sample Mendelian randomisation analyses were conducted to estimate the effect of NT-proBNP and heart function on adiponectin concentrations using publicly-available summary statistics (ADIPOGen; the PLATO trial).

The mean (standard deviation) age was 56 (6) years and mean body mass index was 26 (4) kg/m^2. Per five µg/mL higher adiponectin, the E/A ratio was -0.05 (95% CI: -0.10, -0.01) lower, left ventricle ejection fraction was -0.5% (95% CI: -1.1, 0.1) lower, and right ventricle ejection fraction was 0.5% (95% CI: -0.1, 1.2) higher. Genetically-determined NT-proBNP was causally related to adiponectin concentrations in ADIPOGen: per doubling of genetically-determined NT-proBNP, adiponectin concentrations were 11.4% (95% CI: 1.7, 21.6) higher.

With causal MR methods we showed that NT-proBNP affects adiponectin concentrations, while adiponectin is not associated with heart function parameters. Therefore, reverse causation may explain the adiponectin paradox observed in previous studies.

Introduction

Adiponectin is a protein that is produced by adipocytes, and is negatively associated with ectopic fat depots. [145] Serum adiponectin concentrations are decreased in individuals with overweight or obesity, and in particular in individuals with increased visceral adipose tissue (VAT) depots. [93, 145, 146] Several studies suggest that adiponectin has beneficial effects on lipid metabolism, endothelial function and obesity-related low-grade inflammation. [147-151] Also, adiponectin is suggested to have insulin sensitizing properties and to be protective against type 2 diabetes mellitus. [21, 28, 147, 148, 152, 153] However, recent Mendelian randomization studies indicated the absence of a causal relation between adiponectin and type 2 diabetes, and coronary heart disease. [154, 155] This contradicts the findings that high adiponectin levels are associated with an increased risk of heart failure in large observational cohort studies of 3,263 and 5,574 individuals. [156, 157]

Several explanations for this so-called 'adiponectin paradox' have been explored in previous studies. One potential explanation is that previous studies investigated the association between adiponectin and cardiovascular disease in a population that was selected for a high risk of cardiovascular disease, which could lead to selection bias. [158] But more likely, previous results may be explained by reverse causation, i.e. subclinical cardiovascular disease, specifically heart failure, may have affected adiponectin concentrations. In this explanation, the strong heart failure marker N-terminal-pro-brain natriuretic peptide (NT-proBNP) may cause an increase in adiponectin production and serum concentrations. [159-162]

Mendelian randomization (MR) is an effective method to investigate the causality and causal direction of an observed association, because instead of an observed exposure, the genetic predisposition for an exposure is analyzed. This genetic predisposition is set at conception, which excludes the possibility that the outcome or a confounder affects the genetic exposure. [163] Therefore, MR may be a useful tool to investigate the adiponectin paradox.

In this study, a two-sample MR strategy was employed to unravel the directionality and mechanisms of the relations between adiponectin and cardiac function. We used subclinical measures of heart function in this study to reduce the possibility of an 'index event' on which previous studies have selected, which would limit the possibility of collider bias. [164] First, we aimed to investigate the effect of adiponectin on heart function determined by magnetic resonance imaging in a general population using observational analyses. Our second aim was to investigate the reverse causal pathway by determining the effect of genetical-

ly determined heart function on adiponectin concentrations using a two-sample MR strategy in two large publicly-available datasets. Thirdly, we aimed to investigate a specific mechanism in the reverse pathway by determining the effect of genetically-determined NT-proBNP concentrations on adiponectin concentrations using two-sample MR in large publicly-available datasets.

Methods

Study design and study population

The first part of this study is a cross-sectional analysis of the Netherlands Epidemiology of Obesity (NEO) study, a population-based, prospective cohort study of 6,671 men and women aged between 45 and 65 years. The study design and population are described in detail elsewhere. [59] All inhabitants with a self-reported body mass index (BMI) of 27 kg/m^2 or higher and living in the greater area of Leiden, the Netherlands were eligible to participate in the NEO study. In addition, all inhabitants aged between 45 and 65 years from one adjacent municipality (Leiderdorp, the Netherlands) were invited to participate irrespective of their BMI, allowing for a reference distribution of BMI. Prior to the study visit, participants completed questionnaires at home with respect to demographic, lifestyle, and clinical information. Participants visited the NEO study centre after an overnight fast for an extensive physical examination including blood sampling. In a random subgroup of participants without contraindications (body circumference ≥ 170 cm, implanted metallic devices, or claustrophobia) magnetic resonance imaging (MRI) of abdominal fat was performed. Research nurses recorded current medication use by means of a medication inventory.

For the present analyses, we included 1,150 randomly selected participants without contra-indications for MRI. For observational analyses, we excluded 12 participants with missing adiponectin concentration, and consequently 1,138 participants were included in the present study. For genetic analyses, we additionally excluded related participants, participants with poor genotyping quality and of non-European descent (n=132). [165]

The Medical Ethical Committee of the Leiden University Medical Centre (LUMC) approved the protocol. All participants gave their written informed consent.

Further analyses presented in this study used genetic instruments discovered in genome-wide association studies on NT-proBNP concentrations [64, 65], and publicly-available summary statistics of genome-wide association studies on measures of heart function [66] and on adiponectin [64], available in GRASP. [166]

Blood sampling

During the visit to the NEO study centre, venous blood samples were obtained from the antecubital vein after a >10 hour overnight fast. Fasting serum total cholesterol, HDL-cholesterol, triglycerides, C-reactive protein, and plasma glu-

cose and insulin were determined in the fasting blood samples at the central clinical chemistry laboratory of the LUMC using standard assays. LDL cholesterol concentrations were calculated using the Friedewald equation. [128] The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as the product of fasting glucose and insulin concentrations divided by 22.5. [129] Furthermore, aliquots of plasma and serum were stored after centrifugation at -80°C. Serum adiponectin concentration was determined in a fasting blood sample that had undergone one previous freeze-thaw cycle using a turbidimetric immunoassay (Cat Nr A0299, Randox Laboratories Limited, United Kingdom) on an automated analyser (Roche Modular P800, Roche, Switzerland). Samples with a concentration above 28 µg/L were diluted 2x with water before re-analysis. Analytical variation was calculated based on 88-94 runs over 35 days and was 2.8% at a level of 1.9 mg/L and 1.9% at a level of 11 mg/L using a commercial serum based unassayed control material (Randox Immunoassay Level 1, Randox Laboratories Limited, United Kingdom) and a human pooled serum.

Heart function

In a random sample of the NEO study population without contra-indications for MRI (n=1,150), the heart was imaged in the short-axis orientation by using electrocardiographically gated breath-hold balanced steady-state free precession imaging by magnetic resonance imaging (MRI, 1.5 Tesla MR imaging, Philips Medical Systems) to assess left ventricle (LV) and right ventricle (RV) dimensions and mass. An electrocardiographically gated gradient-echo sequence was performed with velocity encoding to measure blood flow across the mitral valve to determine diastolic function. We used the ejection fraction (EF) as the systolic parameter. Diastolic parameters included peak filling rates of the early filling phase (E) and atrial contraction (A) and their ratio (E/A ratio). Image postprocessing was performed using in-house–developed software packages (MASS and FLOW; Leiden University Medical Center, Leiden, the Netherlands).

Genotyping and selection of genetic instruments

As instruments for measures of LV function, we selected 12 genetic variants from a GWAS that was performed in 44,203 primarily Caucasian individuals included in studies in the EchoGen consortium, in which analyses were adjusted for age, sex, height, weight, and study site (when applicable). [66] Instruments for NT-proBNP were selected from a GWAS on NT-proBNP that was performed in 9,232 patients with acute coronary syndrome of whom 99% were of European descent, in which analyses were adjusted for the first four principal components of ancestry. [65] The reported values for explained variance did not exceed 1% for any of the phenotypes of interest. We extracted the effect estimates (betas)

and their standard errors of the instruments for heart function and NT-proBNP on adiponectin concentrations from summary statistics of the ADIPOGen consortium (n=29,347) that were made publicly available via GRASP. [64-66, 166] Therefore we extracted the summary statistics for the variants rs806322, rs6702619, rs17696696, rs7127129, rs17608766, rs2649, rs4765663, rs11207426 (aortic diameter), rs12541595, rs10774625 (left ventricular diastolic internal dimension), rs1454157 (left ventricle mass), and rs9470361 (fractional shortening), and rs13107325, rs198389, and rs4842653 (NT-proBNP) from the ADIPOGen summary data. For aortic diameter, the variant rs806322 was dropped during harmonisation of the exposure and outcome data due to ambiguous alleles, leading to the use of 7 variants. The variant rs4842653 was used as a proxy (D': 1.0, R^2:1.0) for the original variant rs11105306 from the GWAS on NT-proBNP, as this variant was not available in the ADIPOGen summary data. The overlap between the GWAS for LV function and the ADIPOGgen data was 35% (of the ADIPOGen data), and 0% for the GWAS of NT-proBNP.

Other variables

Self-reported level of education was classified into low (none, primary school or lower vocational education) or high (other). Participants reported their medical history of diabetes and cardiovascular disease. Pre-existing cardiovascular disease was defined as myocardial infarction, angina pectoris, congestive heart failure, stroke, or peripheral vascular disease. Diabetes was defined as self-reported diabetes, use of glucose-lowering medication, or fasting plasma glucose concentrations of 7.0 mmol/L or higher. Tobacco smoking was reported in three categories: never smoker, former smoker or current smoker. Participants reported their physical activity during leisure time, which was expressed in metabolic equivalent hours per week.

Total body fat (%) was estimated using a bio-impedance device (TBF-310, Tanita International Division, UK). Visceral adipose tissue area (VAT) was quantified by MRI using a turbo spin echo imaging protocol in all participants with a cardiac MRI.

Statistical analysis

In the NEO study, individuals with a BMI of 27 kg/m^2 or higher were oversampled. To correctly represent associations in the general population adjustments for the oversampling of individuals with high BMI were made. [60] This was done by weighting all participants towards the BMI distribution of participants from the Leiderdorp municipality, [167] whose BMI distribution was similar to the BMI distribution of the general Dutch population. [16] All results were based

on weighted analyses. Consequently, the results apply to a population-based study without oversampling of individuals with a BMI ≥ 27 kg/m^2.

Data were summarized by sex as mean (SD; normally distributed data only), median (25^{th}, 75^{th} percentiles; non-normally distributed data only), or as percentage (categorical data).

First, for our observational analyses within the NEO study, we used linear regression to estimate the associations between adiponectin concentration and heart function, and their 95% confidence intervals. Analyses were performed crude, and adjusted for potential confounding factors age, sex, total body fat, visceral fat, smoking status, HOMA-IR, triglyceride and C-reactive protein concentrations, use of antihypertensive and glucose lowering and lipid lowering medication.

Second, we conducted two-sample Mendelian randomization analysis to quantify the causal effects of NT-proBNP on adiponectin concentrations, and of left ventricle function on adiponectin in publicly-available summary statistics.

The effects of the three SNPs for NT-proBNP on adiponectin in publicly-available data were estimated using weighted linear regression of the SNP-NT-proBNP associations (betas) on the SNP-adiponectin betas in the R package *TwoSampleMR*. [168]. The regression was weighted towards the inverse of the standard error of the SNP-adiponectin betas, and the intercept was constrained to zero. Because both NT-proBNP and adiponectin have been log-transformed to the natural logarithm in the original GWASs, we reported coefficients that can be interpreted as the percentage change in adiponectin concentration per doubling of genetically-determined NT-proBNP concentration. Similarly, the effects of the variants for aortic root diameter (n=7), LV diastolic internal diameter (n=2), LV mass (n=1), and factional shortening of the LV (n=1) on adiponectin concentration were estimated using weighted regression (aortic root diameter, LV diastolic internal diameter) or Wald estimation (LV mass, fractional shortening). The resulting coefficients can be interpreted as the percentage difference in adiponectin concentration per unit of the heart function parameter.

Results

Baseline characteristics

Table 1 shows baseline characteristics of participants (47% men) with a mean age of 56 years (standard deviation, SD: 6), a BMI of 26 kg/m^2 (SD: 4) and of whom 22% were using antihypertensive medication. The mean LVEF was 64 % (SD: 6) and the mean E/A ratio was 1.26 (0.46) in men and 1.37 (0.52) in women. Adiponectin concentrations were higher in women (11.2 µg/ml, SD: 5.0) than in men (6.5 µg/ml, SD: 2.8). Baseline characteristics of the subpopulation with cardiac MRI were comparable with those of the total population (data not shown).

Observational associations between adiponectin and heart function

The associations between adiponectin concentration and E/A ratio and LV systolic function are presented in Table 2. In adjusted analysis, adiponectin was associated with a -0.05 (95% confidence interval: -0.10, -0.01) lower E/A ratio. We observed no further associations between adiponectin and LVEF and RVEF, nor that the associations differed between women and men (data not shown).

Table 1 – Baseline characteristics of participants of the NEO study that underwent MRI examination of the heart (n=1,138).

	Total population	Men (47 %)	Women (53 %)
Demographic/anthropometric			
Age (y)	56 (6)	56 (6)	56 (6)
Total body fat (%)	31 (8)	25 (6)	37 (6)
BMI (kg/m2)	26 (4)	27 (3)	26 (4)
Visceral adipose tissue (cm2)	92 (56)	117 (58)	71 (44)
Tobacco smoking (% never)	42	41	44
Alcohol intake (g/d)	10 (2-22)	17 (4-29)	8 (1-15)
Total energy intake (MJ/d)	9.5 (3.1)	10.8 (3.2)	8.3 (2.5)
Physical activity (METhours/week)	32 (17-53)	34 (17-54)	30 (16-52)
Education level a (% high)	47	50	44
Biomarkers			
Total cholesterol (mmol/L)	5.7 (1.1)	5.6 (1.0)	5.9 (1.1)
Triglycerides (mmol/L)	1.3 (0.8)	1.5 (0.9)	1.1 (0.6)
Fasting glucose (mmol/L)	5.5 (1.0)	5.7 (1.2)	5.3 (0.9)
Fasting CRP (mg/L)	1.2 (0.6-2.3)	1.1 (0.5-1.9)	1.3 (0.7-2.8)
Adiponectin (µg/mL)	9.0 (4.7)	6.5 (2.8)	11.2 (5.0)
Comorbidity and medication			
Diabetes b (%)	4	5	3
Glucose lowering medication (%)	3	4	2
Lipid lowering medication (%)	10	14	6
Antihypertensive medication (%)	22	22	23
Measures of heart function			
LV Ejection fraction (%)	64 (6)	63 (7)	64 (5)
RV Ejection fraction (%)	55 (6)	53 (6)	57 (6)
E/A ratio	1.32 (0.49)	1.26 (0.46)	1.37 (0.52)

BMI, body mass index; CRP, C-reactive protein; E/A ratio, early/atrial filling ratio; LV, left ventricle; MET, metabolic equivalents of task; MJ, megajoule; MRI, magnetic resonance imaging; NEO, Netherlands Epidemiology of Obesity;

Results are based on analyses weighted towards the BMI distribution of the general population (n = 1,138). Data are shown as mean (SD), median (IQR) or percentage.

a; Low education: none, primary school or lower vocational education as highest level of education
b; Self-reported diabetes or use of glucose-lowering medication or insulin

Associations between genetically-determined measures of left ventricular function, NT-proBNP and adiponectin

We observed wide confidence intervals around the associations between genetically-determined measures of left ventricular function and adiponectin concentrations in the publicly-available summary statistics of genetic variants on adiponectin concentrations (Table 3). However, we observed that a doubling of genetically-determined NT-proBNP was associated with 11.4 (95% CI: 1.7, 21.6) increased adiponectin concentrations.

Table 2 – Results of linear regression analyses to estimate the difference in MRI measures of heart function (ratio or percentage, 95% confidence interval) per 5 µg/mL difference in adiponectin concentration in men and women participating in the Netherlands Epidemiology of Obesity study (n=1,138).

	Per 5 µg/mL adiponectin concentration
E/A ratio	
Crude	-0.00 (-0.04, 0.04)
Multivariate	-0.05 (-0.10, -0.01)
LVEF (%)	
Crude	0.1 (-0.3, 0.5)
Multivariate	-0.5 (-1.1, 0.1)
RVEF (%)	
Crude	1.1 (0.5, 1.6)
Multivariate	0.5 (-0.1, 1.2)

Results are based on analyses weighted towards the BMI distribution of the general population (n=1,138) and presented as difference in MRI measure of heart function per 5 µg/mL of adiponectin concentration. Multivariate: age, sex, total body fat, visceral fat, smoking status, type II diabetes, fasting glucose, triglyceride and C-reactive protein concentrations, use of antihypertensive and glucose lowering and lipid lowering medication.

E/A ratio, early/atrial filling phase ratio; LVEF, left ventricular ejection fraction; MRI, magnetic resonance imaging; NEO, Netherlands Epidemiology of Obesity study; RVEF, right ventricular ejection fraction; SD, standard deviation

Table 3 – Two-sample Mendelian randomization analysis using inverse variance weighted regression or Wald estimation in summary statistics of adiponectin in ADIPOGen (n=24,044-29,347).

Per exposure	% difference in adiponectin (95% CI)	n (SNPs)	Method
NT-proBNP (doubling)	11.4 (1.7, 21.6)	3	IVW
Aortic root diameter (cm)	9.8 (-10.1, 34.1)	7	IVW
LV diastolic internal dimension (cm)	-26.0 (-54.9, 21.3)	2	IVW
LV mass (g)	0.2 (-0.5, 0.9)	1	Wald
Fractional shortening (%)	5.2 (-1.0, 11.9)	1	Wald

Results are presented as percentage difference in adiponectin concentration per exposure.
CI, confidence interval; IVW, inverse variance weighting; LV, left ventricle; NT-proBNP, N-terminal-pro-brain natriuretic peptide; SNP, single nucleotide polymorphism

Discussion

In this study, we combined observational analyses with two-sample Mendelian randomisation analyses in order to unravel the previously described adiponectin paradox. We observed a weak association between higher adiponectin concentrations and lower E/A ratio in our observational analyses, but no associations with left and right ventricular function. Genetic determinants of left ventricular function did not affect adiponectin concentrations directly. Notably, our results indicated a causal relation between NT-proBNP and serum adiponectin concentrations.

The results of our observational analyses of the associations between subclinical measures of heart function and adiponectin in a general population of the NEO study are discordant with previous studies that found an observational association between high adiponectin concentrations and a higher risk of developing heart failure in populations at high-cardiovascular risk. [148, 152, 157]

We observed an association between genetically-determined NT-proBNP and adiponectin concentrations. This confirmed the hypothesis that was generated by previous studies, and may well explain the paradoxical findings in previous observational studies. [157, 169, 170] NT-proBNP strongly affected adiponectin concentrations, as for a doubling of NT-proBNP, adiponectin concentrations increased by 11.4%. A doubling of NT-proBNP is clinically relevant as NT-proBNP concentrations may range by 5-fold between the median and the 97.5th percentile in middle-aged men. [171] This finding gives additional insight in the intricate neurohumoral regulation of heart function and its feedback mechanisms. Adiponectin may serve as a downstream effector as a response of the heart to increased cardiac burden by increasing vascular compliance. This is supported by studies that show a reduction in vascular tone and anticontractile effects as a consequence of increased adiponectin. Therefore further studies into the effects of adiponectin on vascular function may be of large importance. [172]

The main strength of our study is that we investigated the associations of interest in an observational study with participants who were not at high cardiometabolic risk. Thereby we aimed to mitigate the effects of reverse causation. Furthermore, previous studies that have selected a population at high risk of the outcome may have introduced a selection bias: collider stratification bias. Collider stratification bias is the phenomenon that the association between two causes of one disease is biased in the diseased population, or in a population at high risk of the disease. [159, 164] In this case, as a marker of visceral fat, adiponectin may seem a risk factor in a selected population already at high risk of cardiovascular disease, because due to the selection it may become inversely

associated with other strong risk factors for heart failure, such as smoking or hyperlipidaemia. As a consequence, the high adiponectin concentrations accompanying low visceral fat may be paradoxically associated with high risk of mortality in individuals selected on their high risk of mortality. In our study, we aimed to decrease the risk of collider stratification bias by including individuals from the general population and using subclinical variation in measures of heart function. A third strength of the present study is the focus on subclinical measures of heart function instead of clinical cardiovascular disease. In general, our observational results suggest that adiponectin is not associated with heart function except for a marginal association with E/A ratio. However, this association may still be explained by residual confounding. While previous studies suggested that adiponectin has modest effects on heart function through beneficial effects on insulin sensitivity and endothelial function, our results do not support this hypothesis. [28, 147, 151, 173, 174]

This study also has limitations. First, publicly-available summary statistics did not include sex-specific results. Large differences between women and men exist with regard to prevalence of risk factors for cardiovascular disease, in particular visceral fat and adiponectin, and also in markers like NT-proBNP. [146] Therefore, the possibility remains that effects of adiponectin on heart function or vice versa are different between women and men. Second, due to the limited number of genetic variants, it was not possible to perform sensitivity analyses such as MR-Egger and median- or modal-estimator methods. [175]

In conclusion, we observed no associations between adiponectin concentrations and measures of heart function, and no reverse causal relation, but we showed that a potential explanation for the adiponectin paradox could be that NT-proBNP raises circulating adiponectin concentrations.

SECTION II

LIPIDS

CHAPTER 6

MENDELIAN RANDOMISATION ANALYSIS OF CHOLESTERYL ESTER TRANSFER PROTEIN AND SUBCLINICAL ATHEROSCLEROSIS: A POPULATION-BASED STUDY

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Abstract

Background

Several trials to prevent cardiovascular disease by inhibiting cholesteryl ester transfer protein (CETP) have failed, except REVEAL. Thus far, it is unclear to what extent CETP is causally related to measures of atherosclerosis.

Objective

To study the causal relation between genetically-determined CETP concentration and carotid intima media thickness (cIMT) in a population-based cohort study.

Methods

In the Netherlands Epidemiology of Obesity study, participants were genotyped, and cIMT was measured by ultrasonography. We examined the relation between a weighted genetic risk score for CETP concentration, based on 3 SNPs that have previously been shown to largely determine CETP concentration and cIMT using Mendelian randomisation in the total population and in strata by sex, Framingham 10-year risk, (pre)diabetes, HDL-cholesterol, triglycerides and statin use.

Results

We analysed 5,655 participants (56% women) with a mean age of 56 (range 44-66) years, BMI of 26 (range 17-61) kg/m^2 and serum CETP of 2.47 (range 0.68-5.33) µg/mL. There was no evidence for a causal relation between genetically-determined CETP and cIMT in the total population, but associations were differently directed in men (16µm per µg/mL increase in genetically-determined CETP; 95% CI: -8, 39) and women (-8 µm; -25, 9). Genetically-determined CETP appeared to be associated with cIMT in normoglycemic men (26 µm; -1, 52) and in (pre)diabetic women (48 µm; -2, 98).

Conclusion

In this population-based study, there was no causal relation between genetically-determined CETP concentration and cIMT in the total population, although we observed directionally differing effects in men and women. Stratified results suggested associations in individuals with different cardiometabolic risk factor profiles, which require replication.

Introduction

Recent studies to improve cardiovascular risk prevention have focussed on cholesteryl ester transfer protein (CETP) inhibitors since they increase HDL-c and decrease non-HDL-c concentrations. [20, 176, 177]

CETP facilitates the migration of cholesteryl esters from HDL to LDL and very low-density-lipoproteins (VLDL). A high CETP concentration is therefore hypothesized to contribute to an atherogenic lipoprotein profile by increasing (V) LDL-c and decreasing HDL-c.[178] Several observational studies have suggested that lower concentrations of CETP are associated with reduced CVD risk. [179, 180] Most recent efforts to lower CETP concentration pharmacologically with the purpose of reducing CVD risk have been unsuccessful, except for the REVEAL trial, in which CETP inhibition with anacetrapib successfully lowered the risk of major coronary events in high-cardiovascular risk patients. [48, 181] The effect of genetically determined CETP has been subject to considerable discussion in recent literature, but in general a detrimental effect of high CETP, if any, appears to be restricted to men [49, 50, 176, 178, 181-189] Close inspection of previous studies on the association of CETP with CVD risk suggested that in addition to sex, other factors potentially modulate the effects of CETP on CVD risk, including HDL-c or triglyceride concentrations, insulin resistance, or the use of statins or fibrates. [20, 50, 51, 190, 191] This suggests that CETP inhibition could be effective in specific subgroups of the population. To provide more insights in the role of CETP on cardiovascular risk , we aimed to study the causal effect of genetically determined higher CETP concentration on atherosclerosis in the general low-risk population, as well as specific subgroups, using a genetic risk score for CETP concentration as determinant.

Methods

Study design and study population

The Netherlands Epidemiology of Obesity (NEO) study is a population-based, prospective cohort study of 6,671 men and women aged between 45 and 65 years. The study design and population are described in detail elsewhere.[59] All inhabitants with a self-reported body mass index (BMI) of 27 kg/m^2 or higher and living in the greater area of Leiden, the Netherlands were eligible to participate in the NEO study. In addition, all inhabitants aged between 45 and 65 years from one adjacent municipality (Leiderdorp, the Netherlands) were invited to participate irrespective of their BMI, allowing for a reference distribution of BMI. Participants visited the NEO study centre for extensive baseline measurements, including blood sampling and cIMT. Research nurses recorded current medication use by means of a medication inventory. Prior to the study visit, participants completed questionnaires at home with respect to demographic, lifestyle, and clinical information.

The Medical Ethical Committee of the Leiden University Medical Centre (LUMC) approved the protocol. All participants gave their written informed consent.

For the present analyses, we excluded participants from non-European ancestry or with poor genotyping quality (n=927) : when the sample call rate was <98%, there was a sex mismatch, heterozygosity rate was not within ±3 SD of mean heterozygosity rate, participants differed based on the first two principal components (PCs) (±3.5 SD), samples were duplicates, or concordance with another DNA sample was >0.25 (related individuals). Furthermore, we excluded participants with missing CETP (n=31) and cIMT measurement (n=58).

Blood sampling

During the visit to the NEO study centre, venous blood samples were obtained from the antecubital vein after a >10 hour overnight fast. Fasting serum total cholesterol and TG concentrations were measured with enzymatic colorimetric assays (Roche Modular P800 Analyzer, Roche Diagnostics, Mannheim, Germany) and fasting serum HDL-c concentrations with third generation homogenous HDL-c methods (Roche Modular P800 Analyzer, Roche Diagnostics, Mannheim, Germany). LDL-c concentrations were calculated using the Friedewald equation.[128] Furthermore, aliquots of plasma and serum were stored at -80°C after centrifugation. DNA was extracted and genotyping was performed by the Centre National de Génotypage (Evry Cedex, France), using the Illumina HumanCoreExome-24 BeadChip (Illumina Inc., San Diego, California, United States of

America). Subsequently, genotypes were imputed to the 1000 Genome Project reference panel (v3 2011) using IMPUTE (v2.2) software. [104, 105] Three variants within the CETP gene were discovered in a genome wide association study in this population: rs12720922, rs247616 and rs1968905 were used for the present study, of which rs12720922 and rs1968905 were imputed variants (Blauw et al, submitted).

CETP concentrations were measured in serum that had undergone one previous freeze-thaw cycle with enzyme-linked immune sorbent assay (ELISA) kits according to the manufacturer's instructions (DAIICHI CETP ELISA, Daiichi, Tokyo, Japan; coefficient of variation 11.7%).

Subclinical atherosclerosis

Carotid intima media thickness (cIMT) was used as a measure of subclinical atherosclerosis. cIMT was assessed by ultrasonography of the common carotid arteries (CCA). A 15 mm long section 10 mm proximal of the CCA bifurcation was measured while the subject was in supine position. cIMT was measured using a 7.5–10 MHz linear-array probe and the Art.Lab system in B-mode setting and using a wall-track system (ART.LAB version 2.1, Esaote, Maastricht, The Netherlands) to detect boundaries between lumen and intima, as well as between media and adventitia. cIMT was measured during six heart beats in angles of 180, 135 and 90 degrees (right CCA) and 180, 225 and 270 degrees (left CCA). We calculated the mean IMT for each participant by averaging the 36 cIMT measurements within each individual. Measurements of cIMT were validated by analysing repeated measurements in 169 randomly selected participants, which resulted in an intra-observer CV of 5.8% and an inter-observer CV of 9.0%. Detailed results of the validation study are presented in Appendix 1.

Other variables

On the questionnaire, participants reported their highest level of education in ten categories according to the Dutch education system, which was further reclassified into low (none, primary school or lower vocational education) or high (other). Participants reported their medical history of diabetes and cardiovascular diseases. Pre-existing CVD was defined as myocardial infarction, angina pectoris, congestive heart failure, stroke, or peripheral vascular disease. In addition, all use of medication in the month preceding the study visit was recorded. Tobacco smoking was reported in three categories: never smoker, former smoker or current smoker. Participants reported their physical activity during leisure time, which was expressed in metabolic equivalent hours per week. Menopausal state was categorized in pre-, and postmenopausal state according to informa-

tion on ovariectomy, hysterectomy and self-reported state of menopause in the questionnaire. The Framingham 10-year risk of cardiovascular disease was calculated by summation of component scores based on age, LDL-c, HDL-c, diastolic and systolic blood pressure, diabetes, and smoking. [192]

Statistical analysis

The present study is a cross-sectional analysis of the baseline measurements of the NEO study. In the NEO study, individuals with a BMI of 27 kg/m^2 or higher were oversampled. To represent baseline associations in the general population adjustments for the oversampling of individuals with a BMI ≥ 27 kg/m^2 were made.[60] This was done by weighting all participants towards the BMI distribution of participants from the Leiderdorp municipality.[167] The BMI distribution of the participants from Leiderdorp was similar to the BMI distribution of the general Dutch population.[16] All results were based on weighted analyses.

Data were summarized by sex as mean (SD; normally distributed data only), median (25^{th}, 75^{th} percentiles; non-normally distributed data only), or as percentage (categorical data).

We examined the observational association between CETP concentration and cIMT using multivariate linear regression analysis. Results are presented as beta coefficients with 95% confidence intervals (CI) which can be interpreted as difference in cIMT (µm) per unit (µg/mL) of measured CETP concentration. These regression models were adjusted for age and sex, LDL-c concentrations, ethnicity, education, physical activity and smoking. Adjustment for LDL-c was performed to evaluate the association between CETP and IMT through HDL-c. Because it is not completely known whether the following factors are common causes of both CETP and cIMT, and therefore their role as confounding factors is doubted, we performed separate observational analyses additionally adjusted for statin use, diabetes and hypertension.

We calculated a weighted genetic risk score (GRS) by summation of the products of the number of alleles per SNP and the effect size per allele. We estimated the association of the separate SNPs and the GRS with CETP concentrations using linear regression analyses.

We estimated the causal relation between the separate SNPs for CETP concentration and cIMT using linear regression analyses, and we examined the causal association between CETP concentration and cIMT using an instrumental variable two-stage-least-squares regression analysis in which CETP concentration was instrumented by a genetic risk score composed by the three genetic vari-

ants in the *CETP* gene. The regression coefficients from these analyses can be interpreted as difference in cIMT (μm) per unit (μg/mL) in genetically-determined CETP concentration.

The analyses were performed in the total population, and separately for pre-specified subgroups based on sex, high CVD risk profile (defined as a Framingham predicted 10-year risk of 10% or higher), (pre)diabetes (defined as having fasting glucose concentrations ≥6.1 mmol/L, self-reported diabetes or using glucose-lowering medication), high HDL-c concentration (with a cut-off value of 1.04 mmol/L in men, and 1.30 mmol/L in women), triglyceride concentration (with a cut-off value of 1.7 mmol/L), statin use, and menopausal status. [193-195]

Analyses were performed in Stata (version 14, StataCorp. 2015)

Results

Baseline characteristics

Table 1 – Baseline characteristics of the participants of the Netherlands Epidemiology of Obesity study, men and women aged between 45 and 65 years (n=5,655).

	Total population (n=5,655)	CETP ≤ median (2.60 µg/mL)	CETP > median (2.60 µg/mL)
Age (y)	56 (6)	56 (6)	56 (6)
Sex (male)	44	52	36
BMI (kg/m2)	26 (4)	26 (4)	26 (4)
Tobacco smoking (% never)	38	36	41
Physical activity (MET h/week)	30 (16-50)	30 (16-50)	30 (16-50)
Education level (% high)	47	49	46
(Pre)diabetes (%)	14	17	11
Hypertension (%)	34	34	34
CVD (%)	5	7	3
Statin use (%)	10	15	5
Framingham 10-year risk ≥10%	24	24	23
Total cholesterol (mmol/L)	5.7 (1.0)	5.5 (1.0)	5.9 (1.0)
HDL cholesterol (mmol/L)	1.6 (0.5)	1.6 (0.5)	1.6 (0.5)
LDL cholesterol (mmol/L)	3.5 (1.0)	3.3 (0.9)	3.8 (0.9)
Triglycerides (mmol/L)	1.2 (0.9)	1.2 (0.8)	1.2 (0.9)
CETP (µg/mL)	2.47 (0.65)	1.96 (0.32)	2.98 (0.47)
Coding allele rs247616 (%)	56	66	46
Coding allele rs12720922 (%)	17	11	23
Coding allele rs1968905 (%)	82	80	84

Results were based on analyses weighted towards the BMI distribution of the general population (2,715 men and 2,940 women). Results are shown as mean (SD), median (25th, 75th percentiles) or percentage.

BMI, body mass index; CETP, cholesteryl ester transfer protein; cIMT, carotid intima media thickness; HDL, high density lipoprotein; LDL, low density lipoprotein; MET, metabolic equivalent of task; NEO, Netherlands Epidemiology of Obesity.

Baseline characteristics of the study population are presented in Table 1, stratified by sex. The mean (SD) age of the participants was 56 (6) years, and 56% were women. The mean BMI was 26 (4) kg/m^2, mean CETP concentration was 2.47 (0.65) µg/mL, and the mean cIMT was 616 (92) µm.

Serum CETP concentration and atherosclerosis

Table 2 – Associations of observed CETP concentrations (adjusted), CETP SNPs, and genetically-determined CETP concentrations with the intima media thickness in the total population of the Netherlands Epidemiology of Obesity study (n = 5,655) and in men and women separately.

	Difference in cIMT in µm (95% CI)		
cIMT	Total population	Men (44%)	Women (56%)
CETP concentration (per µg/mL) (crude)	-2 (-7, 4)	8 (-1, 17)	-0 (-7, 6)
CETP concentration (per µg/mL) (adjusted)	-1 (-6, 5)	10 (1, 19)	-7 (-14, -1)
CETP concentration (per µg/mL) (additionally adjusted)	-1 (-6, 5)	10 (1, 19)	-7 (-14, -0)
rs1968905-G (per risk-increasing allele)	2 (-5, 9)	-2 (-13, 10)	3 (-7, 12)
rs247616-C (per risk-increasing allele)	0 (-5, 6)	-4 (-12, 4)	4 (-4, 11)
rs12720922-A (per risk-increasing allele)	2 (-5, 8)	7 (-3, 17)	-3 (-11, 5)
Genetically-determined CETP (µg/mL)	2 (-12, 16)	16 (-8, 39)	-8 (-25, 9)

Results were based on analyses weighted towards the BMI distribution of the general population (n=5,655), and were derived from beta coefficients (95% CI) from linear regression and expressed as difference in cIMT µm. Results for the genetic risk score were presented as µm difference in cIMT per µg/mL genetically-determined CETP concentration. Observational analyses were adjusted for age, LDL-c concentration, ethnicity, education, physical activity, and smoking. The additionally adjusted model was additionally adjusted for statin use, diabetes and hypertension. The observational analyses in the total population was additionally adjusted for sex.
CETP, cholesteryl ester transfer protein; cIMT, intima media thickness; LDL-c, low density lipoprotein cholesterol; SNP, single nucleotide polymorphism.

In observational analyses in the total population, CETP concentration was weakly associated with cIMT (adjusted coefficient: -1 µm per µg/mL CETP; 95% CI: -6, 5). Table 2 reports stratified analyses with a weak positive association in men (10 µm per µg/mL higher CETP concentration; 95% CI: 1, 19) and a weak negative association in women (-7 µm per µg/mL CETP; 95% CI: -14, -0)

Associations between genetically-determined CETP and atherosclerosis

The genetic risk score (GRS) of three independent CETP SNP risk alleles rs247616-C, rs12720922-A, and rs1968905-G accounted for 14.7% of variation in CETP concentrations in the participants of the NEO study.

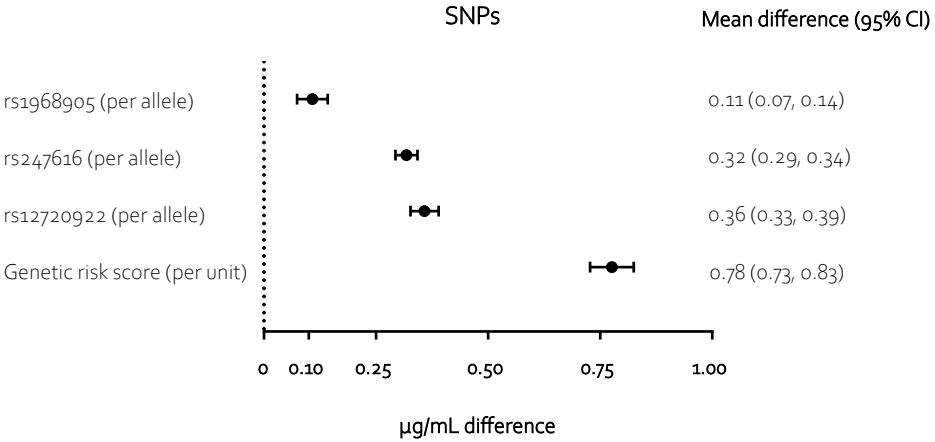


Figure 1 – Associations with 95% confidence intervals of SNPs rs1968905, rs247616, and rs12720922 and the genetic risk score composed from these SNPs with serum CETP concentrations in the participants of the Netherlands Epidemiology of Obesity study, men and women aged between 45 and 65 year (n=5,655). Associations are expressed in µg/mL CETP per allele of the SNPs or per unit of the genetic risk score.

Figure 1 reports the difference in CETP concentration per CETP-increasing SNP allele and per point increase in GRS: 0.78 µg/mL (95% CI: 0.73, 0.83). Table 2 reports causal relations between variations in the *CETP* gene and cIMT. CETP was not causally related to cIMT in the total study population. We observed a causal relation between CETP and cIMT in men of 16 µm (95% CI: -8, 39) difference in cIMT per µg/mL genetically-determined CETP in men and -8 µm (95% CI: -25, 9) in women.

Subgroup analyses

Men and women were stratified for CVD risk profile, (pre)diabetes, fasting HDL-c, triglycerides and statin use. The results of the stratified analyses reported in Figure 2 and Figure 3 demonstrate that in men with a low 10-year Framingham risk, one µg/mL genetically-determined CETP was related to 12 µm (95% CI: -12, 36) thicker cIMT, while in men with a 10-year risk ≥10% the relation was 24 µm (95% CI: -25, 72) per µg/mL genetically-determined CETP. In men without

(pre)diabetes one µg/mL genetically-determined CETP was related with 26 µm (95% CI: -1, 52) thicker cIMT per µg/mL, and with -24 µm (95% CI: -66, 18) in men with (pre)diabetes. In men with normal HDL-c, normal TG concentrations, or not using statins, the associations ranged between 19 and 21 µm per µg/mL genetically-determined CETP concentration, while in men with low HDL-c CETP was inversely related to cIMT (-20 µm per µg/mL CETP; 95% CI: -67, 27).

One µg/mL genetically-determined CETP was related with a -17 µm (95% CI: -36, 1) difference in cIMT in women with a low 10-year Framingham risk, while in women with a 10-year Framingham risk ≥10% this relation was 23 µm (95% CI: -14, 59) per µg/mL genetically-determined CETP. In women with (pre)diabetes, one µg/ml genetically-determined CETP was related with a 48 µm (95% CI: -2, 98) thicker cIMT, and with -13 µm (95% CI: -31, 5) difference in cIMT in women without (pre)diabetes. We observed relations close to the null in women with low HDL-c, high TG concentrations, or who used statins. In women with normal HDL-c, fasting TG, or not using statins, the relations ranged between -9 and -14 µm per µg/mL CETP. In premenopausal women, one µg/mL genetically-determined CETP was related with a -25 µm (95% CI: -59, 8) difference in cIMT, and -0 (-19, 18) in postmenopausal women.

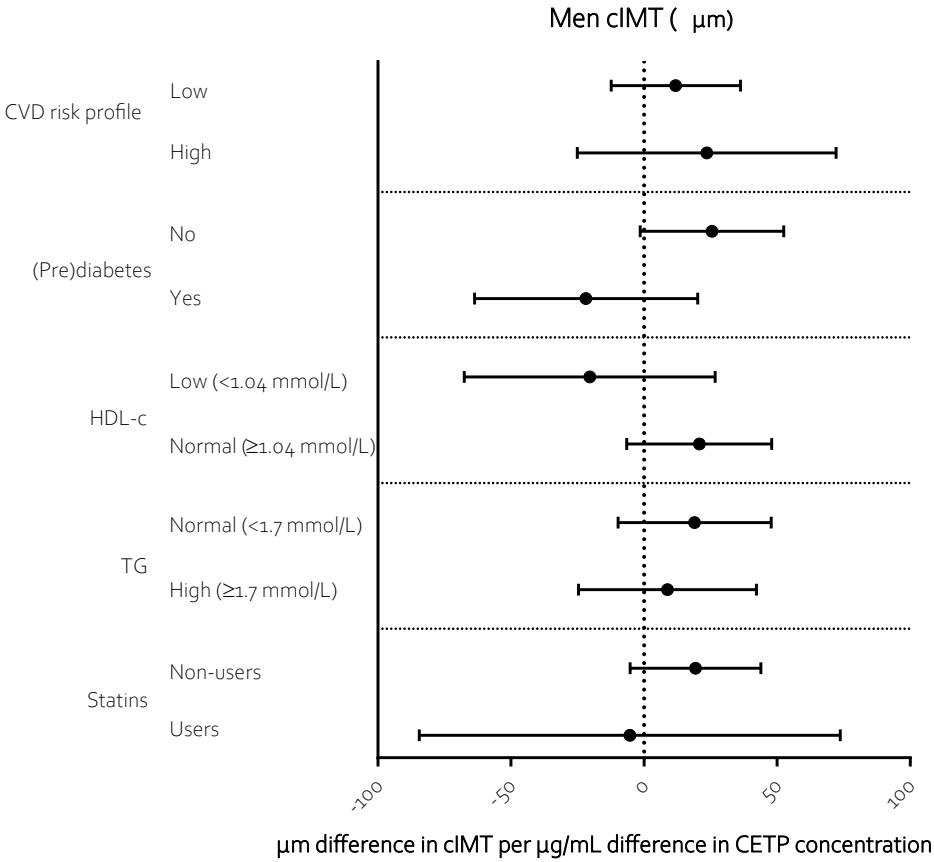


Figure 2 – Associations between (genetically determined) serum CETP concentrations (µg/mL) and intima media thickness (µm) in men participating in the Netherlands Epidemiology of Obesity study, aged 45-65 years (n=2,715). CETP, cholesteryl ester transfer protein; cIMT, carotid intima media thickness; CVD, cardiovascular disease; HDL-c, high density lipoprotein cholesterol; TG, triglycerides.

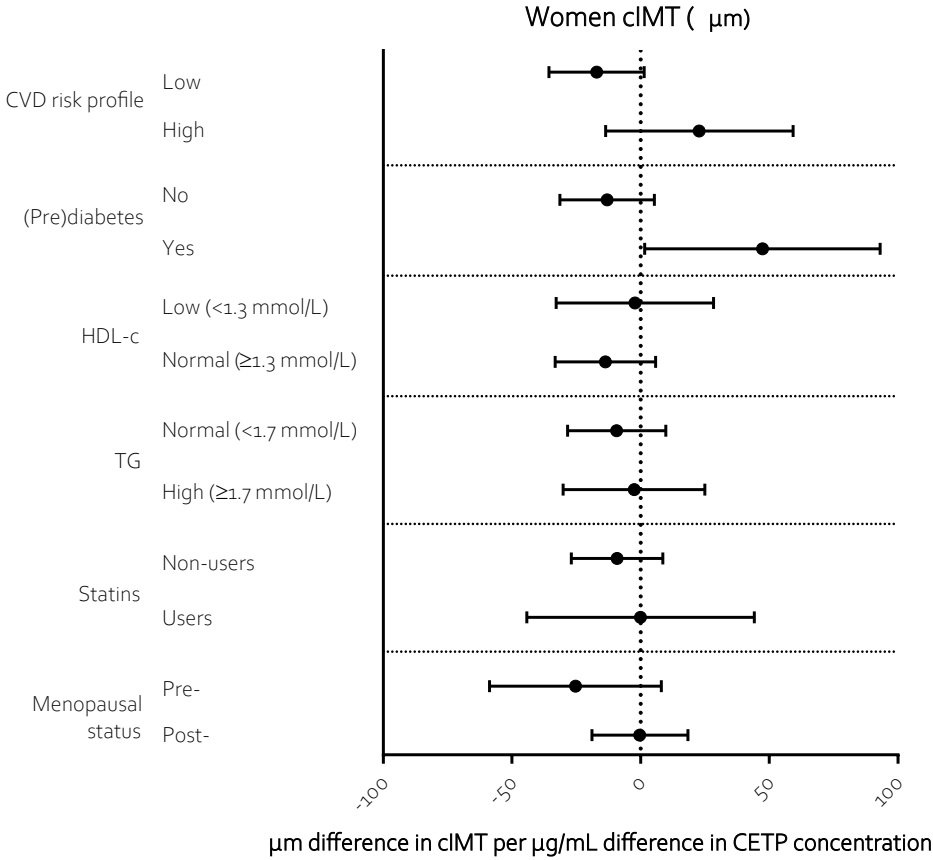


Figure 3 – Associations between (genetically determined) serum CETP concentrations (µg/mL) and intima media thickness (µm) in women participating in the Netherlands Epidemiology of Obesity study, aged 45-65 years (n=2,940). CETP, cholesteryl ester transfer protein; cIMT, carotid intima media thickness; CVD, cardiovascular disease; HDL-c, high density lipoprotein cholesterol; TG, triglycerides.

Discussion

In 5,655 men and women, we investigated the causal associations between CETP and measures of subclinical atherosclerosis. We showed that there was a marginal observational association between CETP and subclinical atherosclerosis in the study population, while a genetic propensity towards higher CETP concentrations was not associated with cIMT. However, stratified analyses in prespecified subgroups of sex, cardiovascular risk, (pre)diabetes, HDL-c, triglycerides and statin use suggested a marginal association between higher CETP and thicker cIMT in men, and a minimal association in the opposite direction in women, albeit with wide confidence intervals.

The null results in the total study population of this study are consistent with findings in the total population in previous Mendelian randomisation studies and randomised controlled trials, however some Mendelian randomisation studies did not measure CETP concentrations. [49, 181, 186-188] Our findings indicate that if a relation between CETP concentrations and subclinical measures of CVD is present, this may be restricted to men, and to women with a moderate-to-high cardiovascular risk or (pre)diabetes in a middle aged population. Our results in high-cardiovascular risk individuals are consistent with the results of the REVEAL trial, that showed a small decrease in incidence of major coronary events in patients with atherosclerotic vascular disease treated with anacetrapib, compared with placebo. [48] These findings are consistent with the positive relations between CETP and subclinical and clinical CVD that were observed mainly in studies in predominantly men.[20, 50, 176, 179, 189] Our findings in women with (pre)diabetes are supported by a smaller previous study that reported that higher observed CETP concentrations were associated with CVD risk in women with type 2 diabetes, but we observed subgroup effects in men that were not observed by this previous study.[196]

CETP mediates the transfer of cholesteryl ester from HDL towards LDL, which is generally assumed to be detrimental with respect to the development of CVD. [197, 198] The surprising absence of an association between CETP and subclinical atherosclerosis on a population level is consistent with the failure of the recent ILLUMINATE[199], dal-OUTCOMES[46], and ACCELERATE[47] clinical trials of pharmacologic CETP inhibition, due to futility or inferiority. However, the ineffectiveness of drugs that target an increase in HDL-c via CETP in the general population may be explained by opposing effects in subgroups that may be masked in summary effect estimates. Although most clinical trials may likely not be powered to perform stratified analyses, reporting stratum-specific effect estimates may reveal different relations in subgroups.

The observed sex differences may be explained by a difference in the relative contribution of a detrimental lipoprotein profile to atherosclerosis in men and women of the same age. This may be an explanation for the observation that women develop atherosclerosis and CVD later than men.[200] It has been shown that while cholesterol is a slightly stronger risk factor in men, diabetes is a stronger cardiovascular risk factor in women than in men.[201] Therefore, women of the same age may need an accumulation of multiple risk factors that are detrimental to the vascular endothelium to develop atherosclerosis. This may be due to vasoprotective effects of endogenous oestrogens,[202] which is consistent with the marginally negative effects that we observed in premenopausal women, and no relation in postmenopausal women. Consequently, the underlying mechanisms of differing relations between CETP and cIMT may reach further than changes in lipid metabolism that are associated with endogenous oestrogens.

Strengths of this study are the large study population with measurements of CETP, cIMT and genetic information to perform a Mendelian randomisation study in the general population. Moreover, extensive phenotypic data was available. Therefore, we were able to perform several subgroup analyses based on sex, menopausal status, medication use and cardiovascular risk factors. The present study also has some limitations that need to be considered. First, the use of cIMT as a measure of subclinical atherosclerosis may limit interpretation of the results in terms of cardiovascular risk. However, previous research has indicated that cIMT is strongly associated with the incidence of future cardiovascular disease in the general population.[203] Second, performing several subgroup analyses increases the risk of chance findings. Further research is needed to replicate the observed relations. Third, this study was performed in a white middle-aged population. Genetic variants or the effects of CETP on atherosclerosis may be specific for this kind of population, and the results therefore need to be confirmed in other ethnicities.

The causal role of CETP is subject to controversy and at the moment of conceptualizing this study, three of four experimental studies of CETP inhibition had been halted due to a lack of protective effect. [204-206] If additional research confirms the potential subgroup effects of CETP, this may indicate that personalisation of pharmacotherapy may be a sensible strategy in the primary and secondary prevention of (recurrent) cardiovascular disease. Further research may elucidate the efficacy of intervening on CETP in subgroups of clinical trials that have been performed. We conclude that despite the absence of a relation between CETP and atherosclerosis in a population of men and women, this relation may be present in men with normal glucose, HDL-c and triglyceride concentrations, and women with a high cardiovascular risk profile or impaired

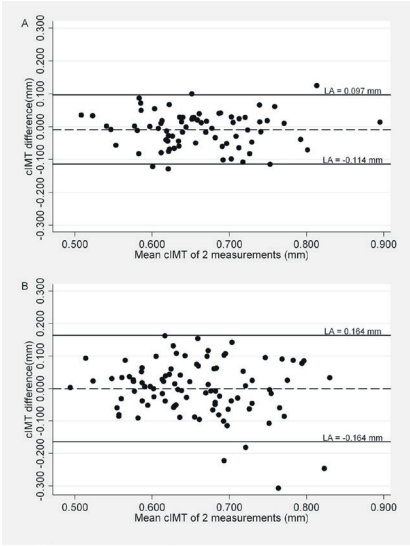
fasting glucose. Further research may give insight in potential sex differences in the aetiology of atherosclerosis.

Appendix

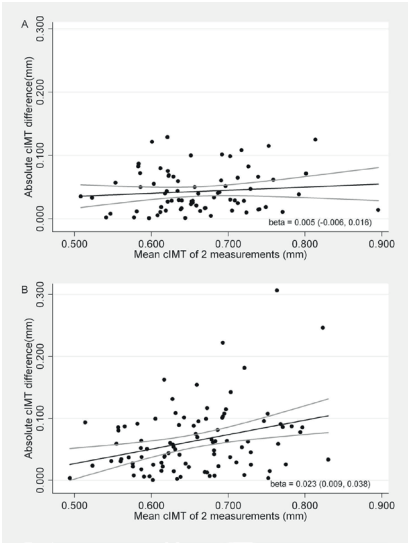
Within the NEO study, cIMT was determined in all participants. Precision of cIMT measurements is crucial to prevent misclassification, decreased study power and diluted effect estimates. We examined the reproducibility of the cIMT measurements in the common carotid artery. We performed paired scans of the cIMT in a random sample of participants at the baseline of the study. Of 169 participants with repeated measurements, 76 participants were scanned twice by the same sonographerm, and 93 participants were scanned twice by two different sonographers. Sonographers were blinded for the scan of the other sonographer. All sonographers were right-handed research nurses who recently had been trained to measure cIMT by an experienced sonographer according to standardized procedures.

To study agreement within and between sonographers we constructed Bland-Altman plots and calculated 95% limits of agreement (LA), intraclass correlation coefficients (ICCs) and coefficients of variation (CVs) for intra-observer and inter-observer measurements separately. For the Bland-Altman plots we calculated the mean cIMT of 2 paired measurements and the difference between 2 paired cIMT measurements by subtracting the second measurement from the first measurement. The ICC explains how much of the total variance between measurements is caused by true inter-subject variability. An ICC of one represents perfect agreement and ICC of zero implies no agreement at all. The CV expresses the standard error between paired measurements as a percentage of the sample mean, where a CV of 0% equals perfect agreement and a CV of 100% signifies no agreement at all. We calculated the ICC and CV for the intra-observer measurements, as well as for the inter-observer measurements.

The mean difference for the intra-observer measurement was -0.009 mm (SD 0.054, 95% LA -0.114 mm to 0.097 mm, Supplementary figure 1, Panel A) and the mean absolute difference was 0.043 mm (SD 0.033). Sixty-nine (91%) intra-observer measurements had an absolute cIMT difference between paired measurements of less than 0.100 mm. The intra-observer ICC was 0.74 (95% CI 0.62 to 0.83) and the CV was 5.8%. The inter-observer measurements had a mean difference of 0.000 mm (SD 0.084, 95% LA -0.164 mm to 0.164 mm, Supplementary figure 1, Panel B) and the mean absolute difference was 0.064 mm (SD 0.054). Seventy-seven (83%) inter-observer measurements had an absolute cIMT difference between paired measurements of less than 0.100 mm. The inter-observer ICC was 0.49 (95% CI 0.33 to 0.63) and the CV was 9.0%.



Supplementary figure 1 – Bland-Altman plots representing the difference between repeated cIMT measurements by the absolute mean cIMT of the repeated measurement. Solid lines represent 95% limits of agreement (LA). Panel A presents the intra-observer variation, panel B presents the inter-observer variation.



Supplementary figure 2 – Plots representing the absolute difference in repeated cIMT measurements by the mean of the repeated measurements. Grey solid lines represent the 95% confidence intervals of the regression line. Panel A represents the intra-observer variation, panel B represents the inter-observer variation.

CHAPTER 7

THE ROLE OF TRIGLYCERIDES IN THE ASSOCIATION BETWEEN HDL-CHOLESTEROL AND CORONARY ARTERY DISEASE: A TWO-SAMPLE MULTIVARIABLE MENDELIAN RANDOMIZATION STUDY

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In preparation

Abstract

Background

Whilst lower levels of high-density lipoprotein cholesterol (HDL-c) strongly predict cardiovascular disease, this relationship is unlikely to be causal. The extent to which the association between HDL-c and coronary artery disease is explained by other correlated cardiometabolic factors such as fasting triglyceride (TG), postprandial TG response and low-density lipoprotein cholesterol (LDL-c) concentrations is unclear.

Aim

To investigate whether the association between HDL-c and coronary artery disease is explained by the residual postprandial TG in addition to other plasma lipoproteins, we performed a multivariable Mendelian Randomisation study.

Methods

HDL-c, LDL- c, fasting TG and postprandial TG concentrations were determined in 5,490 participants of the Netherlands Epidemiology of Obesity (NEO) study. We performed a two-sample multivariable Mendelian randomization analysis, using genetic variants reported to be associated with plasma lipoproteins by the Global Lipids Genetics Consortium (GLGC). Firstly, we estimated the association between these genetic variants and HDL-c, fasting TG concentrations, postprandial TG response and LDL-c in the NEO study. Then, we obtained the estimates of association between the same genetic variants and coronary artery disease from the publicly available summary statistics from the CARDIoGRAMplusC4D consortium. These estimates were combined using two-sample and multivariable Mendelian randomization methodology, including genetically influenced concentrations of all other plasma lipoproteins in the multivariable Mendelian randomization analyses.

Results

We observed a 19% risk reduction (95% confidence interval (CI): 5 – 30%) in the association between HDL-c and coronary artery disease, which attenuated to 10% (95% CI: -8 – 24 %) after inclusion of genetically-influenced fasting TG, and to 2% (95% CI: -16 – 18%) after additional inclusion of genetically-influenced LDL-c. Additional inclusion of the genetically-influenced residual postprandial TG response, neither in addition to other lipids, nor in its own, did not change the risk reduction.

Discussion

Our results confirm that the association between HDL-c and coronary artery disease is almost totally explained by LDL-c and fasting TG. We could not distinguish an effect of the residual postprandial TG response.

Introduction

Imbalances in circulating lipids have extensively been described as risk factors for atherosclerosis and coronary artery disease (CAD), which is a major cause of death. [1, 207, 208] Notably, high concentrations of high density lipoprotein cholesterol (HDL-c) are strongly associated with a lower cardiovascular risk in observational studies and in naïve Mendelian randomisation studies [45]. However, multivariable Mendelian randomisation studies have indicated that HDL-c itself is unlikely to exert a causal role in the development of CAD but this association is rather explained by LDL-c, TG and apolipoprotein B [58, 209], which supports clinical trials of therapeutic raising of HDL-c showing a likely non-causal role of HDL-c in CAD risk. [48, 199] High concentrations of low density lipoprotein cholesterol (LDL-c), very low density lipoprotein cholesterol (VLDL-c) and remnant particles are considered likely causal factors in the development of atherosclerosis and CAD. [210, 211] A potential explanation for the apparent observational association between HDL-cholesterol and CAD is that a high HDL-cholesterol concentration is a marker of a favourable flux of catabolism components of triglyceride-rich lipoproteins by lipoprotein lipase (LPL). [212-214] This hypothesis implies that HDL-c concentrations are a proxy for the physiological capacity to clear dietary triglycerides from the circulation; therefore the strong observational association between HDL-c and CAD may be explained by postprandial TG concentrations (ppTG). However, previous work showed that postprandial TG concentrations have the same genetic background as fasting TG concentrations. [215] Therefore, the residuals of an orthogonal nonlinear least squares regression of postprandial and fasting TG concentrations were calculated. In this study, we used these residuals to study the effects of the postprandial TG response independent of fasting TG concentrations.

We aimed to explore the role of residual postprandial TG response in addition to LDL-c and fasting TG concentration in the association between HDL-c concentration and CAD, using a two-sample multivariable Mendelian randomization analysis, as visualised in the directed acyclic graph in Figure 1.

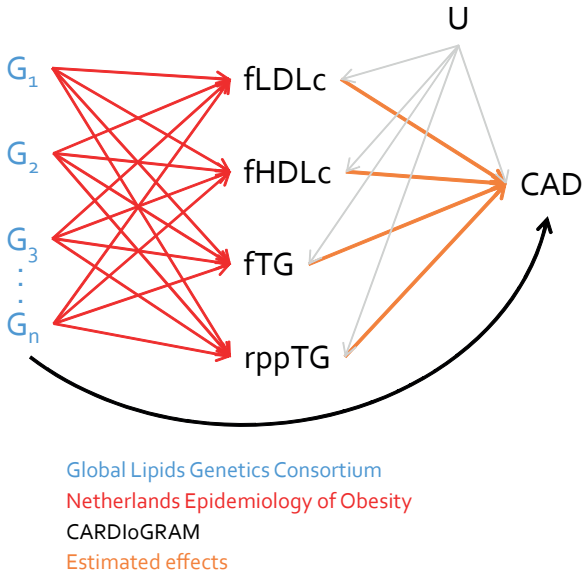

Figure 1 – Directed acyclic graph of the data and analyses used to investigate the assumed causal pathways between genetic variants, blood lipids and CAD. CAD, coronary artery disease; CARDIoGRAM, Coronary ARtery DIsease Genome wide Replication and Meta-analysis (CARDIoGRAM) plus The Coronary Artery Disease (C4D) Genetics; fHDL, fasting high-density lipoprotein; fLDL, fasting low-density lipoprotein; fTG, fasting triglycerides; G, genetic variant; rppTGr, residual postprandial triglyceride response.

Methods

Study design and study population

The Netherlands Epidemiology of Obesity (NEO) study is a population-based, prospective cohort study of 6,671 men and women aged between 45 and 65 years. The study design and population are described in detail elsewhere. [59] Briefly, all inhabitants with a self-reported body mass index (BMI) of 27 kg/m^2 or higher and living in the greater area of Leiden, the Netherlands were eligible to participate in the NEO study. In addition, all inhabitants aged between 45 and 65 years from one adjacent municipality (Leiderdorp, the Netherlands) were invited to participate irrespective of their BMI, allowing for a reference distribution of BMI. Participants visited the NEO study centre for extensive baseline measurements, including blood sampling. Research nurses recorded current medication use by means of a medication inventory. Prior to the study visit, participants completed questionnaires at home with respect to demographic, lifestyle, and clinical information.

The Medical Ethical Committee of the Leiden University Medical Centre (LUMC) approved the protocol. All participants gave their written informed consent.

Blood sampling

Serum lipids

After 5 minutes rest, fasting blood samples were collected from the antecubital vein after a >10 hour overnight fast. All participants consumed the same liquid mixed meal of 400 mL, that contained 600 kcal of which 16 percent (En%) was derived from protein, 50 En% from carbohydrates and 34 En% from fat. In total, the 400 mL meal contained 75.0 g of carbohydrates and 2.4 g of saturated, 13.7 g of monounsaturated and 6.8 g of polyunsaturated fat. Participants were instructed to drink the mixed meal within 5 minutes. Blood was sampled 30 and 150 minutes after the meal ingestion. Triglyceride concentrations were determined using an enzymatic colorimetric assay using a TG GPO-PAP kit (11730711216, Roche) on on an automated analyser (Roche Modular P800, Roche Diagnostics, Almere, The Netherlands). The postprandial TG response was calculated by using the residuals of the TG concentration distribution of 150 minutes adjusted for fasting TG concentrations. The specific method has been described elsewhere. [215] Fasting serum total cholesterol concentrations were measured with enzymatic colorimetric assays (Roche Modular P800 Analyzer, Roche Diagnostics, Mannheim, Germany) and fasting serum HDL-c concentrations with third generation homogenous HDL-c methods (Roche Modular P800 Analyzer,

Roche Diagnostics, Mannheim, Germany). LDL cholesterol concentrations were calculated using the Friedewald equation. [128]

Furthermore, aliquots of plasma and serum were stored after centrifugation at -80°C. DNA was extracted and genotyping was performed by the Centre National de Génotypage (Evry Cedex, France), using the Illumina HumanCore-Exome-24 BeadChip (Illumina Inc., San Diego, California, United States of America). Subsequently, genotypes were imputed to the 1000 Genome Project reference panel (v3 2011) using IMPUTE (v2.2) software. [104, 105] Known genetic variants for HDL-c, LDL-c, fasting TG concentration and TG response reported by Global Lipids Genetics Consortium study (n=171) were extracted using a p-value threshold of $p<5*10^{-8}$ and a linkage disequilibrium threshold of $r^2<0.001$. [67, 215, 216]

CARDIoGRAMplusC4D

Outcome measures were obtained from publicly-available data from the CARDIoGRAMplusC4D consortium. The CARDIoGRAMplusC4D consortium included 60,801 CAD cases and 123,504 controls of European ancestry. [61] In general, CAD was defined as fatal or nonfatal coronary artery disease, percutaneous coronary intervention, coronary artery bypass graft surgery, stenosis >50%, or angina. Specific definitions have been described previously. [217]

The NEO study did not overlap with the GLGC and CARDIoGRAMplusC4D consortia, but there was an overlap of 10.3% between GLGC and CARDIoGRAMplusC4D.

Statistical analysis

We performed a two-sample multivariable Mendelian randomization study, using the baseline measurements of the NEO study as the exposure sample and the publicly available summary statistics of the CARDIoGRAMplucC4D consortium as outcome sample.

For the present analyses, we excluded participants of the NEO study with poor genotyping quality, using criteria described elsewhere. [109] We also excluded participants with missing HDL-c, LDL-c and fasting and postprandial TG measurements. Furthermore, participants with meal irregularities (who arrived at study site not fasting, did not or not completely drink meal, ate between postprandial blood samples) were excluded. Baseline characteristics were summarized as mean (SD; normally distributed data only), median (25^{th}, 75^{th} percentiles; non-normally distributed data only), or as percentage (categorical data). We as-

sumed all genetic effects to be additive. Four principal components of ancestry were calculated to be able to correct for population stratification.

Two-sample multivariable Mendelian randomization

We estimated the effects of HDL-c, fasting TG concentrations, TG response, and LDL-c with CAD using two-sample multivariable Mendelian randomization analysis. [58]

First, we created composite instruments for the four lipid measurements in the NEO study: fasting LDL-c, HDL-c, and TG concentrations, and postprandial TG response at 150 minutes. These were adjusted for four principal components of ancestry, and weighted towards a BMI distribution of the reference cohort.

Second, we constructed a single composite instrument CAD from CARDIoGRAMplusC4D. We then used two-sample Mendelian randomization methodology to estimate the causal effect of each of the plasma lipoproteins individually on CAD. Therefore we used inverse variance weighted meta-analysis in which the inverse of the variance of the effect of the variant on the outcome was used to weight the causal estimates of the variant.

In addition, we estimated adjusted beta coefficients between HDL-c and CAD by including genetically influenced fTG, postprandial TG response and LDL-c in the multivariable Mendelian randomization analyses using regression-based methods. We report odds ratios (ORs) and 95% confidence intervals (CIs) for the effects of HDL-c on CAD, and after inclusion of combinations of the other plasma lipids. The genetic variants that were used may influence other phenotypes, i.e. horizontal pleiotropy. To investigate the potential effects of horizontal pleiotropy, and to adjust for these effects, we performed multivariable MR-Egger as a sensitivity analysis using the R-packages *MendelianRandomization* and *TwoSampleMR,* and the code from the seminal multivariable MR-Egger paper. [218]

Results

Baseline characteristics

In total, 6,671 participants were included in the NEO study. Consecutive exclusion of related participants, participants of non-European descent, or with insufficient genotyping quality (n=927), participants with missing HDL-c, fasting TG measurements or TG response (n= 148), participants with meal irregularities (not fasting, did not or not completely drink meal, or ate between blood samples, n= 33), resulted in a final study population of 5,490 participants.

Participants had a mean age of 56 years, 45% were men and 16% were current smokers. Participants had a mean BMI of 26 kg/m^2 (SD: 4) and HDL-c of 1.6 mmol/L (SD: 0.5). Further baseline characteristics are presented in Table 1.

Table 1 – Baseline characteristics of the participants of The Netherlands Epidemiology of Obesity study, men and women aged between 45 and 65 years of whom the triglyceride response was assessed after a mixed meal challenge (n = 5,490).

Age (y)	56 (6)
Sex (% men)	45
BMI (kg/m^2)	26 (4)
Alcohol intake (g/d)	10 (3-22)
Physical activity (MET-h/week)	30 (16-50)
Education level (% high*)	47
Total cholesterol (mmol/L)	5.7 (1.1)
HDL-c (mmol/L)	1.6 (0.5)
LDL-c (mmol/L)	3.6 (1.0)
Fasting TG (mmol/L)	1.0 (0.7-1.5)

Results are based on analyses weighted toward the BMI distribution of the general population (n = 5,490). Data are shown as mean (standard deviation), median (interquartile range), or weighted percentage.

BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MET-h, metabolic equivalents of task; TG, triglycerides

*****Low education: none, primary school, or lower vocational education as highest level of education.

Mendelian randomization analyses

In univariate Mendelian randomization analyses, each 1 mmol/L higher level of HDL-c decreased the risk of CAD by 19% (95% CI: 5-30%). Inclusion of fasting TG in the multivariable Mendelian randomization almost halved the risk reduction to 10% (95% CI: -7-24%), which was similar to the risk reduction after additional inclusion of genetically influenced LDL-c concentration (risk reduction: 8%; 95% CI: -7-20%). Simultaneous inclusion of genetically influenced fTG and LDL-c concentrations completely attenuated the risk reduction to 2% (95% CI: -15–18%). Additional inclusion of the residual postprandial TG response in the multivariable Mendelian randomization did not change these results individually or in combination with the other plasma lipoproteins (results not shown).

Table 2 – Two-sample multivariable Mendelian randomization analysis of the odds ratio of HDLc on the risk of coronary artery disease. Odds ratios are based on the effects of genetic variants on lipid concentrations in The Netherlands Epidemiology of Obesity study (n=5,490) and their effects on coronary artery disease in the CARDIoGRAMplusC4D consortium (60,801 CAD cases and 123,504 controls).

	Odds ratio per 1 mmol/L (95%CI)	Additionally included in the multivariable MR model
HDLc	0.81 (0.70, 0.95)	none
	0.92 (0.80, 1.07)	LDLc
	0.90 (0.76, 1.07)	fTG
	0.80 (0.68, 0.93)	rppTGr
	0.98 (0.83, 1.15)	LDLc, fTG
	0.90 (0.77, 1.05)	LDLc, rppTGr
	0.88 (0.74, 1.05)	fTG, rppTGr
	0.96 (0.81, 1.14)	LDLc, fTG, rppTGr

Results are based on weighted analyses of the genetic effects on lipid concentrations in the NEO-study (n=5,490). Data are shown as odds ratios of coronary artery disease per 1 mmol/L increase in HDL-c concentration and their 95% confidence intervals.

fTG, fasting triglycerides; HDLc, high-density lipoprotein cholesterol; LDLc, low-density lipoprotein cholesterol; MR, Mendelian randomization; rppTGr, residual postprandial triglyceride response.

Discussion

This study is a proof of principle of a two-sample multivariable Mendelian randomization strategy to improve understanding of the causal link between HDL-c and CAD. We confirmed that fasting TG and LDL-c combined almost totally explain the association between low HDL-c and increased risk of CAD. In addition, our results suggested that fasting TG and LDL-c have a similar magnitude of effect on the effect of HDL-c on CAD. The genetic instrument for residual postprandial TG response was not strong enough to estimate its role in the relation between HDL-c and CAD.

Our analyses provided strong evidence for the absence of a causal effect of HDL-c on CAD, after inclusion of LDL-c and fTG in the multivariable analyses. [58] As previous studies in small samples have shown that HDL-c is correlated with the postprandial TG response, [212] we hypothesized that postprandial TG response would partly explain the underlying mechanism of the strong observational associations between HDL-c and CAD. The biological explanation for this may be that ingested lipids are first taken up by VLDL-c or chylomicrons, which are a major determinant of measured serum TG concentrations. Its lipolyzed products are transferred to HDL-c, while leaving atherogenic remnant particles. [210, 219, 220] As a consequence, HDL-c concentration is a marker of the capacity to clear alimentary fat. Both fasting and postprandial TG concentrations are determined by the capacity to clear TGs from the serum by LPL, CETP, and PLTP, and therefore the genetic determinants are similar. [212, 221]

Because fasting TG concentrations and postprandial TG response have a strongly similar biological and genetic background, we aimed to investigate isolated effects of the postprandial TG response by using a measure of the response that excluded effects of fasting TG. [212, 215, 222, 223] In recent work we discovered one genetic variant that determined postprandial TG response independent from fasting TG concentration, of which a proxy that was used for the genetic instrument in this study. [215] The genetic instrument for postprandial TG response appeared not to be strong enough to distinguish from fasting TG concentration and to estimate its causal effects. [215]

Two possible pathways are hypothesized for the likely causal effect of fTG and LDL-c CAD risk, via remnant particles and via lipolytic toxins. [224, 225] Triglyceride-rich lipoproteins metabolised by LPL, leading to an abundance of remnant particles, which in turn affect monocyte activation [226] and endothelial inflammation [227]. The lipolytic toxins hypothesis suggests that oxidized free fatty acids that result from lipolysis contribute to the susceptibility of the vascular wall to macrophage and LDL-c infiltration. [228-230] In combination with the

role of other TG-mediated pathways, this may be an explanation for the strong observational association between HDL-c concentrations as a marker of TG and its clearance, and CAD risk [231-233]

This study uniquely uses a two-sample multivariable Mendelian randomization analysis to investigate the relation between a phenotype that is subject to genetic pleiotropy and is expensive or difficult to measure and a rare outcome. the present study are the large number of participants, who were well phenotyped in a fasting and postprandial state.

This study also has some limitations that need to be considered. First, the mixed meal that was used, was not standardized towards body size or composition, and contained 34 energy-percent of fat. This may not fully represent the usual daily feeding behaviour of the participants. Second, using a two-sample Mendelian randomisation analysis requires additional assumptions: the causal relationships should be identical in both samples, the covariance matrix should be the same in both samples and the error variances should be known. Also the exposure should be measured without error. However, heterogeneity tests require more power than available in the present study, while measurement error can not be ruled out. Therefore the possibility remains that there is residual bias in these results.

In conclusion, this study confirms that in the general population, genetically-determined fasting TG and LDL-c almost totally explain the relationship between HDL-c and CAD. Further studies are needed to investigate the determinants of the capacity to increase and to clear serum lipids, and which metabolites of this clearance influence the risk of developing atherosclerosis or cardiovascular disease.

CHAPTER 8

ASSOCIATION OF FASTING TRIGLYCERIDE CONCENTRATION AND POSTPRANDIAL TRIGLYCERIDE RESPONSE WITH THE CAROTID INTIMA MEDIA THICKNESS IN THE MIDDLE AGED: THE NEO STUDY

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Abstract

Background

People are in a postprandial state for the majority of the day, postprandial triglyceride response may be more important in the etiology of atherosclerosis than fasting triglycerides.

Objective

To investigate the associations of fasting triglyceride concentration (TGc) and postprandial TG response after a meal challenge with subclinical atherosclerosis, measured by intima media thickness (IMT) in a middle aged population.

Methods

5,574 participants (57% women) with a mean (SD) age of 56 (6) years were included in this cross-sectional analysis of baseline measurements of the Netherlands Epidemiology of Obesity study. Serum TGc was measured fasting, and 30 and 150 minutes after a liquid mixed meal and the incremental area under the curve (TGiAUC) was calculated. With linear regression analyses, we calculated the differences in IMT with 95% confidence intervals (CI), adjusted for confounding factors, and additionally for TGc or TGiAUC.

Results

Per standard deviation (SD) of TGc(0.82 mmol/L), IMT was 8.5 μm(2.1, 14.9) greater after adjustment for TGiAUC and confounding factors. Per SD of TGiAUC(24.0 mmol/L*min), the difference in IMT was -1.7 μm(-8.5, 5.0) after adjustment for fasting TG and confounding factors.

Conclusion

The association between TG response after a mixed meal and IMT disappeared after adjusting for TGc. The association between fasting TG concentration and IMT persisted after adjustment for postprandial TG response. These findings imply that it is not useful to perform a meal challenge in cardiovascular risk stratification. Our results support use of fasting TGc instead of postprandial TG responses for cardiovascular risk stratification in clinical practice.

Introduction

Atherosclerosis is a vessel disease that predisposes for the development of chronic or acute coronary heart disease and stroke [234]. Because clinically relevant atherosclerosis is present in almost half and coronary artery calcifications in one fifth of the middle-aged population, many individuals are at risk for the development of cardiovascular disease (CVD), the leading cause of death worldwide [1, 235, 236].

The progression of atherosclerosis is influenced by multiple cardiovascular risk factors, such as smoking, diet, body composition and genetic predisposition [234]. High fasting and non-fasting plasma TG concentrations (TGc) are well-known additional risk factors for cardiovascular disease [52]. The recently updated consensus statement by the European Atherosclerosis Society indicated that measuring TGc in a fasting state does not improve CV risk prediction compared to measurement of non-fasting TGc [194]. This implies that for clinical prediction of CVD, measuring fasting and non-fasting TGc may be exchangeable. However, the mechanisms that link fasting TGc with CVD may be different from the mechanism relating non-fasting TGc and CVD.

Since Zilversmit first proposed in 1979 that atherogenesis may be a predominantly postprandial process [53], studies have been undertaken to elucidate the underlying mechanisms. It has been hypothesized that the relation between postprandial hypertriglyceridemia and atherosclerosis depends on the size and TG content of lipid-transporting lipoprotein particles [211, 237]. Another mechanism between the TG response to a meal and atherosclerosis is the accumulation of lipoprotein remnant particles, which are responsible for TG transport [238]. The concentration of lipoprotein remnant particles is associated with postprandial hyperlipidemia [219] and strongly associated with coronary heart disease [220, 239]. Atherogenic effects of postprandial hypertriglyceridemia may be even more prominent when the vascular endothelium is exposed to oxidative stress as a result of smoking [240] or hyperglycemia [241]. Therefore smokers and (pre)diabetics may be more susceptible to the development of atherosclerosis as a result of hypertriglyceridemia.

To quantify the effects of TG metabolism on (subclinical) CVD in epidemiological studies, fasting TGc are often used as exposure measurement. However, people in the Western world are in a postprandial state for the largest part of the day [53], which may lead to prolonged high plasma TGc. We hypothesized that postprandial hypertriglyceridemia is associated with a larger carotid IMT as a measure of subclinical atherosclerosis and that this association might be stronger in strata of risk factors that are associated with deterioration of the vascular en-

dothelium. Therefore, the aim of the present study was to examine the associations of fasting TGc and postprandial TG response after a liquid mixed meal challenge with subclinical atherosclerosis in the total population and in subgroups based on sex, smoking status and fasting glucose.

Methods

Study design and population

The Netherlands Epidemiology of Obesity (NEO) study is a population-based, prospective cohort study designed to investigate pathways that lead to obesity-related diseases. The NEO study started in 2008 and includes 6,671 individuals aged 45–65 years, with an oversampling of individuals with overweight or obesity. The study design and population are described in detail elsewhere [5].

Men and women living in the greater area of Leiden (in the West of the Netherlands) were invited through letters sent by general practitioners, by local advertisements and via registries of municipalities surrounding Leiden. They were invited to participate if they were aged between 45 and 65 years and had a self-reported BMI of 27 kg/m^2 or higher. In addition, all inhabitants aged between 45 and 65 years from one municipality (Leiderdorp) were invited to participate irrespective of their BMI, allowing for a reference distribution of BMI. Participants were invited for a baseline visit at the NEO study center of the Leiden University Medical Center (LUMC) after an overnight fast.

Prior to the study visit, participants completed a general questionnaire at home to report demographic, lifestyle and clinical information. The participants were asked to bring all medication they were using in the month preceding to the study visit. Research nurses recorded names and dosages of all medication. All participants underwent an extensive physical examination, including anthropometry, blood sampling and a meal challenge. In the present analysis, we excluded participants with a history of CVD (defined as angina pectoris, myocardial infarction, stroke, arrhythmias or congestive heart failure), missing values for fasting and postprandial TG and IMT. Because it is unknown how the TG response and atherosclerosis are influenced by glucose lowering medication, we additionally excluded participants that used this medication. Furthermore, participants that did not drink the meal challenge were excluded.

The Medical Ethical Committee of the Leiden University Medical Center (LUMC) approved the design of the study and all participants gave their written informed consent.

Data collection

Fasting and postprandial triglycerides

After 5 minutes rest, fasting blood samples were collected from the antecubital vein. All participants consumed the same liquid mixed meal of 400 mL, that contained 600 kcal of which 16 percent (En%) was derived from protein, 50 En% from carbohydrates and 34 En% from fat. In total, the 400 mL meal contained 75.0 g of carbohydrates, 2.4 g of saturated, 13.7 g of monounsaturated and 6.8 g of polyunsaturated fat. Participants were instructed to ingest the mixed meal within 5 minutes. Blood was sampled 30 and 150 minutes after the meal ingestion. Triglyceride concentrations were determined using an enzymatic colorimetric assay using a TG GPO-PAP kit (11730711216, Roche) on on an automated analyser (Roche Modular P800, Roche Diagnostics, Almere, The Netherlands). Postprandial TG response was calculated as the TGiAUC using the trapezoidal method and was represented as mmol/L*min.

Postprandial triglyceride concentrations peak approximately 4 hours after the meal [242]. In the present study, the response was measured up until 150 minutes after the meal, a duration that may be too short to capture the complete triglyceride response. To validate whether 150 minutes is sufficient, we validated this measurement in a randomly selected subgroup of participants. Therefore, blood was sampled at 240 minutes after ingestion of the mixed meal (n=14) to investigate the agreement in TG response between 0-150 minutes and 0-240 minutes.

Plasma glucose and insulin concentrations, serum total cholesterol and high-density lipoprotein (HDL)-cholesterol were determined in the fasting blood samples at the central clinical chemistry laboratory of the LUMC using standard assays.

Impaired fasting glucose was defined as a fasting glucose concentration ≥6.1 mmol/L. The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as the product of fasting glucose and insulin concentrations divided by 22.5 [129].

Carotid intima media thickness

The carotid IMT was measured in the far wall of the left common carotid artery (CCA) along a section with a length of 15 mm, located 10 mm proximal of the bifurcation with the participant in supine orientation. A 7.5-10 MHz linear array transducer in B-mode setting was used to visualize the distal CCA and the lu-

men-intima and intima-media limits were detected using an online wall-track system (Art.Lab version 2.1, Esaote, Maastricht, The Netherlands). IMT was measured in the transversal angle during six heartbeats.

Population characteristics and other variables

Ethnicity was self-identified in the questionnaire and was regrouped into white (reference) and other. Highest completed level of education was reported in ten categories according to the Dutch education system and regrouped in two categories: low education (no education, primary education or lower vocational education) and high education (other). Participants reported the frequency, type, and duration of their usual physical activity in the past 4 weeks on the Short Questionnaire to Assess Health-enhancing physical activity, a method previously validated in the Dutch population [243, 244]. We calculated the energy expended during physical activity in leisure time in hours per week of metabolic equivalents. Participants reported their history of CVD, defined as myocardial infarction, angina, congestive heart failure, stroke or peripheral vascular disease. Smoking status was self-reported and grouped in three categories: never smoker, former smoker and current smoker. Quantification of long-term tobacco use was expressed in pack-years of smoking, which was defined as the product of the number of (20 cigarette)-packs per day and the number of years the person smoked. Habitual alcohol intake in grams per day was assessed using a semi-quantitative food frequency questionnaire (FFQ), which has been validated in the Dutch population [245], and calculated from the 2011 version of the Dutch food composition table [246].

Height was measured without shoes using a calibrated, vertically fixed tape measure. Body weight and percent body fat were measured by the Tanita bio impedance balance (TBF-310, Tanita International Division, UK) without shoes and one kilogram was subtracted to correct for the weight of clothing. Body mass index (BMI) was calculated by dividing body mass in kilograms by body height in meters squared. Waist circumference was measured in the middle of the distance between the crista iliaca and the lowest rib using a flexible steel tape measure. Blood pressure was measured seated on the right arm with a validated automatic oscillometric device (OMRON, Model M10-IT, Omron Health Care Inc, IL, USA). Three measurements with 5 min rest in between measurements were performed and the mean systolic and diastolic blood pressure were calculated

Statistical analysis

Baseline characteristics were presented as means with standard deviation, medians with 25th and 75th percentiles or as percentages stratified for fasting TGc. The cut-off value for increased fasting TGc was defined as 1.7 mmol/L [194]. In the NEO study, individuals with a BMI of 27 kg/m^2 or higher were oversampled. To correctly represent baseline associations in the general population, adjustments for the oversampling of individuals with a BMI ≥27 kg/m^2 were made. This was done by inverse probability weighting of all participants towards the BMI distribution of participants from the Leiderdorp municipality [60], whose BMI distribution was similar to the BMI distribution of the general Dutch population in the age range of 45–65 years [16].

We calculated Pearson's correlation coefficients between fasting TGc and TGc at 150 minutes and fasting TGc and TGiAUC. Fasting TGc and the TGiAUC were standardized to a mean of zero and a standard deviation of 1.We performed weighted linear regression analyses to examine the associations between fasting TGc and the TGiAUC as the exposure, with the IMT as the outcome variable and calculated regression coefficients with 95 % confidence intervals (CI). The regression coefficients were expressed as the difference in IMT in micrometers (µm) per standard deviation higher fasting TG or TGiAUC. Crude analyses were first adjusted for age and sex, then we added either the fasting TGc or TGiAUC response, and finally we adjusted all models for the potential confounding factors total body fat, physical activity, smoking status, pack years of smoking, alcohol consumption, use of alpha and beta blockers, use of lipid lowering drugs (fibrates, niacin or statins), fasting LDL-cholesterol and HOMA1-IR.

To investigate whether the associations were different between men and women, smokers and non-smokers and persons with and without hyperglycemia, we tested for interaction by including a product interaction term (TGiAUC*stratification factor) in the regression model. Subsequently, we performed all analyses stratified by sex, fasting glucose concentrations and smoking status.

We furthermore repeated all analyses including the participants with a history of CVD.

Results

Baseline characteristics

In total, 6,671 participants were included in the NEO study. After consecutive exclusion of participants with a history of cardiovascular disease (n=527), missing data on fasting (n=42), 30 minutes (n=90) and 150 minutes (n=72), missing IMT measurement (n=98), meal protocol violations (n=4) or use of glucose lowering medication (n=264), 5,574 participants were included in the analyses.

Baseline characteristics of the NEO study population are presented in Table 1, stratified by fasting TGc. The mean (SD) age of the study population was 56 years, 57% were women and 16% were current smokers. The mean (SD) BMI was 26.1 (4.3) kg/m^2 and the mean IMT was 631 (143) µm, both were higher in the participants with increased concentrations of fasting TG. The population mean (SD) fasting TGc was 1.21 (0.82) mmol/L. The mean population TG response was 29.8 (24.0) mmol/L*min is represented in Figure 1. The correlation coefficient of fasting TGc with TGc at 150 minutes was 0.92 and with TGiAUC 0.36.

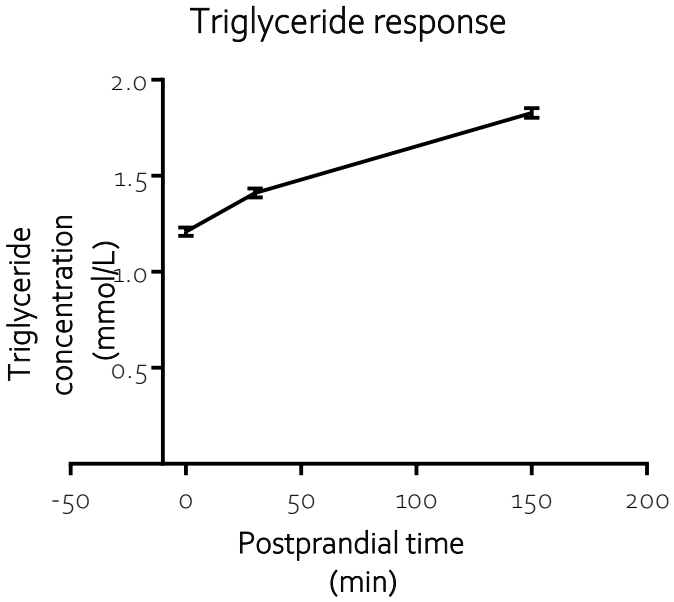


Figure 1 – Postprandial triglyceride response with 95% confidence intervals after a mixed meal in the participants of the Netherlands Epidemiology of Obesity study, men and women aged between 45 and 65 years who did not use glucose lowering therapy (n=5,574). Results are based on analyses weighted towards the BMI distribution of the general population.

In the validation population (n=14), the mean (SD) TGc at 150 minutes was 2.4 (1.1) mmol/L*min and at 240 minutes 2.3 (1.2) mmol/L*min. The area under the curve between 0 and 150 minutes postprandial was highly correlated with the area under the curve between 0 and 240 minutes; r^2=0.99 (Supplementary figure 1)

Table 1 – Baseline characteristics of the participants of the Netherlands Epidemiology of Obesity study, men and women aged between 45 and 65 years without medical history of cardiovascular disease of whom the TG response was assessed after a mixed meal challenge and who did not use glucose- and lipid lowering therapy (n=5,574).

	Fasting TG	
Characteristic	<1.7 mmol/L (83%)	≥1.7 mmol/L (17%)
Age (year)	55.5 (6.1)	55.5 (5.7)
Sex (% men)	39	61
Ethnicity (% whites)	95	95
BMI (kg/m^2)	25.6 (4.1)	28.5 (4.3)
Tobacco smoking (% never/former/current)	41 / 45 / 14	31 / 46 / 22
Pack years (packs*years)		
Current smokers	18 (10-29)	20 (12-32)
Former smokers	8 (3-17)	11 (5-23)
Physical activity (MET hours/week)	31 (17-51)	26 (12-45)
Education level (% high)[a]	48	41
Total cholesterol (mmol/L)	5.6 (1.0)	6.2 (1.2)
HDL cholesterol (mmol/L)	1.7 (0.4)	1.2 (0.3)
LDL cholesterol (mmol/L)	3.5 (0.9)	3.8 (1.1)
Fasting glucose (mmol/L)	5.3 (0.7)	5.7 (0.9)
Fasting glucose ≥6.1 mmol/L (%)	9	22
Fasting insulin (IU/L)	2.5 (2.0-5.6)	6.7 (3.3-11.7)
HOMA1-IR	1.6 (1.1-2.5)	2.9 (2.0-4.3)
Fasting TG (mmol/L)	0.94 (0.35)	2.53 (1.13)
TGiAUC (mmol/L*min)	25.6 (19.4)	50.3 (32.2)
IMT (µm)	625 (142)	663 (145)

BMI, body mass index; IMT, carotid Intima Media Thickness; HDL, high density lipoproteins; HOMA-IR, Homeostasis Model Assessment Insulin Resistance; TGiAUC, incremental area under the curve; LDL, low density lipoproteins; MET, metabolic equivalents of task; NEO, Netherlands Epidemiology of Obesity.

Results are based on analyses weighted towards the BMI distribution of the general population (n = 5,574). Data are shown as mean (SD), median (IQR) or weighted percentage.

a; Low eduation: none, primary school or lower vocational education as highest level of education.

Associations of fasting TG concentrations and carotid intima media thickness

The associations between fasting TGc and IMT (µm) are presented in Figure 2. Per SD of fasting TGc, the difference in IMT was 18.8 (95% confidence interval: 13.4-24.1) µm. After adjustment for age and sex this difference attenuated to 15.3 (10.1-20.5) µm and after additional adjustment for TGiAUC this coefficient was 14.4 (8.9-20.0) µm. After additional adjustment for all confounding factors the coefficient further attenuated to 8.5 (2.1-14.9) µm.

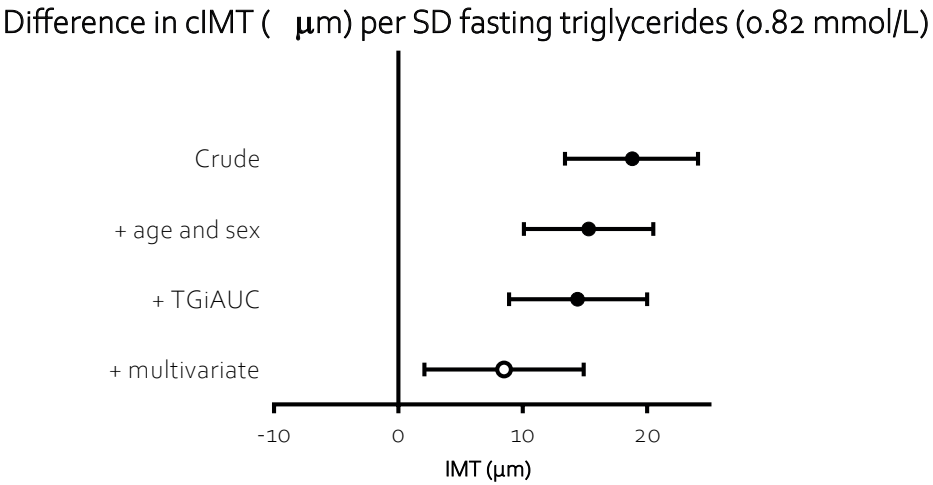


Figure 2 – Difference in carotid intima media thickness (µm) per standard deviation of fasting triglyceride concentrations (0.82 mmol/L) Multivariate: additionally adjusted for LDL-cholesterol, total body fat, alcohol consumption, use of alpha and beta blockers, use of lipid lowering drugs, physical activity, pack years of smoking, HOMA1-IR, TGiAUC. TGiAUC, incremental area under the curve of postprandial triglycerides; cIMT, carotid intima media thickness; LDL, Low density lipoprotein; HOMA-IR, Homeostastis assessment of insulin resistance. Results are based on analyses weighted towards the BMI distribution of the general population.

Associations between postprandial TG response and carotid intima media thickness

One SD of TGiAUC was associated with a 13.2 (7.0-19.4) µm difference in IMT. After adjustment for age and sex this difference attenuated to 7.2 (1.0-13.5) µm per SD. After additional adjustment for fasting TGc the coefficient attenuated to 2.7 (-3.6-9.0) and after full adjustment for confounding factors TGiAUC was not associated with IMT (-1.7, 95% CI: -8.5-5.0 µm per SD). (Figure 3).

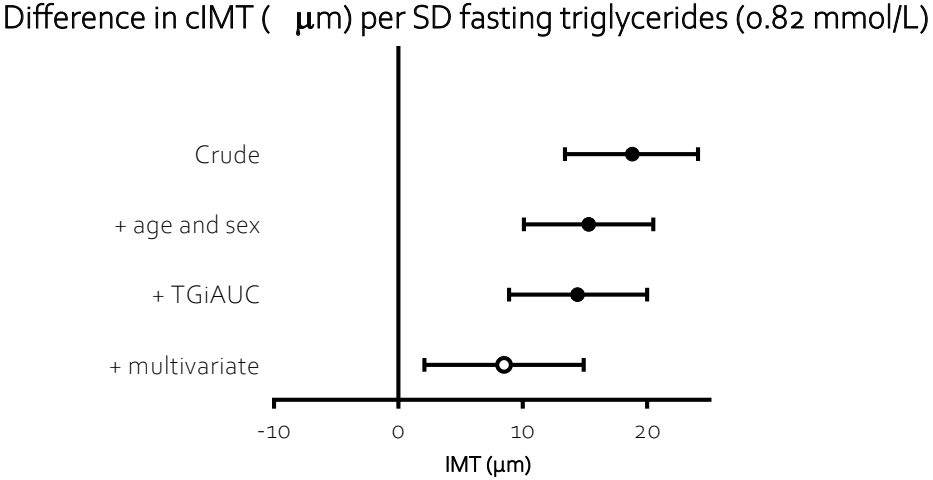


*Figure 3 – Difference in carotid intima media thickness (μm) per standard deviation of incremental area under the curve of triglyceride response (24.0mmol/L*min) Multivariate: additionally adjusted for LDL-cholesterol, total body fat, alcohol consumption, use of alpha and beta blockers, use of lipid lowering drugs, physical activity, pack years of smoking, HOMA1-IR, TGiAUC. TGiAUC, incremental area under the curve of postprandial triglycerides; cIMT, carotid intima media thickness; LDL, Low density lipoprotein; HOMA-IR, Homeostastis assessment of insulin resistance. Results are based on linear regression analyses weighted towards the BMI distribution of the general population.*

Associations between postprandial TG response and carotid intima media thickness in high-risk subgroups

Stratified results are presented in Table 2.

In men, the crude difference of 5.6 (-4.4-15.5) μm IMT per SD TGiAUC attenuated to 4.5 (-5.2-14.1) μm after adjustment for age and fasting TG and to -0.3 (-10.3-9.6) μm after additional adjustment for confounding factors. In women, the crude difference of 12.3 (4.5-20.1) μm IMT per SD TGiAUC disappeared after adjustment for age and fasting TG (0.7; -7.8-9.2 μm) and additional adjustment for confounding factors (-1.3; -10.1-7.5 μm). There was no significant interaction between TG response and sex (p= 0.23).

Crude effects in persons with normal and impaired fasting glucose concentrations of 12.0 (5.0-19.0) and 8.1 (-4.2-20.5) μm per SD attenuated to 6.0 (-1.1-13.1) and 8.7 (-3.1-20.6) μm per SD after adjustment for age and sex. After adjustment for fasting TGc, these coefficients changed to 1.3 (-5.8-8.5) μm per SD in persons with normal fasting glucose and 7.5 (-4.5-19.4) μm per SD in persons with impaired fasting glucose concentrations. After additional adjustment for confounding factors the differences were -2.3 (-10.0-5.3) μm per SD for persons

with normal and 3.2 (-8.9-15.3) µm per SD for persons with impaired fasting glucose concentrations. The interaction between TG response and glycemia was not significant (p=0.35).

The crude differences per SD of TGiAUC in never smokers (15.8; 6.0-25.5 µm), former (8.3; -0.1-16.7 µm) and current smokers (14.8; -1.8-31.3 µm) attenuated to 3.5 (-7.1, 14.1) µm (never), -1.7 (-10.1, 6.7) µm (former) and 8.5 (-6.6, 23.6) µm (current) after adjustment for age, sex and fasting TG. Additional adjustment for confounding factors resulted in differences of 0.8 (-10.0-11.6) µm (never), -6.6 (-15.5-2.2) µm (former) and 7.7 (-8.5-23.9) µm IMT (current) per SD of TGiAUC. The interaction between the postprandial TG response and smoking status was not significant (p=0.41 for never vs former smoking and p=0.70 for never vs current smoking).

When we included participants with prior or prevalent cardiovascular disease in the analyses, the results were similar (data not shown).

*Table 2 – Differences in IMT (µm) with 95% confidence intervals per standard deviation of postprandial TG response (24.0 mmol/L*min) in participants of the NEO study stratified by sex, glycaemia and smoking status.*

		IMT		
Postprandial TGiAUC (SD = 24.0 mmol/L*min)		Model	Difference in IMT (µm)	95% Confidence interval
Men	(43%)	Crude	5.6	-4.4, 15.5
		Adjusted for age and sex	7.9	-2.2, 17.9
		+fasting TG	4.5	-5.2, 14.1
		Multivariate adjusted	-0.3	-10.3, 9.6
Women	(57%)	Crude	12.3	4.5, 20.1
		Adjusted for age and sex	6.6	-0.9, 14.1
		+fasting TG	0.7	-7.8, 9.2
		Multivariate adjusted	-1.3	-10.1, 7.5
Fasting glucose <6.1 mmol/L	(89%)	Crude	12.0	5.0, 19.0
		Adjusted for age and sex	6.0	-1.1, 13.1
		+fasting TG	1.3	-5.8, 8.5
		Multivariate adjusted	-2.3	-10.0, 5.3
Fasting glucose ≥6.1 mmol/L	(11%)	Crude	8.1	-4.2, 20.5
		Adjusted for age and sex	8.7	-3.1, 20.6
		+fasting TG	7.5	-4.5, 19.4
		Multivariate adjusted	3.2	-8.9, 15.3
Never smoker	(39%)	Crude	15.8	6.0, 25.5
		Adjusted for age and sex	8.5	-1.1, 18.2
		+fasting TG	3.5	-7.1, 14.1
		Multivariate adjusted	0.8	-10.0, 11.6
Former smoker	(45%)	Crude	8.3	-0.1, 16.7
		Adjusted for age and sex	2.6	-5.4, 10.5
		+fasting TG	-1.7	-10.1, 6.7
		Multivariate adjusted	-6.6	-15.5, 2.2
Current smoker	(16%)	Crude	14.8	-1.8, 31.3
		Adjusted for age and sex	10.9	-5.9, 27.8
		+fasting TG	8.5	-6.6, 23.6
		Multivariate adjusted	7.7	-8.5, 23.9

IMT, carotid Intima Media Thickness; TGiAUC, incremental area under the curve;
Multivariate: additionally adjusted for LDL-cholesterol, total body fat, alcohol consumption, use of alpha and beta blockers, use of lipid lowering drugs, physical activity, pack years of smoking, HOMA1-IR and fasting TGc in additional models
Results are based on analysis weighted towards the BMI distribution of the general population.

Discussion

In this population-based study of men and women without prevalent CVD we observed a clear association between fasting TGc and IMT, which persisted after adjustment for postprandial TG response over 150 minutes. The association between the TG response and IMT however disappeared after adjusting for fasting TGc. These findings imply that in general it is not useful to perform a meal challenge in order to estimate a person's risk of atherosclerosis and thereby add to the ongoing debate on the importance of fasting TGc measurements in cardiovascular risk. Although our results have no implications for random non-fasting TGc, the association between fasting TGc and IMT persisted beyond postprandial TG response.

Previous studies showed that postprandial TG response is a risk factor for cardiovascular disease and atherosclerosis in certain subgroups of the general population [247-251]. It has been shown that smoking and diabetes aggravate the effect of other cardiovascular risk factors [251, 252]. Also, the mechanisms behind the additional risk in the presence of the atherogenic risk factors smoking and diabetes has been studied in smaller experimental studies [219, 253]. Because the vascular endothelium is exposed to elevated TGc in the postprandial range for a large part of the day, we hypothesized that the postprandial TG response would be stronger associated with (subclinical) atherosclerosis than fasting TGc. However, our findings suggest that fasting TGc are responsible for the observed crude association between postprandial TG response and IMT. However, the suggestion of a remaining association between TG response and IMT in smokers and (pre)diabetics after adjustment for fasting TGc may indicate that these conditions increase the susceptibility of the endothelial wall to either postprandial TG response or higher concentrations of remnant particles.

Besides the importance of TG response in certain subgroups prone to atherosclerosis, our findings indicate an important role of fasting TGc in the etiology of atherosclerosis beyond TG response. These results are in line with clinical practice, but are not in complete agreement with the recent consensus statement [194], because the association between fasting TGc and IMT persists after adjustment for TG response.

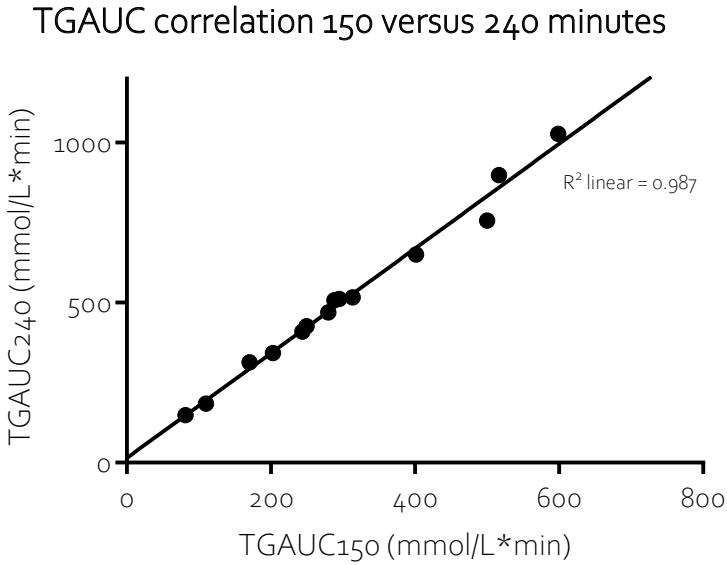
Not all modulators of fasting TGc are completely known. Excess of TG and cholesterol in the diet most likely play a role, as well as a limited hydrolysing capacity of lipoprotein lipase (LPL) due to low systemic LPL expression [222] or other lipoproteins competing for hydrolysis by LPL [52, 254, 255]. The present study, together with other studies indicate that TG response [241] and its effects on IMT [252] are different between persons with and without hyperglycemia.

Strengths of this study are that all participants were challenged to a mixed meal to represent daily dietary challenges instead of a meal challenge with isolated carbohydrates or lipids. Other strengths of this study are the availability of extensive phenotypic data regarding atherosclerosis and confounding variables in a large study population.

There are also some limitations that need to be considered. First, the TG response was assessed during a 150 minute period, while the peak of TGc after a meal may occur even 4 hours after a meal. Therefore a residual association may exist between a longer TG response and subclinical atherosclerosis. However, in a subgroup of participants we showed that the TG response over 240 minutes strongly correlated with the TG response over 150 minutes. Second, we assessed the TG response to one single meal with 34 energy percent from fat, which was not individualized with regard to body surface area, which may not fully represent daily exposure to postprandial TG or provoke a substantial triglyceride response. Elevated TGc due to multiple meals during the day may be more important in vascular wall damage than the TG response to one meal. The results did not change when additionally adjusting for body surface area (data not shown). Future studies are needed to investigate the relation between daily TGc and atherosclerosis and cardiovascular disease. Third, the confidence intervals of the observed associations in the subgroups were large and include null indicating large variation or insufficient power in these specific subpopulations. However, the calculated association between TG response and IMT in smokers and persons with increased fasting glucose concentrations did not reach zero after adjustment for other confounding factors, implicating a loss of power in the subgroup analyses rather than chance findings. Fourth, the cross-sectional design of this study precludes causal inference. As a result of the observational design, residual confounding may be present due to remaining unknown, unmeasured or inaccurately measured confounding factors, such as the use of dietary supplements.

In conclusion, this study shows that in the general population, the association between TG response after a mixed meal and subclinical atherosclerosis disappeared after adjusting for fasting TGc. Our findings suggest that non-fasting and fasting TGc may not be exchangeable but that a higher fasting TGc is related to a larger IMT beyond TG response. These results confirm the clinical practice of using fasting TGc for cardiovascular risk stratification in the general population. More research is needed to specifically study the effect of the postprandial TG response during the day, in particular in susceptible individuals as smokers and (pre)diabetics.

Supplementary tables and figures



Supplementary figure 1 – Correlation plot of the area under the blood concentration curve of triglycerides measured over 150 minutes (TGAUC150) versus the area under the blood concentration curve of triglycerides measured over 240 minutes of time (TGAUC240) after a mixed meal challenge in 14 participants of the Netherlands Epidemiology of Obesity study.

SUMMARY AND GENERAL DISCUSSION

Summary of main findings

The aim of this thesis was to unravel a selection of a multitude of potential causal pathways that may underlie the association between excess body fat and cardiovascular disease, such as adipokines, inflammation, HDL-cholesterol and postprandial triglyceride response, and cholesteryl ester transfer protein (CETP).

In the first section of this thesis, we focused on factors that have only recently been regarded as risk factors for cardiovascular disease, like inflammation and adipokines. In the second section we focused on lipids and lipoproteins that were analysed using novel methods, such as metabolomics and Mendelian randomization. Here we summarize and interpret the findings of our studies, discuss their relevance in the field of cardiovascular risk factors, and describe the used methods, their application and potential pitfalls. In this thesis, we showed that body fat and its distribution are strongly related to serum concentrations of atherogenic lipoproteins, inflammatory markers such as C-reactive protein and glycoprotein acetyls, and the adipokines leptin and adiponectin. In addition, we showed that the association between body fat (distribution) and subclinical atherosclerosis was not mediated by inflammation as measured by C-reactive protein and glycoprotein acetyls. Also we showed that the adipokines leptin and adiponectin are unlikely to be causally related to subclinical measures of cardiovascular disease. Furthermore, we showed that the postprandial triglyceride response is not associated with subclinical atherosclerosis beyond fasting triglycerides. In addition, we found that CETP may have differential effects on subclinical atherosclerosis in different subgroups based on sex and cardiometabolic risk factors, while there is no effect in the total population.

In the middle of the 20th century the interrelated risk factors smoking, hypertension and LDL-cholesterol were the strongest contributors to cardiovascular disease. [13, 255] Despite reasonably successful efforts to reduce the burden of these factors by medication and life-style interventions, the decline in incidence rate of cardiovascular disease has slowed with the emergence of (childhood) obesity and diabetes. [15, 256, 257] This implies that the relative contribution of causal mechanisms may have shifted from the more traditional risk factors LDL-cholesterol and hypertension towards the well-known risk factor diabetes and novel factors like adipokines, inflammation and lipids and lipoproteins other than LDL-cholesterol. In this thesis, we mainly focused on the novel risk factors.

Atherosclerosis was previously regarded as a purely lipid-caused disease, but lesions were found to also contain pro-inflammatory macrophages containing oxidized LDL-cholesterol, suggesting that inhibiting inflammation could reduce the rate of progression of atherosclerosis. [3] Therefore it is not surprising that

the latest development in this field was the publication of the results of the CANTOS trial that aimed to assess the effects of the anti-inflammatory drug canakinumab, which is a monoclonal antibody against interleukin-1β. [39] Indeed, treatment with canakinumab reduced the incidence of cardiovascular disease, which was offset by an increased mortality due to infectious diseases. However, the role of inflammation as an intermediate pathway between obesity and cardiovascular disease has been less studied. Therefore in **Chapter 2** we studied the promising target of inflammation as a potential mediating factor between body fat, in specific visceral fat, and carotid intima media thickness as a marker of subclinical atherosclerosis. We observed strong associations between overall and visceral body fat and markers of systemic inflammation in the general population, but these markers were not associated with intima media thickness as a marker of subclinical atherosclerosis in men and women. Due to the absence of the association between inflammation and intima media thickness, no mediation was observed. The absence of the association between inflammation and subclinical atherosclerosis was in contrast with results from previous studies, and mainly the CANTOS trial. A potential explanation may be that the NEO study population is relatively healthy as compared to other study populations, and therefore the extent of atherosclerosis was limited. Also, this study could not exclude the possibility that inflammation contributes to cardiovascular disease via effects on other factors, such as coagulation or endothelial wear. In addition, other markers than C-reactive protein and glycoprotein acetyls may be more specific for the inflammation of visceral adipose tissue.

The second class of potential novel intermediate risk factors between obesity and cardiovascular disease are adipokines. Adipokines are signal molecules that are predominantly produced by adipose tissue. [258, 259] While adiponectin is mainly produced by visceral adipose tissue and modulates insulin sensitivity and fat oxidation [260], leptin is mainly produced by subcutaneous adipose tissue and signals to the brain to regulate food intake. [123] In recent years, adiponectin and leptin have both been linked to detrimental as well as advantageous effects on cardiovascular disease outcomes, but studies doing so were of diverse quality. [30, 33, 91, 127, 144, 154, 157, 159, 261-263] Therefore, we performed three studies to investigate the effect of sex on the association between body fat and circulating adipokine concentrations, and to assess the effects of these adipokines on (sub)clinical cardiovascular disease outcomes, while adjusting for the collateral effects of body fat.

In **Chapter 3** we confirmed that women had higher adiponectin and leptin concentrations than men. Our results suggest that the sex difference in leptin concentrations can be fully explained by the difference in total body fat, whereas the sex difference in visceral fat did not completely explain differences in adi-

ponectin concentrations. Previous studies have suggested that the sex difference in adiponectin and leptin concentration exceeded the difference that could be expected due to body fat. [55, 97, 101] However, most studies did not take into account that white adipose tissue is the main producer of leptin, and in general only compared linear associations or partial correlation coefficients between BMI and leptin concentrations in women and men. Similarly, previous studies have used waist circumference as a proxy for visceral fat, which is the main determinant of adiponectin concentrations. [97] In addition, we showed that in the general population there are women with extreme leptin concentrations, without apparent symptoms except excess body fat. A genetic variant in a potential regulatory area of the *LEP* gene was overrepresented in these women.

In **Chapter 4** we studied the relation between (genetically-determined) leptin concentration and measures of subclinical cardiovascular disease in thee domains: heart function, ECG parameters, and atherosclerosis. Also, we investigated whether observed associations could be explained by confounding effects of total body fat. We also performed two-sample Mendelian randomisation studies for the effect of leptin on pulse wave velocity and coronary heart disease in summary statistics of the UK Biobank and CARDIoGRAMplusC4D. [62, 63, 134, 168, 264] Most associations between leptin and measures of subclinical cardiovascular disease were explained by potential confounding effects of total body fat, and we did not find different results in women and in men. Also, genetically-determined leptin was not related to any of the measures of cardiovascular disease in the NEO study and the UK Biobank. In addition, we found no evidence of a strong genetic effect of leptin on the risk of coronary heart disease. However, the width of confidence intervals of these estimations could indicate heterogeneity in these effects. Previous observational studies provided conflicting results regarding the direction of the effect of leptin on cardiovascular disease. Leptin was assumed to have beneficial mechanistic effects on subclinical markers of cardiometabolic health, but an overall detrimental effect was shown in meta-analysis of studies that were adjusted for BMI. [91] However, our two-sample Mendelian randomisation study indicated that it is unlikely that leptin has a strong detrimental effect on coronary heart disease. Altogether, any effects of leptin on cardiovascular may be of limited clinical relevance and heterogeneous.

In **Chapter 5**, we attempted to disentangle the adiponectin paradox, which is fuelled by contradicting studies that on the one hand associate adiponectin with non-causal beneficial effects on insulin sensitivity and diabetes, while on the other hand observational studies show that individuals with increased adiponectin are at higher risk to develop cardiovascular disease. [155, 157, 159, 170, 261] The contradiction may be explained by reverse causation or collider stratification bias. Collider stratification could have occurred because these observational

studies have been performed in individuals at high risk to develop cardiovascular disease. In observational analyses in the NEO study, we observed no association between adiponectin and MRI measures of heart function, also not in analyses stratified by sex. However, using two-sample Mendelian randomisation, we found that a genetic predisposition towards a higher NT-proBNP concentration, which is a major biomarker of deteriorated left heart function, affects adiponectin concentrations. This findings suggests that previous observations are possibly explained by reverse causation through increased NT-proBNP concentrations due to deteriorated heart function.

Section II of this thesis includes chapters that go deeper into the relations between lipid and lipoprotein profiles and subclinical atherosclerosis. Extensive efforts to improve measurement of lipid and lipoprotein subfractions by metabolomics, and to discover genetic variants that influence the lipoprotein landscape by GWAS have made it possible to look further into the mechanisms that determine lipoprotein classes, their response to a meal challenge, and their associations with atherosclerosis.

While large-scale MR studies and clinical trials with CETP inhibitors have shown that CETP does not affect the risk of cardiovascular disease through the increase of HDL-cholesterol concentrations, early genetic studies on CETP suggested effects on the risk of cardiovascular risk. [49, 176, 178] These early studies have often been performed in specific subgroups of the population, such as men at high risk of cardiovascular disease, or women with diabetes. The combination of these results suggests that CETP has heterogeneous effects on atherosclerosis and cardiovascular disease. Therefore, in **Chapter 6** we performed a MR study of CETP on subclinical atherosclerosis in subgroups of the general population using uniquely strong genetic instruments. We confirmed that in the general population of the NEO study, genetically-determined CETP was not associated with intima-media thickness as a measure of subclinical atherosclerosis. However, our results suggested differential effects in men and women, and in individuals at high and low cardiometabolic risk. These results are coherent with the findings of previous studies that reported observational and MR results in specific groups of individuals. This may suggest that CETP-inhibiting treatment could be effective in subgroups of the population, potentially through their LDL-cholesterol lowering effects.

In **Chapter 7**, we investigated the hypothesis that the postprandial triglyceride response may explain the strong predictive value of HDL-cholesterol for the risk of coronary artery disease using a multivariable two-sample Mendelian randomisation strategy. We investigated the effects of genetically-determined HDL-cholesterol, LDL-cholesterol, fasting triglycerides, and the residual post-

prandial triglycerides on coronary heart disease using data from the GLGC consortium, the NEO study and the CARDIoGRAMplusC4D consortium. Our results indicate that LDL-cholesterol and fasting triglycerides together explain the association between HDL-cholesterol and coronary artery disease. The genetic instrument used to assess explanation by residual postprandial triglycerides was too weak to make solid inferences about this mechanism.

Previous research suggested that increased postprandial serum triglyceride excursions are a stronger risk factor for the development of atherosclerosis and cardiovascular disease than fasting triglyceride concentrations because the vascular wall is exposed to high postprandial triglyceride concentrations during the majority of the day and night. However, the main limitation of previous studies that investigated the relation between postprandial hypertriglyceridemia and atherosclerosis is that fasting triglyceride concentrations have not been taken into account adequately. We showed in **Chapter 8** that the association between the postprandial triglyceride response, defined as the incremental area under the curve, and subclinical atherosclerosis was completely explained by fasting triglyceride concentration. This may be due to inadequate reflection of daily triglyceride exposure by our meal challenge, or due to the same mechanism underlying the regulation of fasting and postprandial triglyceride concentrations. Our results suggest that in subgroups of the population with a damaged or susceptible vascular wall, such as patients with diabetes or smokers, there may be an association between higher triglyceride response and increased subclinical atherosclerosis in addition to the association with fasting triglycerides. However, the sample size in our subgroups was too small to draw solid conclusions.

Causal inference in observational studies

In epidemiologic research, one can distinguish prediction and aetiology (i.e. causal research). For example, one can partly predict the risk of cardiovascular disease once a patients' HDL-cholesterol and C-reactive protein concentration is known, but an intervention to alter these factors will render futile as these are most likely to be mere predictors and not causal factors. In this thesis we aimed to estimate causal effects of several risk factors for atherosclerosis and cardiovascular disease. While there are two main limitations of using only observational studies for causal inference, we used several strategies to mitigate these limitations.

A first limitation may be that at this point in time, the Netherlands Epidemiology of Obesity (NEO) study includes only cross-sectional data. Therefore the temporal relation between the variables that are regarded as exposure and as outcomes cannot be determined. This leaves the possibility that the assumed out-

come in fact causes the assumed exposure. This phenomenon is referred to as reverse causation. In this thesis, we aimed to investigate associations in the NEO study that were unlikely to have a reverse causal relation, based on previous research. When possible, we added Mendelian randomisation analyses to reduce the risk of reverse causation.

Second, the NEO study was designed as an observational study. The exposures of interest were therefore not allocated by chance, as is the case in a randomised controlled trial, but may also be determined by other factors. Some of these factors may also be determinants of the outcomes of interest and therefore constitute confounding, provided they are not part of the causal mechanism underlying the relation between exposure and outcome (mediation). [265] To mitigate the effects of confounding factors, one can apply different strategies in the design and analysis of a study. In this thesis, we used restriction in the design of the study (**all Chapters**), stratification (**Chapters 3, 4, 6**), and statistical adjustment (**all Chapters**). Still, a main limitation of observational studies is that confounding factors can not all be measured, and if so, measured well. This poses observational studies at risk for residual confounding, which may lead to biased results. Therefore, in this thesis, Mendelian randomisation analyses are used when possible, to prevent problems with residual confounding.

Mendelian randomisation studies

In recent years, a novel methodology has been developed to mitigate the aforementioned problems of reverse causation and the potential bias as a result of residual confounding: Mendelian randomisation analysis (after Georg Mendel, the founder of modern genetics). This method makes use of the attribute of genetic variants that they are assigned randomly at conception, and inherited independently from other genetic variants.

When a genetic variant is a determinant of an exposure of interest, one can use this information to infer a causal relation between an exposure and outcome of interest, given the validity of three assumptions. [163] First, the genetic variant should be associated with the exposure of interest. Second, the genetic variant is independent of other determinants of the outcome of interest, and third, the genetic variant is only associated with the outcome of interest through the exposure of interest. The first assumption can and should be checked within the data of the study using a simple regression analysis. The second assumption may be explored, but not tested, by investigating the effects of the genetic variants on known potential confounding factors of the association between the exposure and outcome of interest. Testing the third assumption is also infeasible, because it is often impossible to distinguish whether the genetic variant influences the

outcome via the exposure of interest or via a parallel pathway. These assumptions are under pressure from several potential mechanisms, of which pleiotropy, population stratification, and linkage have the largest potential impact. [266]

Pleiotropy is the phenomenon in which a genetic variant not only affects one, but multiple factors. This may pose a problem for a successful Mendelian randomisation analysis, as the effect on the outcome cannot be assigned to one of the factors for which the genetic variant predisposes. This may violate the second assumption for Mendelian randomisation analysis, when the genetic variant affects the factor that is not of interest directly as opposed to through the factor of interest. A potential way to circumvent this problem is to perform Mendelian randomisation with multiple genetic variants for multiple risk factors, to estimate the separate effects of these risk factors instrumented by genetic risk scores on the outcome of interest. [58] This is specifically useful in situations where multiple risk factors are themselves highly intertwined, such as lipoprotein species. Therefore we aimed to perform a multivariable Mendelian randomisation analysis in **Chapter 7**.

Potentially, a genetic variant is not distributed completely at random over the population, but influenced by other factors like ancestry. This may constitute one condition of potential confounding. Often, ancestry is in some way related to the outcome of interest. This may violate the third assumption for Mendelian randomisation analysis, and lead to confounding that needs to be adjusted for. Therefore genetic analyses should be adjusted for population stratification. In our analyses, we performed our analyses adjusted for four markers of ancestry to avoid confounding by population stratification. Also, genetic variants are assumed to be inherited independently of each other. However, often genetic variants inherit with other variants more often than expected by chance, which is described by the term linkage. This may result in a form of pleiotropy, as several treats may be affected at the same time as a consequence of linked genetic variants. This would violate the second assumption for Mendelian randomisation analysis. Alternatively, linkage may be used to one's advantage when the genetic variant of interest has not been measured, but can be estimated using a linked variant. This is common practice in Mendelian randomisation analyses, as different genome analysis kits include different sets of genetic variants. Also, for many phenotypes no genetic instruments are known, because the phenotype is too difficult to measure or has a too low prevalence to perform a genome-wide association study. This limits the application of Mendelian randomisation studies in terms of rare causes of disease. For other phenotypes only weak instruments are known. The use of weak instruments requires large sample sizes. Therefore, a major strength of this thesis is that in **chapters 4, 5, and 7** we made use of publicly-available summary statistics of several large genome-wide association

studies to look up effects of genetic variants that predispose for our exposures of interest. One drawback of the use of these summary statistics is that it limits the possibilities of performing stratified analyses, e.g. to investigate effect measure modification. Also, the assumptions regarding pleiotropy and independence of the genetic variants or risk scores with potential confounders could not be tested more extensively than customary in Mendelian randomisation studies.

Mediation analyses

In **Chapter 2** a mediation study of the association between body fat and subclinical atherosclerosis is presented. Mediation is a concept in causal inference indicating that the relation between a cause and an outcome is completely or partly actuated through a mediator. [84] The use of directed acyclic graphs such as in Chapter 2, Figure 1, is a straightforward way to visualise the concept of mediation.

The Baron and Kenny method is a straightforward way of mediation analysis. Using this method, several assumptions should hold. There should be associations between exposure and mediator, and between mediator and outcome. Also, interaction between exposure and mediator should be absent. Assuming continuous and normally distributed exposure, mediator and outcome, this method consist of a linear regression analysis between exposure and outcome, performed without and with additional adjustment for the mediator. The difference between the regression coefficients represents the proportion of the association mediated by the mediator. The main limitations of this method are the sensitivity to bias due to measurement error, and strong assumptions regarding confounding. [85, 267] In most situations it is not possible to test these assumptions, but it is possible to perform sensitivity analyses using bias formulas. [85] While the Baron and Kenny method requires only simple analyses, it may give biased results in the case of interaction between exposure and mediator. More advanced methods have been developed to investigate interactions between exposure and mediator in their effects on the outcome. The most prominent models are the structural equation models by Vanderweele. [268, 269] As we did not observe an association between the mediators and the outcome in **Chapter 2**, we only used the Baron and Kenny method of mediation analysis to confirm the absence of mediation. Independent of which method is used to study mediation, it is essential to carefully consider strategies to fulfil assumptions regarding confounding and measurement error.

Clinical implications and future perspectives

A major challenge in current clinical practice is the early identification of individuals at a high risk of cardiovascular disease. This would allow for improved treatment strategies based on the characteristics of the patient, indicating the most intensive (preventive) treatment in individuals at the highest risk, while individuals with a lower risk could be treated less intensively. This strategy would reduce side effects and lower costs. To be able to identify individuals at the highest risk, studies should be performed that investigate the combined effect of risk factors. The recent setup of several large population-based cohorts and our ability to extensively genotype and phenotype participants are major opportunities to improve our risk stratification. The pursuit of extensions of such risk categorization models fits into the trend towards personalized medicine. Personalized medicine has a multitude of challenges, including but not limited to ethical, legal, practical, financial and social. However, these challenges are often reduced to a consideration of efficacy, effectiveness, and (cost-)efficiency. Major limiting factors are the economic capacity of a society and the willingness to allocate a substantial part of its budget to health. This may challenge researchers in exploring fields with a smaller chance of success, but might also lead to creative strategies and collaborations.

In this thesis we focused on the risk stratification within a study that included participants who were already at a higher risk of cardiovascular disease due to their high body mass index. In this thesis we observed heterogeneity in the associations between the different risk factors and measures of subclinical cardiovascular disease. This indicates the presence of unknown underlying mechanisms and suggests that effect modification may be present. While there are many potential explanations for heterogeneity in results of large studies, in this thesis we attempted to investigate subgroup differences as a source of variation. Therefore, when sample size allowed this, analyses were performed separately for women and men or for subgroups of the population with different baseline risk factors. Our results suggested that different risk factors may contribute to measures of subclinical cardiovascular disease to a different extent in women than in men. For example, in the Mendelian randomisation analysis of the effect of CETP on subclinical atherosclerosis the point estimates for women and men were in the opposite direction. While in large clinical trials CETP inhibition did not affect the risk of cardiovascular disease, our results were consistent with previous studies that detected effects of CETP on the risk of cardiovascular disease only in men or in women with diabetes. In addition, the cardiovascular effects of CETP inhibition are likely to differ by the genotype of the rs1967309 variant (*ADCY9*), with the AA genotype benefiting the most from treatment with dalcetrapib, and the

GG genotype being harmed by treatment, while heterozygous individuals had a slightly reduced risk of cardiovascular disease. [270] Analogously, the associations that we observed between fasting and postprandial triglyceride concentrations, and subclinical atherosclerosis may be more prominent in individuals at high risk of cardiovascular disease. This may indicate an interaction between risk factors and suggest that it may be useful to screen for additional risk factors in individuals at high risk for cardiovascular disease.

Extrapolating, we suggest that more studies should include investigations into sex differences and subgroup effects in the pathophysiology and treatment of cardiovascular disease.

However, we recognise that this will come at the cost of statistical power, and indeed larger sample sizes are needed to reliably draw conclusions about subgroup effects. This limitation will be amplified when subgroup analyses are employed in Mendelian randomisation studies, as by using genetic instruments power generally decreases. In the future, more large cohort studies, such as the UK Biobank, will be able to perform well-powered analyses using clinical and genetic data. Also, to investigate relations between the uniquely measured risk factors in the NEO study and clinical manifestations of disease, the use of follow-up data from this population is crucial. Also, future studies may investigate other underlying mechanisms that may mediate the relation between overweight and cardiovascular disease, such as sympathetic nervous system activation, coagulation and endothelial wear.

In conclusion, in this thesis we elucidated various pathways that may connect body fat distribution and cardiovascular disease. In **Section I** we showed that hs-CRP and GlycA as measures of inflammation, adiponectin, and leptin are not associated with clinical and subclinical cardiovascular disease in the general population. However, all may be relevant markers of disease risk. In addition, in the **Section II** we showed that postprandial triglyceride excursions, genetically-determined CETP and HDL-cholesterol, while not related with subclinical atherosclerosis in the general population, may be interesting targets to pursue in women and men separately, and in subgroups of individuals at high-cardiovascular risk. Therefore, there is still a remaining part of the association between overweight and cardiovascular disease that needs to be elucidated. Studying the interplay between different risk factors seems indispensable to advance our knowledge of the development of cardiovascular disease.

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NEDERLANDSE SAMENVATTING

Wereldwijd zijn hart- en vaatziekten een veelvoorkomende oorzaak van sterfte en zorgafhankelijkheid. Overgewicht en obesitas dragen in belangrijke mate bij aan het voorkomen van hart- en vaatziekten. De belangrijkste manieren waarop overgewicht en obesitas hart- en vaatziekten veroorzaken zijn via LDL-cholesterol, hoge bloeddruk en diabetes. Deze mechanismen verklaren echter slechts de helft van het verband tussen obesitas en hart- en vaatziekten. Het doel van dit proefschrift is om inzicht te verkrijgen in onderliggende mechanismen die de overige helft van het verband tussen overgewicht en hart- en vaatziekten verklaren.

In dit proefschrift wordt voornamelijk gebruik gemaakt van gegevens die zijn verzameld tijdens het eerste bezoek van deelnemers aan de Nederlandse Epidemiologie van Obesitas (NEO)-studie. De 6671 deelnemers waren bij inclusie tussen de 45 en 65 jaar oud, en 5000 van hen hadden een body mass index van 27 kg/m^2 of hoger. 1671 van hen zijn niet geselecteerd op body mass index, en hun gegevens worden daarom gebruikt om de resultaten te kunnen generaliseren.

In het eerste deel van dit proefschift worden risicofactoren voor hart- en vaatziekten onderzocht, die slechts recent als mogelijke risicofactor zijn beschreven: een chronische staat van ontsteking en hormonen die hun oorsprong vinden in vetweefsel. Vetweefsel past zich aan wanneer het groeit. Er worden immuuncellen aangetrokken, die zich nestelen tussen de vetcellen. Deze cellen worden geactiveerd wanneer het vetweefsel 'overvuld' raakt, en zorgen voor een verhoogde staat van ontsteking in het vetweefsel. Hierbij scheiden deze cellen ook signaalstoffen af, die elders in het lichaam kunnen zorgen voor ontstekingsreacties. In een vaatwand die aangetast is door aderverkalking vindt ook een ontstekingsreactie plaats. Het wordt gedacht dat de signaalstoffen vanuit de ontstekingsreactie in het vetweefsel de ontsteking in de vaatwand kan versterken, waardoor de ontwikkeling van aderverkalking versneld wordt. **Hoofdstuk 2** beschrijft in hoeverre een staat van chronische ontsteking het verband tussen lichaamsvet(verdeling) en aderverkalking medieert. De resultaten laten een sterk verband zien tussen lichaamsvet en de mate van ontsteking. Dit geldt voornaamelijk voor het viscerale vet, het vet in de buikholte. Desondanks zagen wij geen verband tussen twee maten van ontstekingsstaat en de mate van aderverkalking. Een mogelijke verklaring is dat de NEO-studie een relatief gezonde populatie is, waarin de mate van aderverkaling nog beperkt is.

Vetweefsel produceert ook hormonen, waarbij de productie afhangt van de hoeveelheid lichaamsvet. In dit proefschrift worden twee van deze hormonen nader onderzocht: leptine en adiponectine. De functie van leptine is het onderdrukken van de prikkel om te eten. Resistentie tegen leptine zorgt daarom voor ernstig overgewicht. Van adiponectine wordt gedacht dat het de ontwik-

keling van diabetes remt. Adiponectine wordt juist minder geproduceerd bij een grotere hoeveelheid visceraal vet. Het is opmerkelijk dat vrouwen hogere concentraties van zowel leptine als adiponectine in het bloed hebben dan mannen. Een mogelijke verklaring is dat vrouwen gemiddeld meer onderhuids vet, maar minder visceraal vet hebben dan mannen. In **hoofdstuk 3** onderzochten wij of het geslachtsverschil in concentratie leptine en adiponectine proportioneel is met het verschil in hoeveelheid lichaamsvet. De resultaten lieten geen verschil zien tussen vrouwen en mannen voor wat betreft de concentratie leptine bij gelijkblijvende hoeveelheid lichaamsvet, maar suggereerden wel dat vrouwen bij gelijkblijvende hoeveelheid visceraal vet hogere concentraties adiponectine in het bloed hebben. Daarnaast identificeerden wij 44 vrouwen met extreem hoge leptineconcentraties in het bloed. Zij hadden allen ernstig overgewicht, maar bij een deel van deze vrouwen ontdekten wij dat er ook sprake was van een genetische variant in de buurt van het gen dat codeert voor de leptinereceptor (***LEPR***) die geassocieerd is met verhoogde leptineconcentraties. Het is opmerkelijk dat deze variant alleen in vrouwen voor verhoogde leptineconcentraties zorgt.

Van leptine werd gedacht dat het naast een effect van negatieve terugkoppeling op de voedselinname, tevens bij-effecten heeft op hart- en vaatziekten. In **hoofdstuk 4** onderzochten wij door middel van een Mendeliaanse randomisatiestudie binnen de NEO-studie de effecten van leptine op drie categorieën van subklinische hart- en vaatziekten: de pompfunctie van het hart, de elektrische geleiding in het hart, en aderverkalking. Daarnaast onderzochten wij in een grote Britse populatiestudie (UK Biobank) en het CARDIoGRAMplusC4D-consortium het effect van leptine op de vaatstijfheid, en het risico op hart- en vaatziekten. De eerder beschreven verbanden tussen leptine en hart- en vaatziekten lijken naar aanleiding van dit onderzoek voornamelijk verklaard te worden door de overmaat aan lichaamsvet, die sterk gerelateerd is aan de leptineconcentraties in het bloed. De klinische relevantie van de resterende effecten lijkt beperkt te zijn.

Aan het hormoon adiponectine worden voornamelijk oorzakelijke gunstige effecten op insulinegevoeligheid en de ontwikkeling van diabetes toegeschreven. Het is daarom opmerkeljk dat in verschillende studies de observatie wordt gedaan dat mensen met hoge concentraties adiponectine in het bloed een verhoogd risico hebben op hart- en vaatziekten, en in het bijzonder hartfalen. Deze paradoxale situatie zou verklaard kunnen worden doordat deze studies zijn uitgevoerd in een populatie die geselecteerd is op een hoog risico op hartfalen. Mensen met een hoge concentratie adiponectine in het bloed lijken metabool gezonder dan mensen met een lagere concentratie. Daarom kan het voorkomen dat wanneer een individu met een hoge adiponectineconcentratie

én een hoog risico op hartfalen heeft, sterke andere risicofactoren zal moeten hebben voor dit hartfalen. Hierdoor kan het lijken alsof een normaal gesproken gunstige conditie een negatief effect heeft op ziekte. Dit heet ***collider stratification bias***. Een andere mogelijkheid is dat tijdens de fase van hart- en vaatziekten waarin een patiënt nog geen klachten heeft, er factoren zijn die invloed hebben op adiponectineconcentraties. Dit wordt ***reverse causation*** genoemd. In **hoofdstuk 5** onderzochten wij binnen de NEO-studie het verband tussen adiponectine en maten van hartfunctie. Daarnaast onderzochten wij door middel van Mendeliaanse randomisatie of, en in welke richting adiponectine en hart- en vaatziekten zich mogelijk verhouden. Wij vonden hierbij dat genetische varianten die invloed hebben op NT-proBNP, een hormoon dat door het hart wordt geproduceerd wanneer hartfalen zich ontwikkelt, ook invloed heeft op adiponectineconcentraties in het bloed. Dit suggereert dat het verband tussen adiponectineconcentraties en hart- en vaatzieken mogelijk waargenomen wordt door ***reverse causation***.

Het tweede deel van dit proefschrift behandelt de relatie tussen diverse categorieën van vet- en cholesteroldeeltjes in het bloed en de ontwikkeling van aderverkalking. Hiervoor worden nieuwe technieken gebruikt die erop gericht zijn om de diverse vet- en cholesteroldeeltjes in het bloed beter te onderscheiden, en technieken die effectiever gebruik maken van beschikbare genetische informatie over verschillende populaties heen.

De rol van CETP in de ontwikkeling van aderverkalking is onduidelijk. CETP transporteert cholesterolesters van het 'goede' HDL-cholesterol naar het 'slechte' LDL-cholesterol. Hierdoor zou de concentratie van LDL-cholesterol stijgen, wat vervolgens het risico op aderverkalking zou vergroten. Dit mechanisme is in verschillende genetische studies aangetoond, waarbij het opvallend is dat effecten slechts waargenomen worden in specifieke patiëntgroepen zoals vrouwen met diabetes of mannen met een zeer hoog risico op hart- en vaatziekten. Desondanks zijn de meeste pogingen om door middel van medicatie de activiteit van CETP te remmen er niet in geslaagd om daarmee ook in de totale populatie het risico op hart- en vaatziekten te verlagen. Daarom onderzochten wij in **hoofdstuk 6** met Mendeliaanse randomisatie het effect van CETP op de mate van aderverkalking in de halsslagader in afgebakende groepen in de populatie. Hoewel wij geen effect van CETP op de mate van aderverkalking zagen in de volledige NEO-studiepopulatie, leken er effecten in tegengestelde richting te zijn in mannen en in vrouwen, en in deelnemers met een hoog en een laag cardiometabool risico. Dit komt overeen met het beeld in de literatuur, en suggereert dat therapieën mogelijk wel effectief zouden kunnen zijn in bepaalde groepen in de populatie.

Lang werd gedacht dat hoge concentraties HDL-cholesterol in het bloed beschermend zouden werken tegen hart- en vaatziekten. Recent is in genetische associatiestudies aangetoond dat dit verband voor een groot deel berust op onderliggende lagere concentraties LDL-cholesterol en triglyceriden, die wel daadwerkelijk een oorzakelijk verband houden met een lager risico op hart- en vaatziekten. Desondanks is het nog niet duidelijk of het verband tussen HDL-cholesterol en hart- en vaatziekten deels verklaard wordt door de nuchtere waarden van triglyceriden, of de waarden daarvan na een maaltijd. Dit wordt ook ***postprandiaal*** genoemd. In **hoofdstuk 7** onderzochten wij door middel van een Mendeliaanse randomisatiestudie de gecombineerde effecten van HDL-cholesterol, postprandiale en nuchtere triglyceriden, en LDL-cholesterol op hart- en vaatziekten. Hiervoor combineerden wij gegevens van de NEO-studie en het CARDIoGRAMplusC4D-consortium. Hoewel de resultaten met betrekking tot nuchtere triglyceriden, HDL- en LDL-cholesterol bevestigd werden, bleek het genetische instrument voor postprandiale triglyceriden niet sterk genoeg om uitspraken te kunnen doen over mogelijke verklarende effecten hiervan.

Omdat mensen in het dagelijks leven een groot deel van de dag niet nuchter zijn, bestaat de hypothese dat het nuttiger is om voor het bepalen van het risico op hart- en vaatziekten te kijken naar de concentratie triglyceriden na een maaltijd dan naar nuchtere triglyceridenwaarden. Daarom hebben wij in **hoofdstuk 8** onderzocht of er een verband was tussen postprandiale triglyceriden en de vaatwanddikte in de halsslagader, bovenop het verband tussen nuchtere triglyceriden en vaatwanddikte. De postprandiale concentraties bleken niet meer te zeggen dan de nuchtere. Het vermoeden bestond dat wanneer de vaatwand beschadigd is door bijvoorbeeld roken, deze wel extra gevoelig is voor langdurig hoge concentraties triglyceriden zoals na een maaltijd. Daarom hebben wij het verband tussen postprandiale triglyceriden en vaatwanddikte tevens onderzocht in subgroepen van de studiepopulatie. Hiervoor hebben wij de populatie opgedeeld op basis van geslacht, nuchtere glucosewaarde, of hun rookgedrag. In deze analyse konden geen statistische interacties worden aangetoond, hoewel de richting van de verbanden vooral na correctie voor nuchtere triglyceridenwaarden uit elkaar bleek te lopen tussen de verschillende subgroepen.

Mendeliaanse randomisatie

In verschillende studies in dit proefschrift is gebruik gemaakt van Mendeliaanse randomisatie. Mendeliaanse randomisatie is gebaseerd op de aanname dat genetische informatie willekeurig verdeeld is over een populatie. Deze genetische informatie is een bepalende factor voor allerlei eigenschappen, waaronder risicofactoren voor hart- en vaatziekten. Mendeliaanse randomisatie maakt gebruik van de aanname dat er geen verband is tussen de genetische informatie en

omgevingsfactoren. Een voorbeeld: genetische informatie die LDL-cholesterol verhoogt, bepaalt niet de kans dat iemand begint met roken. Daarom is het mogelijk om een groep mensen mét een bepaalde genetische variant te vergelijken met een groep mensen zonder die genetische variant. Hierbij is zo min mogelijk sprake van verstoring door omgevingsfactoren. Door deze aanname hebben wij onderzoek kunnen doen naar risicofactoren die sterk samengaan met andere risicofactoren voor dezelfde ziekte: leptine in hoofdstuk 4, adiponectine in hoofstuk 5, CETP in hoofdstuk 6, en LDL- en HDL-cholesterol en triglyceriden in hoofdstuk 7.

Conclusies

1. De maten voor ontsteking die in dit proefschrift zijn onderzocht, lijken geen rol van betekenis te spelen in de voorklinische stadia van hart- en vaatziekten in de populatie.
2. Hoewel postprandiale triglyceriden, CETP en HDL-cholesterol in de totale populatie geen verband lijken te hebben met voorklinische stadia van hart- en vaatziekten, kunnen dit wel relevante metingen zijn in subgroepen van de populatie met een verhoogd risio op hart- en vaatziekten door andere eigenschappen.
3. Een groot deel van het verhoogde risico op hart- en vaatziekten dat mensen met overgewicht of obesitas lopen, is nog onverklaard.

Toekomstperspectief

Overgewicht en obesitas veroorzaken een hoger risico op hart- en vaatziekten. De tussenliggende factoren diabetes, LDL-cholesterol en hypertensie verklaren samen de helft van dit verhoogde risico, maar het overige deel is nog onverklaard. In verder onderzoek kan de rol van het immuunsysteem, hormonen, de bloedstolling en de vaatwand binnen dit verband bestudeerd worden.

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Curriculum vitae

Tim Christen werd geboren op 30 maart 1989 te Amsterdam. Hij groeide op in Leiderdorp en in 2006 behaalde hij zijn gymnasiumdiploma aan het Stedelijk Gymnasium te Leiden. In datzelfde jaar startte hij met zijn studie Biomedische Wetenschappen aan de Universiteit Leiden, waar hij in 2013 zijn Bachelor of Science-diploma behaalde. Aansluitend startte hij zijn master Biomedical Sciences met als specialisatie Research aan de Universiteit Leiden.

Tijdens zijn master raakte hij voornamelijk geïnteresseerd in het vóórkomen van ziekte in populaties, onder andere door een onderzoeksstage bij de Afdeling Klinische Epidemiologie van het Leids Universitair Medisch Centrum en het volgen van enkele cursussen bij deze afdeling.

Aansluitend aan het behalen van het Master of Science-diploma startte Tim in december 2014 met zijn promotieonderzoek bij de Afdeling Klinische Epidemiologie en de Afdeling Hartziekten van het Leids Universitair Medisch Centrum, onder supervisie van prof. dr. Wouter Jukema, dr. Stella Trompet en dr. Renée de Mutsert. Tim presenteerde de resultaten van dit onderzoek op verschillende medische congressen. Het promotieonderzoek waarvan de resultaten zijn beschreven in dit proefschrift werd afgerond in november 2018. Voor de registratie als Epidemioloog B volgde Tim verschillende epidemiologische cursussen.

Tim is nu werkzaam als onderzoeker bij Zorg en Zekerheid.