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Genetic determinants of cholesterol and energy metabolism : implications for cardiometabolic health

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Genetic determinants of cholesterol and energy metabolism

Implications for cardiometabolic health

Lisanne L. Blauw

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Genetic determinants of cholesterol and energy metabolism

Implications for cardiometabolic health

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1

Aim and general introduction

1

Scientific progress is the basis for our ever-increasing understanding of the world surrounding us. The desire to find answers and keep asking questions is the heart of science. Regarding the specific area of science that covers human biology, there is an ultimate goal: understanding human biology to increase our life span, spent in good health. Looking back at the past century, we can convincingly state that science succeeded, as life expectancy in developing countries increased with around thirty years.[1] In the 21st century, we long outlive reproductive age. The human genome has been modified over hundred thousands of years to make us the prosperous species that we are now, but in the current era of welfare evolution cannot keep up with our rapidly changing lifestyle and increasing longevity. While most infectious diseases have been combatted in the past centuries, mankind is now facing epidemics of non-communicable diseases.[2] This epidemiological transition is strikingly reflected in the global rise of cardiometabolic disorders. The prevalence of obesity has been doubled in over seventy countries since 1980.[3] By 2015, overweight and obesity were estimated to contribute to 4 million deaths worldwide, with cardiovascular disease (CVD) being the leading cause of death (2.7 million deaths), followed by diabetes (0.6 million deaths).[3] Although the numbers are evident, the underlying biological mechanisms and the risk factors of these adverse cardiometabolic phenotypes are far less understood.

The interplay between physiological factors, environmental factors, and genetic predisposition determines the individual risk of developing cardio- metabolic disease. Amplified insight in these factors will facilitate the search for preventive and curative strategies. Therefore, the aim of this thesis is to further disentangle the interwoven physiological, environmental and genetic factors that determine cholesterol and energy metabolism to increase our understanding of their contribution to cardiometabolic health, thereby coming one step closer to the ultimate goal of biomedical science.

Physiological factors

The total individual risk for developing CVD is multifactorial and highly heterogeneous between individuals. In clinical practice, the preventive treatment of CVD is therefore adapted to an individual risk estimation, which is assessed with the general systematic coronary risk estimation (SCORE) chart.[4] The SCORE chart was designed to estimate an individual's 10-year risk of fatal CVD, based on their age, sex, smoking habits, plasma total cholesterol concentration and systolic blood pressure.[5] Dependent on risk prediction clinicians can advise lifestyle changes for individuals at low to moderate risk (SCORE<5%), recommend intensive lifestyle changes or consider drug treatment for individuals at high risk (SCORE ≥5% and <10%), or start drug treatment for individuals at very-high risk (SCORE ≥10%).[4] Both the pharmacological control of circulating cholesterol concentrations and blood pres-

sure have been proven effective in reducing CVD risk, and are therefore keystones of preventive therapy.[4]

Statins are the most prescribed drugs to lower the risk of CVD.[6] These drugs reduce low-density lipoprotein cholesterol (LDL-C) concentration by inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, which functions as the rate-limiting enzyme in cholesterol synthesis. LDL particles are the main carriers of cholesterol in the circulation, and it has been firmly established that elevated LDL-C concentration fulfils a causal role in the development of CVD.[7] Although an effective reduction of LDL-C concentration by statins lowers CVD risk by around 40%, a significant residual risk is left.[6] For that reason, the search for additional lipid lowering drugs went on, and currently fibrates, bile acid sequestrants, niacin and selective cholesterol absorption inhibitors are available on the market for the treatment of dyslipidaemia.[4] Still, the development of LDL-C lowering medication is ongoing. In the beginning of 2017, the FOURIER trial with the proprotein convertase subtilisin–kexin type 9 (PCSK9) inhibitor evolocumab was completed, showing an additional 59% reduction in LDL-C on a background of statin therapy, and a reduced risk of cardiovascular events (hazard ratio 0.85; 95% confidence interval (CI) 0.79, 0.92).[8] Another potential therapeutic target to further reduce the risk of CVD is cholesteryl ester transfer protein (CETP), which is a main focus of this thesis. Given the failure of the first clinical trials with CETP inhibitors,[9–11] it is crucial to obtain further insight in the physiology of CETP and study the causal role of CETP in lipid metabolism and CVD.

Cholesteryl ester transfer protein

CETP is a protein that is known to transfer neutral lipids between circulating lipoproteins. This protein was isolated and characterized from human plasma back in 1978,[12,13] and its deoxyribonucleic acid (DNA) sequence was determined nine years later.[14] Shortly thereafter, a point-mutation in the *CETP* gene, leading to CETP deficiency, was described in a family that had a marked increase in the amount and size of their high-density lipoprotein (HDL) particles.[15] From then on, the role of CETP in lipid metabolism has been extensively studied.

The molecular shape of CETP resembles a boomerang with two pores at the far ends of the molecule that give access to a central cavity along its long-axis.[16–18] Via this central cavity, CETP is able to transfer cholesteryl esters from HDL towards apolipoprotein B (ApoB) containing triglyceride-rich lipoproteins, mainly very low-density lipoproteins (VLDL). In exchange, triglycerides are transferred from VLDL towards triglyceride-poor particles, such as HDL.[17,19,20] By the action of CETP the clearance of HDL is accelerated, as enrichment of HDL with triglycerides makes it a better substrate for hepatic triglyceride lipase.[21]

Catabolism of triglyceride-rich HDL by hepatic triglyceride lipase leads to the formation of very small HDL remnants that are rapidly cleared by the liver and kidneys.[16,21–25] On the other hand, CETP modulates the core composition of VLDL in opposite direction, by increasing its cholesteryl ester content while decreasing the triglyceride content.[20] These cholesteryl esters can subsequently be delivered to the liver via uptake of (V)LDL particles by the LDL receptor, a process referred to as indirect reverse cholesterol transport that is potentially antiatherogenic.[26,27] However, modulation of LDL and HDL particles is regarded to contribute to an overall proatherogenic action of CETP. By transferring triglycerides from VLDL to LDL, CETP modifies the LDL particle composition and enlarges the total LDL pool in plasma. Specifically in the context of hypertriglyceridemia, when more VLDL particles are present that can accept cholesteryl esters from CETP, the net transfer of triglycerides to LDL is high.[26,27] Since triglyceride-enriched LDL particles are better substrates for hepatic triglyceride lipase, LDL are converted into small dense particles that are thought to be especially proatherogenic, as they are more easily deposited in the arterial wall.[26,28,29] Thus, by reducing the amount of HDL, and modifying the size and increasing the amount LDL particles, CETP is able to induce a proatherogenic lipoprotein profile.

CETP as a target to reduce cardiovascular disease

Given the well-established inverse association between HDL-C concentration and CVD,[30] it was reasoned years ago that pharmacological inhibition of CETP could be a novel therapeutic strategy to prevent the development of CVD by raising HDL-C. Indeed, preclinical studies showed that CETP inhibition largely reduces atherosclerotic plaque formation.[31,32] Therefore, pharmaceutical companies have massively invested in the development of compounds that inhibit CETP. Since 2005, four clinical trials with CETP inhibitors have been launched. Unfortunately, the first trial with torcetrapib was terminated, because of increased mortality and morbidity in the torcetrapib-treated participants, possibly due to off-target effects on blood pressure and aldosterone concentrations.[9] The second and third trial with the CETP inhibitors dalcetrapib and evacetrapib, respectively, were also terminated, as both drugs lacked efficacy in reducing cardiovascular events on top of statin treatment.[10,11] After the failure of three clinical trials, the effectiveness of CETP inhibition therapy was largely doubted.

The origin of circulating CETP

To fully understand the role of CETP in CVD, its physiology and biological function should be fully unravelled. In addition, elucidating the genetic basis of serum CETP concentration is of importance to provide further insight in the underlying pathways that regulate CETP metabolism, which we will discuss later in more depth.

Although CETP inhibitors have been tested in clinical trials for over a decade, strikingly, it was only recently elucidated which cells in the body are responsible for the production of CETP. Adipose tissue, in particular subcutaneous adipose tissue, and the liver are the most abundant sources of *CETP* mRNA expression, and hence they were both postulated to contribute substantially to the plasma CETP pool.[20,33,34] Lately, it was shown in humans that the number of resident macrophages in the liver, which are referred to as Kupffer cells, correlates strongly with both hepatic *CETP* expression and plasma CETP concentration.[35] In the same study, waist circumference did not associate with plasma CETP concentration.[35] These findings indicate that circulating CETP is predominantly derived from Kupffer cells, and that adipose tissue may not significantly contribute to the circulating pool of CETP. However, it is unclear whether and to what extent different body fat depots contribute to plasma CETP concentration, and this needs to be established in large populations.

Regulation of *CETP* expression and CETP production

Besides its established role in lipid metabolism, accumulating evidence suggests that CETP is involved in immunity and inflammatory processes.[36] This is in line with the primary expression of *CETP* by Kupffer cells, which comprise the largest set of resident tissue macrophages in the body and play a pivotal role in inflammation and host defence against e.g. Gram-negative infections.[37,38] Kupffer cells can detect lipopolysaccharide (LPS), a potent endotoxin released from Gram-negative bacteria, and induce an antibacterial response via the release of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β).[37] Interestingly, LPS reduces liver *CETP* expression and circulating CETP concentrations in CETP-transgenic mice.[39] Similarly, LPS, TNF-α and IL-1β decrease *CETP* expression and CETP concentration in hamsters.[40] Collectively, inflammatory stimuli seem to downregulate CETP expression by Kupffer cells.

Apart from their beneficial role in host defence,[37] Kupffer cells play a detrimental role in the progression of non-alcoholic fatty liver disease (NAFLD) from simple steatosis to non-alcoholic steatohepatitis (NASH), which is characterized by liver inflammation.[41–46] With the growing prevalence of obesity worldwide, an increasing number of individuals suffer from NAFLD.[47] The central role of Kupffer cells in NAFLD is partly explained by excess free cholesterol that cannot be detoxified by esterification, leading to cholesterol crystallization not only within hepatocytes but also within Kupffer cells, and consequently activates inflammatory pathways.[42,48,49] As it has been shown that infection-related stimuli (i.e. LPS, TNF-α and IL-1β) decrease *CETP* expression, it would be interesting to study whether metabolic liver inflammation, as a component of NAFLD, is also associated with a decrease in circulating and hepatic CETP.

A more firmly established regulatory mechanism of *CETP* expression is mediated through the liver X receptor (LXR) via an LXR response element that is located in the promotor region of the *CETP* gene.[50,51] Natural ligands for LXR are oxidized derivatives of cholesterol (oxysterols) and the cholesterol precursor desmosterol.[52–54] Activation of LXR by these ligands, and subsequent binding of LXR to the LXR response element in the *CETP* gene, induces *CETP* expression.[50,51] LXRs regulate a variety of genes to control cholesterol and lipid homeostasis, thereby protecting cells from an overload of toxic free cholesterol.[55] Interestingly, *in vitro* exposure of murine macrophages to LPS, TNF-α or interferon γ (IFN-γ) supresses an LXRα-induced increase in *CETP* expression.[56] Taken together, *CETP* expression is increased by LXRα which may be counteracted by inflammatory stimuli.

Environmental factors

The environment we live in contains major risk factors for the development of cardiometabolic disease. Most well-known risk factors have been extensively described, and include among others excess caloric intake and low physical activity.[57] These risk factors contribute to an adverse positive energy balance by increasing energy intake and decreasing energy expenditure, respectively, consequently leading to weight gain.[58] Total daily energy expenditure is the sum of basal metabolic rate, diet-induced thermogenesis, and physical activity.[58] The basal metabolic rate makes the largest contribution to total energy expenditure (55-65%), and is defined as the rate at which energy is expended when an individual is lying down at rest in the postabsorptive state, in the morning at thermal neutral conditions.[58,59] When the basal metabolic rate is measured under less strict conditions, it is referred to as resting metabolic rate.[59]

One of the many contributors to resting energy metabolism is brown adipose tissue (BAT), which contributes to non-shivering thermogenesis. BAT has been estimated to contribute up to 5% of the basal metabolic rate.[59] In response to cold, BAT combusts energy stored in glucose and triglycerides into heat instead of adenosine triphosphate (ATP), by virtue of the unique presence of uncoupling protein 1 (UCP1) that uncouples β-oxidation from ATP synthesis.[60,61] BAT is located in the thorax around the aorta and subclavian arteries.[60] Apart from these classical BAT depots, so called beige adipocytes are scattered within white adipose tissue.[62] These beige cells have a low basal expression of *UCP1*, but upon cold stimulation the expression is markedly increased and their thermogenic capacity is induced.[62] Prolonged exposure to cold leads to a rise in BAT activity and a consequent modest reduction in fat mass in humans.[63] Also, preclinical studies showed that activation of BAT protects against the development of adiposity, insulin resistance, and atherosclerosis.[61,64] In this thesis, we focus on the associations of two environmental factors, namely

outdoor temperature and smoking, with resting energy expenditure and diabetes, which are potentially explained by changes in BAT activity as described below.

Effects of temperature on cardiometabolic health

As the world is facing an era of climate change, the health effects of the global rise in temperature and extreme weather conditions are an important topic of research.[65] It has been shown that extreme heat increases the hospital admissions and mortality risk for patients with CVD and diabetes, possibly due to a reduced capacity to dissipate heat.[66–69] On the other hand, an interesting recent landmark paper showed that acclimatisation of patients with type 2 diabetes to moderate cold for ten days already improved their insulin sensitivity, as demonstrated by a markedly higher glucose infusion rate during a hyperinsulinemic-euglycemic clamp.[70] It is conceivable that due to cold exposure, an increased flux of fatty acids is directed towards BAT, resulting in a compensatory increased flux of glucose to other metabolically active tissues, thereby improving systemic insulin sensitivity.[71]

It has previously been shown that BAT activity is negatively associated with outdoor temperature and is highest in winter.[72–74] Considering the putative role of BAT in the control of insulin action, combined with the effect of ambient temperature on BAT activity, it is possible that the global increase in temperature contributes to the current epidemic of type 2 diabetes. Recently, a positive association was found between outdoor temperature and glycated haemoglobin (HbA1c),[75] which suggests that systemic glucose homeostasis is influenced by environmental temperature. Therefore, studies on the relation between outdoor temperature and incidence of diabetes are warranted.

Effects of smoking on cardiometabolic health

The harmful effects of cigarette smoking are generally well-established. Half of all smokers die from a smoking-related disease, mainly CVD, lung disease and cancer.[76] In the case of CVD, smoking interacts with other risk factors in a multiplicative fashion, whereby the combination of smoking and either dyslipidaemia, hypertension or diabetes quadruples the risk of CVD.[76] Although the detrimental health effects of smoking are obvious, the fear of gaining weight after cessation is a commonly reported reason to keep on smoking,[77] as smoking cessation is associated with weight gain.[78,79]

Nicotine is thought to be the most important mediator of the effects of smoking on body weight.[78,80] Nicotine acts by various mechanisms on the body's energy balance and affects both the central nervous system and peripheral tissues by regulating the release of a wide range of neurotransmitters and hormones.[78] Studies in mice suggest that the effects of nicotine on energy metabolism mostly involve the central nervous system. More

specifically, nicotine-induced weight loss was shown to be related to inactivated hypothalamic AMP-activated protein kinase (AMPK). Inactivation of AMPK resulted in decreased appetite, while energy expenditure was increased as a result of increased locomotor and BAT activity.[81]

In humans, smoking has been shown to suppress appetite.[80] Smoking also seems to have a direct effect on energy expenditure. A study with eight healthy cigarette smokers showed an increase in total 24-hour energy expenditure after the participants smoked 24 cigarettes in 24 hours, compared with a 24-hour period without cigarette smoking[82]. In another study with 18 male smokers there was an increase in resting energy expenditure demonstrated directly after a nicotine dose via nasal spray, compared with placebo[83]. Further studies have shown that cigarette smoking increases REE in small (i.e. n ≤ 147) selected patient populations.[84–87] Overall, in several studies a positive association between smoking and REE was observed, which may contribute to the lower body weight of smokers and weight gain after smoking cessation. However, such a relationship has not been confirmed as yet in a large cohort, neither have long-term effects of smoking been investigated at the population level.

Genetic predisposition

As environmental factors play a major role in the onset of obesity and CVD, lifestyle interventions are the keystones of prevention. However, a considerable part of the inter-individual differences in obesity and CVD risk have a genetic basis. It is therefore of great importance for future intervention strategies to unravel genetic factors and related biological pathways involved in obesity and CVD.

For obesity, it has been estimated that 40-70% of the inter-individual risk differences are explained by genetics.[88] In addition, a family history of CVD is an established risk factor for developing the disease later in life.[89] Family studies showed that individuals with a positive family history of coronary artery disease had a 1.5-fold to 2.5-fold increased risk of developing the disease themselves.[89–92] Although family aggregation studies give a good estimation of the heritability of disease, the clustering of environmental risk factors within families may induce confounding, which often leads to overestimation of the true genetic component of disease.[89,92] A more in-depth approach to determine genetic risk factors for disease became available with the tremendous progress in genome sequencing techniques. In the beginning of the 21th century, the initial sequencing of the human genome was performed.[93–95] Soon afterwards, whole-genome sequencing data from larger groups of individuals with different ethnic backgrounds were collected by the International HapMap Consortium, aiming to determine the common patterns of DNA sequence variation and to

make these data freely available for future studies.[96,97]

Single nucleotide polymorphisms and genome-wide association studies

Although humans are highly diverse in their appearance, 99.5% to 99.9% of the human genome is identical between individuals.[89,96] All of our unique characteristics are defined by the remaining 0.1-0.5% of the DNA sequence. The total DNA sequence consists of 3.2×10^9 base pairs, and most of the inter-individual heterogeneity is caused by common variations in this sequence, i.e. variations occurring in more than 1% of the population.[98,99] The most commonly occurring variation in the DNA is the substitution of one base for another, which is called a single nucleotide polymorphism (SNP).[89,100] Around 10×106 common SNPs occur in the world's human population, which are estimated to explain 90% of the worldwide population heterogeneity.[96,99] SNPs arise from the incorrect incorporation of a base during DNA replication, and can be inherited when occurring in the germ cell lines.[89] When inherited, SNPs are exposed to selection pressure and depending on potential beneficial, neutral or detrimental effects on survival and fitness, their frequency either increases or decreases over time.[89,101] Until now, a large number of SNPs has been identified that affect health and disease status. For SNPs that associate with diseases that develop after the reproductive age, there is no selection pressure and SNP frequencies do not decline over time. This concept, referred to as the selection shadow, specifically holds for SNPs that associate with CVD.[89,102]

With the publication of the HapMap Project in 2003, which is an extensive database of SNP allele frequencies that cover the entire genome,[96,97] and the development of cost-efficient genotyping arrays, genome-wide association studies (GWAS) were introduced to study the causal effects of genetic variation on phenotypic traits and disease outcomes. GWAS are based on the principle that genetic variation at one locus of the DNA can predict genetic variation at an adjacent locus with high probability, and therefore it is possible to identify common SNPs in the genome that associate with disease phenotypes, by genotyping only around 500,000 well-chosen SNPs out of the total 10×106 SNPs that occur in the world population.[103] The advantage of GWAS over candidate gene approaches, is that with such a hypothesis-free approach it is possible to detect variants in genes that are not yet known to associate with the outcome, while candidate gene studies rely on *a priori* knowledge to select genes of interest.[104] However, the value of GWAS has also been questioned, one of the main reasons being that identified variants often only have small effects on disease outcomes and explain just a small fraction of heritability.[105] Nevertheless, it can be argued that the main reason for performing GWAS is not risk prediction, but rather providing clues for the underlying pathways that lead to disease,[105] which, for example, may assist in finding potential drug targets.

GWAS on cardiometabolic disease: illumination of biological pathways

In 2007, the first GWAS on coronary artery disease was performed by two groups simultaneously, both identifying the top genetic variants for coronary artery disease to be located on the short arm of chromosome 9 (i.e. 9p21).[106,107] The nearest genes for these identified loci were *CDKN2A* and *CDKN2B* that were previously not known to be associated with cardiovascular risk, and therefore the functional relevance of the identified variants remained to be established at that time. Some years later, a potential underlying mechanism was elucidated, as the 9p21 risk allele was shown to increase smooth muscle cell proliferation, which is involved in atherosclerosis and aneurysm formation.[108,109] This sequence of events clearly illustrates the added value of GWAS in identifying novel pathways that underlie disease or phenotypic traits.

In the search for genetic factors contributing to inter-individual differences in obesity risk, genetic variation in the fat mass and obesity-associated gene (*FTO*) was identified through GWAS as being the top common genetic determinant of body mass index (BMI) and obesity risk.[110–112] SNPs that cluster in the first intron of this gene are robustly associated with obesity. The top hit showed an increase in BMI of 0.39 kg/m^2 and a 1.2-fold increased obesity risk, per additional copy of the risk allele.[112] In addition, these SNPs associate with CVD, metabolic syndrome and diabetes, mostly mediated by BMI.[113] Although *FTO* was already identified in 2007 as an obesity susceptibility gene,[110,111] the biological explanation for the association between genetic variation in *FTO* and the risk of developing obesity remained largely unclear. Only recently, a potential underlying mechanism was illuminated. T to -C allele substitution in the rs1421085 SNP mapped to *FTO*, was shown to disrupt the binding of the transcriptional repressor *ARID5B* to *IRX3* and *IRX5*, which increased their expression specifically in pre-adipocytes.[114] This resulted in a developmental shift from energy-combusting beige adipocytes towards energy-storing white adipocytes *in vitro*, characterized by decreased mitochondrial thermogenesis and increased lipid storage, which may explain the increased obesity risk in C-allele carriers.[114] However, the effects of carrying the C-allele on energy metabolism in humans have not been established yet.

The genetic basis of CETP

A GWAS on circulating CETP has never been performed, and therefore the genetic basis of serum CETP concentration in the general population is largely unknown. The most studied *CETP* SNPs are the common variants Taq1B (rs708272) and -629C>A (rs1800775), which were both identified via hypothesis-driven candidate gene approaches.[115,116] With the hypothesis-free approach of GWAS, independent genetic determinants of CETP can be

identified, potentially providing new clues for underlying mechanisms involved in CETP regulation. In addition, GWAS-identified SNPs can be used as suitable genetic instruments in Mendelian randomization, as explained in the next paragraph.

Variation in the *CETP* gene as a genetic instrument in Mendelian randomization

When determining the direct association of a phenotypic trait with a disease outcome in observational studies, confounding factors can conceal a potential causal association. The definition of confounding, as described by Rothman[117] is: "Confounding in the estimation of the effect of a given factor (in producing disease) may be defined as distortion in the estimate attributable to an extraneous variate. To produce confounding, an extraneous variate must be associated -in the subjects actually studied- with the factor (or exposure) under study, and independent of this association, also be a risk indicator for disease". Although confounding factors can be adjusted or stratified for, residual confounding by unknown, unmeasured, or not accurately measured confounding factors may always remain in observational studies. With the identification of SNPs that are associated with phenotypic traits, causal associations with disease outcomes can be assessed, according to the concept of Mendelian randomization. This concept is based on the second law of Mendel, which states that the segregation of alleles during gamete formation is random.[118,119] With Mendelian randomization, genetic variation is used to define an exposed and a control group of a certain phenotypic trait within a population, with the exposed group carrying the risk allele and the control group carrying the non-risk allele of a SNP that is associated with the phenotypic trait.[118,119] Based on the random distribution of alleles in a population, which is not influenced by any environmental factor, there is no confounding in the association of SNPs and disease or trait outcomes. Another advantage of Mendelian randomization analysis is that there is no reverse causality, since the effect direction is known: genetic variation affects the disease or trait outcome and not the other way around. For these reasons, conclusions on causality can directly be drawn when comparing the exposed and unexposed groups on the disease or trait outcome.

It is important to note that for a valid Mendelian randomization analysis, three assumptions should be met: 1) the genotype is associated with the exposure; 2) there are no common causes of the genotype and the outcome; 3) the genotype is only associated with the outcome via the exposure.[120] These assumptions are strong, and violation can lead to significant bias. Therefore, it is of critical importance to carefully select a genetic instrument.[121] When this genetic instrument shows a robust association with the exposure, assumption 1 can be verified. Assumption 2 holds in general, based on the random distribution of alleles in a population. This assumption could be violated, however, in the case that ancestry is

related to both the genotype and the outcome, which is referred to as genetic confounding by population stratification. Assumption 3 is violated when biologic pleiotropy is present, which means that the genotype is associated with the outcome trough phenotypes other than the exposure.

The importance of the causality principal underlying Mendelian randomization is clearly illustrated by studies on the role of HDL-C in CVD. Whereas epidemiological studies showed that a low HDL-C concentration is associated with an increased risk of CVD,[30] Mendelian randomization showed that genetically-determined higher HDL-C concentrations do not lower the risk of myocardial infarction.[122] This implies that the association between HDL-C concentration and CVD is not causal, and that intervening on HDL-C concentration per se, e.g. pharmacological therapy, will not protect against CVD. Similar Mendelian randomization studies for CETP may shed light on the causal effects of CETP on circulating lipid concentrations, lipoprotein composition, and CVD risk.

Interestingly, a clear analogy exists between randomized controlled trials and Mendelian randomization studies (Figure 1.1) that have therefore commonly been referred to as nature's randomized trials.[123,124] Due to this analogy, Mendelian randomization studies can anticipate the results of randomized trials. However, results from Mendelian randomization cannot be directly translated to the results of a randomized trial.[125] The main reason for this, is that most causal exposures have a cumulative effect over time on the associated outcome. Since Mendelian randomization is based on carriage of genetic variants that causally associate with the exposure, risk allele carriers will be subject to life-long exposure, and results from Mendelian randomization will therefore in general overestimate the effect of short-term randomized intervention studies.[125]

Cohort studies and genetic consortia

The studies described in this thesis were performed with data from cohort studies and genetic consortia. The study populations and study design of these cohorts and consortia are summarized below.

The NEO study

To fulfil the aims of the studies described in **Chapters 2, 4, 5, 6, 8 and 9** we used data from the Netherlands Epidemiology of Obesity (NEO) study.[126] The NEO study is a population-based prospective cohort study including 6,671 men and women in the age of 45 to 65 years. This cohort was designed to study pathways that lead to disease in persons with overweight or obesity. Therefore, individuals with a BMI of ≥27 kg/m^2 were oversampled. Participants were recruited in the area of Leiden, The Netherlands, from September 2008

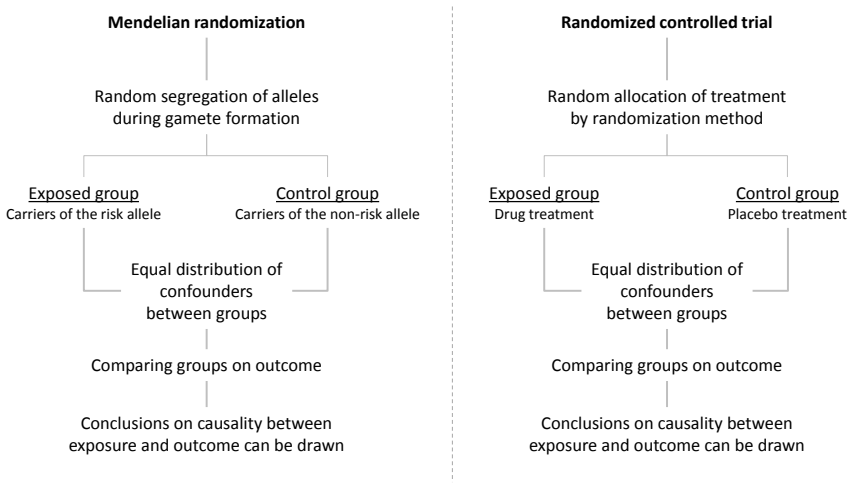


Figure 1.1: Schematic representation of the clear analogy between Mendelian randomization studies and randomized controlled trials. Adapted from Davey Smith G, Ebrahim S. BMJ 2005; 330: 1076-9.

until September 2012. At baseline, participants completed questionnaires for demographic, lifestyle, and clinical information and underwent an extensive physical examination, including anthropometric measurements and blood sampling. All participants were genotyped. A random subgroup of 1,434 participants underwent indirect calorimetry to measure resting metabolic rate.

Bariatric surgery cohort

For **Chapter 3**, we used data from a Dutch bariatric surgery cohort. The study population consisted of 93 severely obese men and women who underwent elective bariatric surgery between 2006 and 2009 at the Department of General Surgery, Maastricht University Medical Center (Maastricht, The Netherlands).[127] Subjects using anti-inflammatory drugs or with acute or chronic inflammatory diseases, degenerative diseases, and subjects reporting alcoholic intake >10 g/day were excluded. Blood samples were drawn on the morning of surgery after 8 hours of overnight fasting. During surgery, wedge biopsies of the liver were taken.

Genetic consortia and GWAS meta-analyses

For the Mendelian randomization studies, which we described in **Chapters 4 and 6**, we used publically available summary data from the genetic consortia CARDIoGRAMplusC4D and GLGC, and the GWAS meta-analysis from the MAGNETIC NMR study. In general, the aim of genetic consortia is to collect data from a large number of genotyped cohorts

to perform GWAS on the outcome of interest, and subsequently combine the results in a meta-analysis to find robust effect estimates. The Coronary ARtery DIsease Genome wide Replication and Meta-analysis plus The Coronary Artery Disease Genetics (CARDIoGRAMplusC4D) consortium is a collaborative effort to perform a GWAS meta-analysis on coronary artery disease, including 60,801 cases and 123,504 controls from 48 studies of mainly European ancestry populations.[128] The Global Lipids Genetics Consortium (GLGC) was set up to perform a GWAS meta-analysis on concentrations of LDL-C, HDL-C, triglycerides and total cholesterol.[129] This meta-analysis was based on 188,577 individuals of European ancestry from 60 different studies. The MAGNETIC NMR GWAS is a GWAS meta-analysis on 123 blood lipid and metabolite concentrations that were quantified by high-throughput nuclear magnetic resonance (NMR) spectroscopy.[130] In total, 24,925 individuals were included from 10 European studies.

Outline of this thesis

The first part of this thesis is focussed on the role of CETP in cardiovascular health, aiming 1) to elucidate the determinants and genetic basis of circulating CETP concentrations, and 2) to determine the causal effects of CETP on the lipoprotein metabolite profile and on coronary artery disease. With the recent identification of Kupffer cells as the main contributors to circulating CETP concentrations, the previously proposed role of adipose tissue in CETP production was questioned. Therefore, the aim of **Chapter 2** was to study the associations of an extensive range of body fat measures with serum CETP concentration in a large population-based cohort. Subsequently, based on the primary expression of *CETP* by cells of the innate immune system and accumulating evidence suggesting that CETP is involved in immunity and host-defences, we hypothesized that the presence of liver inflammation is a determinant of hepatic and circulating CETP. Therefore, in **Chapter 3**, we describe the associations of metabolic liver inflammation with hepatic *CETP* expression and CETP positive cells, using liver biopsy samples collected from a bariatric surgery cohort, and with plasma CETP concentration. Then, to assess the genetic determinants of CETP, we performed the first GWAS and exome-wide analysis on serum CETP concentration to identify common and rare genetic variants that determine circulating CETP, as described in **Chapter 4** and **Chapter 5**. With the GWAS-identified SNPs that independently determine CETP concentration, we were able to mimic a randomized clinical trial design by using Mendelian randomization. With this approach, we investigated the causal effects of a change in CETP concentration on coronary artery disease risk (**Chapter 4**) and on the lipoprotein metabolite profile (**Chapter 6)**, which is relevant to better understand the outcomes of the CETP inhibitor trials that aim to reduce CVD.

The focus of the second part of this thesis is on energy metabolism in cardiometabolic health. Specifically, we aimed to study environmental and genetic factors that were previously described to influence BAT activity, by assessing their association with energy expenditure and disease outcomes. Activation of BAT increases energy expenditure, as this tissue is capable to produce heat in response to cold by combusting energy that is stored in glucose and triglycerides. Based on a recent landmark paper, which showed that a 10 day cold acclimatisation period activates BAT and improves insulin sensitivity in patients with type 2 diabetes, we assessed in **Chapter 7** the association of outdoor temperature with the incidence of diabetes and the prevalence of glucose intolerance, on a country-wide and global scale. Since cigarette smoking reduces body weight, which is determined by the balance between energy intake and energy expenditure, we hypothesized that part of the weight reduction in smokers is explained by a nicotine-induced increase in resting energy expenditure. Therefore, we studied in **Chapter 8** the association between smoking and resting energy expenditure in 1,189 study participants from the NEO study. Finally, based on a landmark paper from 2015 that described a potential genetic basis for BAT, since it was shown that T-to-C substitution in the rs1421085 variant of the *FTO* gene reduced browning of pre-adipocytes *in vitro*, we aimed to study the metabolic effects of genetic variation in this SNP in humans. Humans who carry the C-allele of rs1421085 may have reduced browning of their white fat depots, which possibly leads to a lower energy expenditure and consequently an increased risk of obesity. As brown fat cells selectively enhance the oxidation of fat over glucose, we studied in **Chapter 9** the association between rs1421085-risk allele carriage and whole-body fat oxidation in 1,246 participants of the NEO study.

In the last part of this thesis, the study findings, their implications for future research, and the perspectives for preventive and therapeutic strategies of cardiometabolic disease are discussed (**Chapter 10**).

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2

Serum CETP concentration is not associated with measures of body fat: The NEO study

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2

Abstract

Introduction

Adipose tissue has been postulated to contribute substantially to the serum cholesteryl ester transfer protein (CETP) pool. However, in a recent large cohort study waist circumference was not associated with plasma CETP. The aim of the present study was to further examine associations of accurate measures of body fat and body fat distribution with serum CETP concentration.

Methods

In this cross-sectional analysis of the Netherlands Epidemiology of Obesity study, we examined in 6,606 participants (aged 45-65 years) the associations of total body fat, body mass index (BMI), waist circumference, waist-to-hip ratio (WHR), abdominal subcutaneous (aSAT) and visceral adipose tissue (VAT) assessed with magnetic resonance imaging (n=2,547) and total and trunk fat mass assessed with dual-energy X-ray absorptiometry (n=909) with serum CETP concentration. Regression models were adjusted for age, ethnicity, sex, dietary intake of fat and cholesterol, physical activity, smoking and menopausal status.

Results

Mean (SD) age was 56 (6) years and BMI 26.3 (4.4) kg/m^2, 56% were women. Mean serum CETP concentration was 2.47 μg/mL. The difference in serum CETP was 0.02 μg/mL (95%CI: -0.01, 0.05) per SD total body fat (8.7%), and 0.02 μg/mL (0.00, 0.04) per SD BMI (4.4 kg/m^2). Similar associations around the null were observed for waist circumference, WHR, aSAT, VAT, total and trunk fat mass.

Conclusion

In this population-based study, there was no evidence for clinically relevant associations between several measures of body fat and serum CETP concentration. This finding implies that adipose tissue does not contribute to the CETP pool in serum.

Introduction

Cholesteryl ester transfer protein (CETP) facilitates the net flux of cholesteryl esters from high-density lipoproteins (HDL) towards (very) low-density lipoproteins ((V)LDL), coupled to a net flux of triglycerides (TG) from (V)LDL to HDL.[1] As such, CETP contributes to an atherogenic lipoprotein profile (i.e. high LDL/HDL-cholesterol (C) ratio), as has been extensively studied in both humans and in mice transgenic for human CETP.[2,3]

Because CETP contributes to an adverse lipoprotein profile and high LDL-C levels have been associated with increased cardiovascular disease risk,[4,5] a reduction of CETP activity in plasma has been proposed as a strategy to improve the lipoprotein profile and reduce cardiovascular disease.[6] This could be achieved by inhibition of plasma CETP with small molecules (i.e. CETP inhibitors), of which anacetrapib[7] and TA-8995[8] are currently being tested in clinical trials. Inhibition of CETP expression in tissues contributing to the pool of plasma CETP may be an alternative approach, but the tissues and cell types responsible for determining plasma CETP levels are still not fully elucidated.

Adipose tissue, in particular subcutaneous adipose tissue, and the liver are the most abundant sources of CETP mRNA expression1,[9–11] and hence they have both been postulated to contribute substantially to the plasma CETP pool.[1,12,13] Small studies indeed suggested that adipose tissue contributes to the plasma CETP concentration.[12,14–18] However, most of these studies compared measures of body fat content with plasma CETP activity (assessed by lipid transfer between lipoproteins)[14,16,17], instead of plasma CETP concentration by enzyme-linked immunosorbent assay (ELISA), and plasma CETP activity is highly dependent on the lipoprotein context during the measurement.[19] In addition, a wide variety of methods has been used to measure CETP activity,[19] yielding comparison of CETP activity between different studies difficult. Consequently, CETP activity does not accurately represent plasma CETP concentration and the interpretation of previous study results regarding the contribution of adipose tissue to the plasma CETP pool is therefore questionable. Few studies related plasma CETP concentration to measures of body fat. One study (n=80) found higher CETP concentrations in obese individuals (body mass index (BMI) 30-45 kg/m^2), than in lean participants (BMI 18.5-25 kg/m^2)[15], and in another study (n=44) a positive correlation was observed between total fat mass, measured with bioelectrical impedance analysis (BIA), and plasma CETP concentration.[18]

Interestingly, in a large population-based study (654 men and 780 women, 40-70 year) we recently observed that waist circumference was not associated with the plasma CETP concentration[20], which suggested that central adipose tissue does not contribute to CETP in plasma. However, waist circumference is a crude measure of adipose tissue mass

and does not discriminate between the abdominal subcutaneous and visceral fat depots. Therefore, the contribution of specific adipose tissue depots to plasma CETP remains unresolved. The aim of the present study was to examine the associations between measures of body fat and serum CETP concentration in a large, population-based study, to elucidate the contribution of adipose tissue to CETP in serum.

Materials and Methods

Study design and study population

The Netherlands Epidemiology of Obesity (NEO) study is a population-based prospective cohort study of men and women aged between 45 and 65 years, with an oversampling of persons with a BMI of 27 kg/m^2 or higher. The present study is a cross-sectional analysis of the baseline measurements. Detailed information about the study design and data collection has been described elsewhere.[21]

NEO study participants were recruited from September 2008 until September 2012 in the greater area of Leiden, The Netherlands, to build up a population-based cohort of 6,671 participants. Men and women out of four Dutch municipalities, aged between 45 and 65 years with a BMI of 27 kg/m^2 or higher were eligible to participate. From one municipality (Leiderdorp, The Netherlands) all inhabitants aged between 45 and 65 years were invited to participate regardless of their BMI, in order to obtain a reference distribution for BMI. Participants visited the NEO study center after an overnight fast of at least 10 hours for extensive baseline measurements, including blood sampling and anthropometry. Research nurses recorded current medication use. Prior to the study visit, participants completed questionnaires at home with respect to demographic, lifestyle, and clinical information.

At the study center 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 or claustrophobia). A body circumference of more than 1.70 m was an additional contraindication for undergoing MRI. Of the participants who were eligible for MRI, approximately 35% were randomly selected to undergo direct assessment of abdominal subcutaneous adipose tissue (aSAT) and visceral adipose tissue (VAT). Another random subset, approximately 15% of the total population, underwent dual-energy X-ray absorptiometry (DXA) to measure total fat mass and trunk fat mass. In the present analysis, we excluded study participants with missing data on serum CETP concentration (n=65). The present study population therefore comprises 6,606 participants.

The NEO study was approved by the medical ethics committee of the Leiden University Medical Center (LUMC) and all participants gave written informed consent.

Measures of body fat

Body weight and total body fat were determined with the Tanita bio-impedance balance (TBF-310, Tanita International Division, UK) without shoes and with subtraction of one kilogram (kg) to correct for the weight of clothing. BMI was calculated by dividing the weight in kilograms by the height in meters squared. During physical examination waist circumference was measured mid-way between the lower costal margin and the iliac crest and hip circumference was measured at the maximum circumference of the buttocks. Subsequently the waist-to-hip-ratio (WHR) was calculated.

aSAT and VAT were quantified by MRI. Of the total NEO study population 2,580 participants underwent MRI of the abdomen. MRI images were acquired during a breath-hold using a turbo spin echo imaging protocol (1.5 Tesla MR system, Philips Medical Systems, Best, The Netherlands). Three transverse images with a slice thickness of 10 mm were obtained at the level of the fifth lumbar vertebra. Abdominal subcutaneous and visceral fat depots were quantified by converting the number of pixels to square cm (MASS, Medis, Leiden, the Netherlands). The average of the three slices was used for analyses. In 11 participants the images were of insufficient quality for the quantification of aSAT and VAT, and therefore 2,569 participants had a successful measurement of aSAT and VAT.

DXA was performed in 915 NEO study participants. With a whole body DXA scan (Hologic Discovery A, Tromp Medical BV, Castricum, The Netherlands) total fat mass (kg) was measured. The abdominal region was defined as 20% from pelvis cut to neck cut, and trunk fat mass (kg) comprised the weight of adipose tissue in this region. 912 participants had successful measurements of total and trunk fat mass, as three DXA scans were of insufficient quality.

Serum CETP concentration

During the visit to the NEO study center venous blood samples were obtained from the antecubital vein from all participants after a 10 hour overnight fast. Aliquots of plasma and serum were stored after centrifugation at -80°C. From 11 April until 16 July 2014 CETP concentrations in serum that had undergone one previous freeze-thaw cycle were measured with enzyme-linked immune sorbent assay (ELISA) kits according to the manufacturer's instructions (DAIICHI CETP ELISA, Daiichi, Tokyo, Japan).

Covariates

Questionnaires were sent to all participants and completed at home. The general questionnaire included questions on demographic, lifestyle and clinical data. Smoking status was categorized into never smoker, former smoker and current smoker. Menopausal status

was classified as premenopausal, perimenopausal (menopausal during last year) or postmenopausal. Participants were classified as having pre-existing cardiovascular disease when a medical history of myocardial infarction, angina, congestive heart failure, stroke or peripheral vascular disease was reported via the questionnaire. Participant were classified as having diabetes when the disease was self-reported via the questionnaire, when antidiabetic medication was used, or when a fasting glucose ≥7.0 mmol/L was measured at baseline. Dietary intake of energy, fat, cholesterol and alcohol was assessed with a semi-quantitative food frequency questionnaire (FFQ)[22], and calculated from the FFQ using the 2011 version of the Dutch food composition table (NEVO-2011). Nutrient intake was expressed as energy percentage (En%) of total energy intake. Physical activity during leisure time was evaluated with the short questionnaire to assess health-enhancing physical activity (SQUASH)[23], and expressed as metabolic equivalents of task (MET)*hours/week, with MET indicating the intensity of an activity. 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). Fasting LDL-C concentrations were calculated using the Friedewald equation.[24]

Statistical analyses

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[25], adjustments for the oversampling of individuals with a BMI ≥27 kg/m^2 were made. This was done by weighting all participants towards the BMI distribution of participants from the Leiderdorp municipality[26], whose BMI distribution was similar to the BMI distribution of the general Dutch population in the age range of 45-65 years.[27] In practice, this means that participants with a lower BMI were assigned larger weights than participants with a higher BMI in the analyses. All results are based on weighted analyses, and therefore apply to a population-based study without oversampling of individuals with overweight or obesity. As a consequence, the weighted characteristics of the population are expressed in proportions instead of absolute numbers.

Descriptive statistics of the study population were summarized as mean (standard deviation, SD) or proportions and stratified by sex and total body fat. We considered total body fat assessed with BIA a more accurate measure of body fat than BMI, and categorized a total body fat percentage greater or equal to 25% as high body fat in men, and greater or equal to 35% as high body fat in women.[28–30]

Weighted linear regression analyses were performed to evaluate the associations between standardized z-scores of measures of body fat (i.e. total body fat, BMI, weight circumference, WHR, aSAT, VAT, total fat mass and trunk fat mass) and serum CETP concentration. Differences in serum CETP concentration (beta coefficients from linear regression) were expressed per SD increase in body fat measure. Standardization of body fat measures allowed for comparison of the strengths of the associations between the different body fat determinants. Crude models (model 1) were adjusted for age, ethnicity and sex (model 2). Subsequently, dietary fat intake, dietary cholesterol intake, physical activity, smoking status and menopausal status were added as confounding variables (model 3). As total body fat is strongly related to waist circumference, WHR, VAT and trunk fat mass,[31] the linear regression models of the associations between each of these variables and serum CETP concentration were additionally adjusted for total body fat percentage (model 4). Weighted mean differences and corresponding 95% confidence intervals (CI) were reported. We examined whether associations differed between men and women by including product terms between sex and measures of body fat in the regression models. As there is evidence that lipid-lowering drugs (e.g., statins, fibrates and niacin) lower the plasma CETP concentration32-37, we also examined the presence of interaction between lipid lowering drug use and measures of body fat by including product terms of lipid lowering drug use with measures of body fat into the models. When product terms were statistically significant, stratified analyses were performed.

All analyses were performed using STATA Statistical Software (Statacorp, College Station, Texas, USA), version 12.0.

Results

Population characteristics

Descriptive statistics of the 6,606 included participants are presented in Table 2.1. Serum CETP concentrations were similar for participants with high and lower body fat, both in men and women.

Lipid lowering drugs were used by 10.7% of participants (10.4% used a statin, 0.1% a fibrate, 0.1% both a statin and a fibrate, and 0.1% a bile acid sequestrant, a cholesterol absorption inhibitor or niacin). Of the lipid lowering drug users, 38% were women and mean (SD) age was 59 (5) years. For men mean (SD) total body fat was 27.8 (6.1)% and for women 40.9 (5.8)%. 5,572 participants did not use lipid-lowering drugs, 58% were women and mean (SD) age was 55 (6) years. For men mean (SD) total body fat was 24.5 (5.7)% and for women 36.6 (6.6)%.

Serum CETP concentration was higher in women (2.60 μg/mL, 95% CI 2.57, 2.63) than in men (2.31 μg/mL, 95% CI 2.28, 2.34). Figure 2.1 shows that serum CETP concentrations were lower in users of lipid lowering drugs than in non-users, both in men (1.98 μg/mL, 95% CI 1.90, 2.06 vs 2.37 μg/mL, 95% CI 2.34, 2.40) and in women (2.21 μg/mL, 95% CI 2.11, 2.32 vs 2.63 μg/mL, 95% CI 2.60, 2.66).

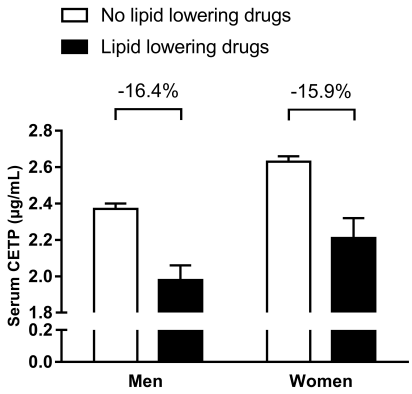


Figure 2.1: Mean serum CETP concentration in non-users and users of lipid lowering drugs, stratified by sex. Results are based on analyses weighted towards the normal BMI distribution, with n=6,606; 1,034 users of lipid lowering drugs, 5,572 non-users. Error bars represent 95% confidence intervals.

Associations between measures of body fat and serum CETP concentration

In Table 2.2 the crude and adjusted differences in serum CETP concentration per SD of the body fat measures are presented. After adjustment for all potential confounding factors total body fat, waist circumference, WHR, aSAT and trunk fat mass were not associated with serum CETP concentration. Per 4.4 kg/m^2 increase in BMI the difference in serum CETP was 0.02 μg/mL (95% CI 0.00, 0.04). Per 56.3 cm^2 increase in VAT the difference in serum CETP was -0.05 μg/mL (95% CI -0.10, -0.01). Per 7.0 kg increase in total fat mass (DXA) the difference in serum CETP was -0.06 μg/mL (95% CI -0.10, -0.02). Figure 2.2 shows the differences in serum CETP concentration per SD of the body fat measures, after adjustment for all confounding factors.

The associations between the measures of body fat and serum CETP concentration did not differ between men and women (p-values of product terms of measures of body fat with sex ranged from 0.154 to 0.988 in adjusted models). Likewise, the associations between body fat measures and serum CETP concentration did not differ between users and non-users of lipid lowering drugs (p-values of product terms of measures of body fat with lipid lowering drug use ranged from 0.053 to 0.934 in adjusted models), except for total body fat. The p-value for the product term of total body fat with lipid lowering drug use was 0.039.

Analyses stratified by use of lipid lowering drugs showed after adjustment for all potential confounding factors, that for users of lipid lowering drugs the difference in serum CETP was -0.02 μg/mL (95% CI -0.11, 0.07) per SD of total body fat (8.8%), and for non-users the difference was 0.06 μg/mL (95% CI 0.03, 0.09) per SD of total body fat (8.6%).

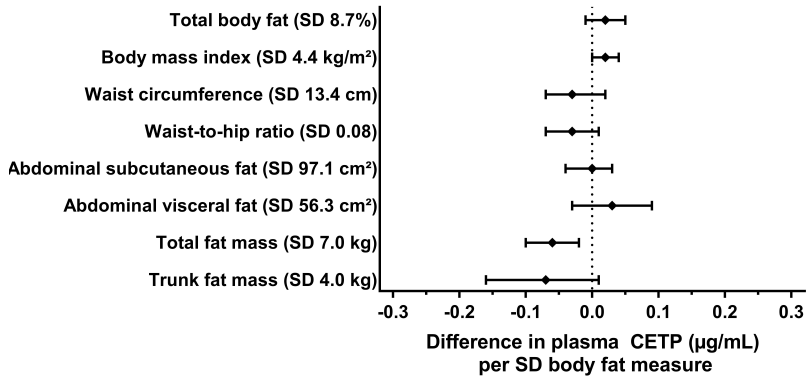


Figure 2.2: Adjusted difference[a] in serum CETP concentration per standard deviation body fat measure, for the total study population. Results are based on analyses weighted towards the normal BMI distribution, with n=6,606; magnetic resonance imaging was performed in a random subsample with n=2,547, dual-energy X-ray absorptiometry was performed in a random subsample with n=909. Error bars represent 95% confidence intervals.

[a] Beta coefficient from linear regression adjusted for age, ethnicity, sex, dietary fat intake, dietary cholesterol intake, physical activity, smoking and menopausal status. Additional adjustment for body fat percentage was restricted to the linear regression analyses of waist circumference, waist-to-hip ratio, trunk fat mass and abdominal visceral fat.

Table 2.1: Demographic and clinical characteristics of the Netherlands Epidemiology of Obesity study population (aged 45-65 years) without participants with missing data on serum CETP concentration, stratified by sex and total body fat.

Characteristics	Men		Women	
	Total body fat (%)[a]			
	<25	**≥25**	**<35**	**≥35**
Proportion of participants (%)	24	20	21	35
Ethnicity (% white)	94	97	93	95
Age (year)	56 (7)	56 (7)	55 (5)	56 (5)
Educational level (% high)[b]	55	40	55	38
Tobacco smoking (% current and former smokers)	60	72	56	60
Physical activity during leisure time (MET hours/week)	40 (37)	37 (39)	40 (26)	34 (28)
Menopausal status (% postmenopausal)	-	-	56	63
Mean daily dietary intake				
Dietary fat intake (En% derived from total fat intake)	33.9 (6.0)	34.5 (6.5)	34.2 (4.9)	34.3 (5.3)
Dietary cholesterol intake (mg/day)	219 (93)	236 (108)	178 (68)	192 (72)
Alcohol intake (g/day)	19 (19)	22 (23)	10 (9)	11 (12)
Measures of body fat				
Total body fat (%)	20.8 (3.2)	29.9 (5.1)	30.1 (3.4)	40.9 (4.0)
Body mass index (kg/m^2)	24.6 (2.2)	29.5 (3.8)	22.1 (1.7)	28.2 (4.4)
Waist circumference (cm)	92 (7)	106 (11)	76 (5)	94 (11)
Waist-to-hip ratio	0.91 (0.06)	0.98 (0.06)	0.80 (0.05)	0.87 (0.06)
Abdominal subcutaneous fat (cm^2)	167 (52)	264 (91)	180 (48)	316 (86)
Abdominal visceral fat (cm^2)	88 (43)	150 (67)	39 (18)	87 (40)
Total fat mass (kg)	20.1 (4.1)	29.6 (8.1)	19.6 (3.2)	31.9 (7.8)
Trunk fat mass (kg)	9.9 (2.6)	16.0 (4.6)	8.2 (2.0)	15.1 (4.1)
Fasting serum concentrations				
CETP (μg/mL)	2.30 (0.63)	2.32 (0.70)	2.58 (0.58)	2.61 (0.63)
Total cholesterol (mmol/L)	5.48 (1.07)	5.56 (1.21)	5.69 (0.87)	5.86 (1.01)
HDL-cholesterol (mmol/L)	1.44 (0.39)	1.24 (0.34)	1.94 (0.40)	1.64 (0.39)
LDL-cholesterol (mmol/L)	3.47 (1.00)	3.52 (1.12)	3.34 (0.81)	3.63 (0.92)
Triglycerides (mmol/L)	1.19 (0.95)	1.67 (1.15)	0.86 (0.45)	1.23 (0.71)
Comorbidity and medication				
Diabetes (%)	3	13	1	6
Cardiovascular disease (%)	6	10	3	6
Lipid lowering drugs (%)	9	22	2	10

Results are based on analyses weighted towards the normal BMI distribution, with n=6,606 (3,134 men; 3,472 women)[c]; magnetic resonance imaging was performed in a random subsample with n=2,547, dual-energy X-ray absorptiometry was performed in a random subsample with n=909.

[a] High body fat was defined as a total body fat percentage ≥25% for men and ≥35% for women. [32]

[b] High educational level: higher secondary education (according to Dutch educational system), higher vocational education, university, PhD.

[c] Missing data: n=13 for ethnicity, n=68 for educational level, n=8 for smoking status, n=3 for menopausal status, n=5 for dietary fat intake, n=4 for dietary cholesterol intake, n=4 for alcohol intake, n=120 for physical activity, n=31 for total body fat, n=7 for waist circumference, n=7 for waist-to-hip ratio, n=20 for total cholesterol, n=21 for HDL-cholesterol, n=22 for LDL-cholesterol, n=21 for triglycerides, n=28 for cardiovascular disease, n=40 for diabetes.

BMI, body mass index; CETP, cholesteryl ester transfer protein; En%, energy percent; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MET, metabolic equivalents of task.

Table 2.2: Difference in serum CETP concentration per standard deviation body fat measure, for the total study population.

Body fat measure	Model	Difference in serum CETP concentration (μg/mL)[a]	95% Confidence interval
Total body fat	1	0.11	0.09, 0.13
SD: 8.7%	2	0.03	0.00, 0.06
	3	0.02	-0.01, 0.05
Body mass index	1	0.01	-0.01, 0.03
SD: 4.4 kg/m^2	2	0.03	0.01, 0.05
	3	0.02	0.00, 0.04
Waist circumference	1	-0.05	-0.07, -0.03
SD: 13.4 cm	2	0.01	-0.01, 0.03
	3	0.01	-0.02, 0.03
	4[b]	-0.03	-0.07, 0.02
Waist-to-hip ratio	1	-0.09	-0.12, -0.07
SD: 0.08	2	-0.01	-0.04, 0.02
	3	-0.01	-0.04, 0.02
	4[b]	-0.03	-0.07, 0.01
Abdominal subcutaneous adipose tissue	1	0.04	0.01, 0.07
SD: 97.1 cm^2	2	0	-0.03, 0.03
	3	0	-0.04, 0.03
Visceral adipose tissue	1	-0.1	-0.13, -0.06
SD: 56.3 cm^2	2	-0.04	-0.08, -0.01
	3	-0.04	-0.08, -0.01
	4[b]	-0.05	-0.10, -0.01
Total fat mass	1	-0.01	-0.05, 0.03
SD: 7.0 kg	2	-0.05	-0.09, -0.01
	3	-0.06	-0.10, -0.02
Trunk fat mass	1	-0.05	-0.09, -0.01
SD: 4.0 kg	2	-0.05	-0.09, -0.01
	3	-0.06	-0.10, -0.02
	4[b]	-0.07	-0.16, 0.01

Results are based on analyses weighted towards the normal BMI distribution, with n=6,606[c]; magnetic resonance imaging was performed in a random subsample with n=2,547, dual-energy X-ray absorptiometry was performed in a random subsample with n=909.
Model 1: crude.
Model 2: adjusted for age, ethnicity and sex.
Model 3: model 2 + adjusted for dietary fat intake, dietary cholesterol intake, physical activity, smoking status and menopausal status.
Model 4[b]: model 3 + adjusted for body fat percentage.

[a] Beta coefficients from linear regression; expressed per SD increase in body fat measure.

[b] Additional adjustment for body fat percentage was restricted to the linear regression analyses of waist circumference, waist-to-hip ratio, trunk fat mass and abdominal visceral fat.

[c] Missing data: n=31 for total body fat, n=7 for waist circumference, n=7 for waist-to-hip ratio, n=13 for ethnicity, n=5 for dietary fat intake, n=4 for dietary cholesterol intake, n=120 for physical activity, n=8 for smoking status, n=3 for menopausal status.

Discussion

Based on the contradiction between findings of initial small studies reporting a positive relation between body fat and plasma CETP[12,14–18], and our recent finding that waist circumference was not associated with plasma CETP concentration[20], we aimed to further examine the contribution of adipose tissue to the serum CETP concentration in a large, population-based study. In 6,606 participants of the NEO study body fat and body fat distribution were extensively characterised using anthropometric measures, BIA, MRI and DXA. There was no evidence for clinically relevant associations between measures of body fat and serum CETP concentration as assessed by ELISA. This finding implies that adipose tissue does not contribute to the CETP pool in serum.

Several previous studies showed a positive association between body fat and plasma CETP[12,14–18], but these studies were relatively small, and mostly measured plasma CETP activity. Various CETP activity assays have been developed that measure either exogenous or endogenous lipid transfer capacity of CETP. Since endogenous CETP activity measurements are dependent on the lipoprotein concentrations in plasma, such assays do not necessarily reflect CETP concentration.[19] Furthermore, several of these studies presented merely crude differences and correlations, and did not account for any potential confounding factors. In the present study total body fat, waist circumference, WHR, aSAT and trunk fat mass were not associated with serum CETP concentration after adjustment for potential confounding factors. Although BMI, VAT and total fat mass (DXA) were statistically significant associated with serum CETP, the associations were weak and in opposite directions. For example, for an individual with a length of 1.80 m, the association of BMI represents a 0.8% increase in mean serum CETP concentration (2.47 μg/mL) following a weight gain of 14.3 kg. Therefore, we consider these associations to be of no clinical relevance.

Since we and others have shown that lipid-lowering drugs, including statins, decrease the plasma CETP concentration[33–38], we tested for interaction between lipid lowering drug use and measures of body fat. Except for total body fat, there was no interaction between measures of body fat and use of lipid lowering drugs. This indicates that, although users of lipid lowering drugs have lower serum CETP concentrations, the associations between these body fat measures and serum CETP concentration do not differ between users and non-users of lipid lowering drugs. The exclusion of users of lipid lowering drugs in the model for total body fat showed a difference of 0.06 μg/mL (95% CI 0.03, 0.09) in serum CETP concentration per 8.6% increase in total body fat. We consider this association to be clinically irrelevant.

The detection of statistically significant, but clinically irrelevant associations may be explained by the large sample size of the present study, making it possible to detect even very small differences. Moreover, BMI and total body fat are less precise measures of body fat than DXA measurements [39], which were not positively associated with serum CETP concentration. We observed a weak negative association between total fat mass (DXA) and serum CETP concentration (-0.06 μg/mL per 7.0 kg higher total fat mass), arguing against the contribution of adipose tissue to CETP in serum.

It has been suggested that there are tissue specific differences in the contribution of adipose tissue to plasma CETP [12,17], as the expression of CETP mRNA in subcutaneous adipose tissue is higher than in visceral adipose tissue11. In this large study population there was no positive associations between either aSAT or VAT with serum CETP concentration (n=2,547), indicating that neither visceral nor subcutaneous adipose depots contributes to the plasma CETP concentration. Recently, we observed in 93 obese individuals that the CETP mRNA expression in the liver largely exceeds the expression in both subcutaneous and visceral adipose tissue. [20] In the liver CETP expression in confined to hepatic macrophages (Kupffer cells) and hepatic Kupffer cell content strongly correlates with plasma CETP concentration. [20]

It is of interest that weight loss was shown to decrease plasma CETP concentration [17,40–42], which at first glance may seem to be contrary to our present findings. However, it is plausible that effects other than a reduction in adipose tissue caused the observed decrease in plasma CETP concentration. Since weight reduction is accompanied by a decrease in hepatic inflammation [43,44], a reduction in Kupffer cell content could be responsible for the decrease of plasma CETP. Another mechanism that might explain a decrease in plasma CETP after weight loss is diminished activation of the liver X receptor α (LXRα). The natural ligands for LXRα are oxysterols and the expression of CETP is mainly under control of this receptor. [45,46] We speculate that a decrease in oxysterols following weight loss might lead to diminished activation of LXRα, and consequently lower CETP production. The main strength of the present study is the availability of several accurate measures of body fat and body fat distribution, in addition to information on multiple potential confounding factors. The observational design is however a limitation, as we cannot exclude the presence of residual confounding by unknown, unmeasured or not accurately measured confounding factors. Besides, the study population was predominantly white and results may therefore not apply to other ethnical groups.

In conclusion, in this large population-based study there was no evidence for clinically relevant associations of total body fat, body mass index, waist circumference, waist-to-hip ratio, subcutaneous adipose tissue (MRI), visceral adipose tissue (MRI), total fat mass

(DXA) and trunk fat mass (DXA) with serum CETP concentration. These findings imply that adipose tissue does not contribute to the CETP pool in serum. To fully understand the role of CETP in cardiovascular disease, future research is needed regarding the physiology and biological function of CETP, especially in light of the termination of the recent clinical trials with the CETP inhibitors dalcetrapib[47] and evacetrapib[48], both due to insufficient efficacy.

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3

Metabolic liver inflammation in obesity does not robustly decrease hepatic and circulating CETP

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3

Abstract

Introduction

We recently showed that plasma cholesteryl ester transfer protein (CETP) is mainly derived from VSIG4-positive Kupffer cells, and that activation of these cells by the bacterial endotoxin lipopolysaccharide (LPS) strongly decreases CETP expression. As Kupffer cell activation plays a detrimental role in the progression of non-alcoholic fatty liver disease (NAFLD), we aimed to study if metabolic liver inflammation, as a component of NAFLD, is also associated with a decrease in hepatic expression and plasma concentrations of CETP in obese individuals.

Methods

We collected plasma and liver biopsy samples at various stages of NAFLD from 93 obese individuals who underwent bariatric surgery. Liver lobular inflammation was histologically determined, and liver sterols, liver triglycerides, liver *CETP* expression, CETP positive cells, circulating CETP concentrations, and liver VSIG4 expression were quantified.

Results

Body mass index ranged from 30.6 to 73.6 kg/m^2. Mean (SD) plasma CETP concentration was 2.68 (0.89) µg/mL. Compared to absence of liver pathology, in the presence of liver lobular inflammation, the age and sex-adjusted difference in hepatic *CETP* expression was -0.03 arbitrary units (95% CI -0.26, 0.20), the difference in the number of hepatic CETP positive cells (range 11 to 140 per mm^2) was -20.0 per mm^2 (95% CI -41.6, 1.9), and the difference in plasma CETP was -0.35 μg/mL (95% CI -0.80, 0.10). Hepatic *VSIG4* expression was not associated with liver inflammation (0.00; 95% CI -0.15, 0.15).

Conclusion

We found no strong evidence for a negative association between metabolic liver inflammation and CETP-related outcomes in obese individuals, although we observed consistent trends. Given that LPS strongly decreases *CETP* expression by Kupffer cells, our results suggest that metabolic and infection-induced liver inflammation have different effects on the expression and production of CETP by Kupffer cells. Further research into the role of CETP in liver inflammation and host defence is therefore warranted.

Introduction

Cholesteryl ester transfer protein (CETP) facilitates the transfer of cholesteryl esters from high-density lipoproteins (HDL) towards (very) low-density lipoproteins ((V)LDL), coupled to a net flux of triglycerides from (V)LDL to HDL, thereby contributing to an atherogenic lipoprotein profile.[1] Recently, we showed in human studies that circulating levels of CETP are mainly determined by resident hepatic macrophages (Kupffer cells),[2] without a contribution of adipose tissue.[3] In fact, hepatic Kupffer cell content strongly correlated with both hepatic CETP expression and plasma CETP concentration.[2]

Besides its established role in lipid metabolism, accumulating evidence suggests that CETP is involved in immunity and inflammatory processes.[4] This is in line with the primary expression of *CETP* by Kupffer cells, which play a pivotal role in inflammation and host defence against e.g. Gram-negative bacterial infections. Kupffer cells can detect lipopolysaccharide (LPS), a potent endotoxin released from Gram-negative bacteria, and induce an antibacterial response via the release of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β).[5] Interestingly, LPS reduces liver CETP expression and circulating CETP concentrations in CETP-transgenic mice.[6] Similarly, LPS, TNF-α and IL-1β decrease *CETP* expression and CETP concentration in hamsters.[7] LPS administration also decreased plasma CETP concentrations in humans.[8] Collectively, inflammatory stimuli seem to downregulate CETP expression by Kupffer cells.

Recently, we showed in *CETP*-expressing mice that hepatic expression of CETP is confined to a specific subset of Kupffer cells that also express *Vsig4*, which is a marker of resting Kupffer cells.[8] Also in humans, hepatic *VSIG4* expression correlated with both liver *CETP* expression and plasma CETP concentration.[Li et al., resubmitted] In this same study, we showed that LPS inoculation of mice markedly reduced liver CETP expression and production, accompanied by a similar loss of the Kupffer cell marker *Vsig4*. Combined, these data indicate that liver CETP expression is exclusively confined to a resting Kupffer cell subset, which loses *CETP* expression when activated by LPS.

Although the mechanisms by which inflammatory stimuli reduce *CETP* expression are not yet fully understood, they may counteract liver X receptor (LXR)-induced *CETP* expression. LXRs regulate a variety of genes to control cholesterol and lipid homeostasis and protect cells from an overload of toxic sterol.[9] *CETP* gene transcription is induced via an LXRα response element in the *CETP* promotor region.[10,11] Natural ligands for LXRα are oxidized derivatives of cholesterol (i.e. oxysterols)[12,13] and the cholesterol precursor desmosterol.[14] Interestingly, *in vitro* exposure of murine macrophages to LPS, TNF-α or interferon γ (IFN-γ) supresses an LXRα-induced increase in *CETP* expression.[15] In ad-

dition, we recently showed that LPS also reduces CETP expression as induced by an LXR agonist in human macrophages.[8] Taken together, LXRα-induced expression of *CETP* by Kupffer cells may be counteracted by inflammatory stimuli.

Apart from their beneficial role in host defence,[5] Kupffer cells play a detrimental role in the progression of non-alcoholic fatty liver disease (NAFLD) from simple steatosis to non-alcoholic steatohepatitis (NASH), which is characterized by liver inflammation.[16–21] With the growing prevalence of obesity worldwide, an increasing number of individuals suffer from NAFLD.[22] The central role of Kupffer cells in NAFLD is partly explained by excess free cholesterol that cannot be detoxified by esterification, leading to cholesterol crystallization not only within hepatocytes but also within Kupffer cells, which consequently activates inflammatory pathways.[17,23,24] The aim of the present study is to determine whether metabolic liver inflammation, as a component of NAFLD, is associated with a decrease in liver *CETP* expression and CETP production similar to LPS. To this end, we collected liver biopsy samples at various stages of NAFLD from a bariatric surgery cohort to histologically determine liver inflammation, and to quantify liver *CETP* and *VSIG4* expression, liver lipids, liver CETP positive cells and circulating CETP concentrations.

Materials and Methods

Study design and study population

The study population consisted of 93 severely obese men and women who underwent elective bariatric surgery between 2006 and 2009 at the Department of General Surgery, Maastricht University Medical Center (Maastricht, The Netherlands).[25] Subjects using anti-inflammatory drugs or with acute or chronic inflammatory diseases, degenerative diseases, and subjects reporting alcoholic intake >10 g/day were not included in this study. There were no specific dietary protocols that the participants had to follow before surgery. Venous blood samples were drawn on the morning of surgery after 8 hours of overnight fasting. During surgery, wedge biopsies of the liver were taken. The study was approved by the medical ethics board of Maastricht University Medical Centre, in line with the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from each participant.

Data collection

Because of the limited samples available, random samples from this study population were selected for data collection. Liver *CETP* and *VSIG4* mRNA expression were determined from microarrays of 82 samples, as described previously.[2,25] Biopsy specimens were formalin-fixed and paraffin-embedded, and subsequently immunohistochemistry was per-

formed. The number of CETP positive cells per mm^2 was obtained (n=44).[2] Plasma CETP concentrations were measured for 73 participants from whom plasma was available with ELISA kits (DAIICHI CETP ELISA, Alpco, Salem, USA), according to the manufacturer's instructions.

Histology

For histological scoring of the liver biopsies, Hematoxylin-eosin and Klatskin (Masson) trichrome stains were used to assess histopathology. Samples were scored for steatosis (n=93), lobular inflammation (n=89), and hepatocellular ballooning (n=89), by an experienced liver pathologist who was blinded for clinical and biochemical parameters, according to the criteria of the NAFLD activity score described by Kleiner et al.,[26] Steatosis was grouped into four categories based on the amount of surface area involved in steatosis (i.e. score 0: <5%, score 1: 5-33%, score 2: >33-66%, score 3: >66%). Lobular inflammation was grouped into four categories based on the number of inflammatory foci per 200X field (i.e. score 0: no foci, score 1: <2 foci, score 2: 2-4 foci, score 3: >4 foci). Ballooning was grouped into three categories based on the number of ballooning cells (i.e. score 0: none, score 1: few, score 2: many). To compose the NAFLD activity score, scores for steatosis, lobular inflammation, and hepatocellular ballooning were summed.[26]

Nuclear magnetic resonance (NMR) spectroscopy

Liver samples (approx. 1 mg) were stored at -80˚C in plastic Eppendorf tubes. After thawing at room temperature, 100 μL of liquid chromatography-mass spectrometry (LC-MS) grade water and 5 stainless steel beads were added and samples were placed in a bullet blender for 3 min. Ten μL of homogenised liver sample was taken and transferred to a fresh 1.5 mL Eppendorf tube for analysis of protein concentration (BCA, ThermoFisher Scientific, Waltham, USA), according to the manufacturer's instructions. Lipids were subsequently extracted from the remaining 90 μL homogenized liver sample, applying protein precipitation as follows. In short, 200 μL isopropanol (Sigma-Aldrich, LC-MS Chromasolv) was added to the sample, vortexed for 5 seconds and centrifuged for 5 min at 16,100×g. Two hundred microliters of the supernatant was transferred into a 2 mL glass vial (Agilent Technologies). The remaining pellet was again homogenised by placing in a bullet blender for 3 min with the addition of 150 μL isopropanol. After centrifugation (5 min at 16,100 ×g), 175 μL of the supernatant was collected and combined with organic extracts. The combined extract was dried under a gentle stream of nitrogen. After drying, the extract was reconstituted in 190 μL deuterated chloroform and transferred to a 3 mm NMR tube.

NMR was used to measure total cholesterol, free cholesterol, cholesteryl ester and triglyceride contents in 68 liver samples. Liver lipids were expressed as nmol per mg protein.

Proton NMR spectra were recorded on a Bruker Avance II 600 NMR spectrometer equipped with a 5 mm TCI cryogenic probe head and a z-gradient system. A Bruker SampleJet sample changer was used for sample insertion and removal. The NMR spectra were recorded using a simple one-pulse experiment. 256 scans of 65,536 points covering a sweep width of 12,336 Hz were acquired. After applying an exponential window function of 0.3 Hz, the time-domain signal was Fourier transformed yielding a spectrum of 32,768 complex data points. The spectrum was imported into a KNIME workflow[27] using a Python 2.7 script that utilizes the Nmrglue module.[28] Subsequently, automatic zeroth-order phase correction and fourth-degree polynomial baseline correction were applied. Relevant sections of the spectra were extracted and individually baseline corrected in order to remove background signals. Well-separated peaks, multiplets, or parts of multiplets were integrated, and divided by the associated number of protons. Peak areas were converted into concentrations using the peak area of the reference material (0.05% Tetramethylsilane). The integration intervals are given in Supplementary table 1.

Biochemical analyses

Plasma concentrations of total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, alanine transaminase (ALT), and aspartate transaminase (AST) were measured, as described before.[25]

Statistical analyses

We assessed the associations of the following determinants with CETP-related outcomes: steatosis score, lobular inflammation score, hepatocellular ballooning score, presence of NASH, plasma ALT concentration, plasma AST concentration, liver total cholesterol content, liver free cholesterol content, liver cholesteryl ester content and liver triglyceride content. For steatosis, lobular inflammation, hepatocellular ballooning, and NASH separately, the presence of disease (score ≥1) was compared with absence of pathology (score 0), as defined by the criteria of the NAFLD activity score.[26] As CETP-related outcome variables we used liver *CETP* expression, CETP protein positive cells, and plasma CETP concentration. Linear regression analyses were used to determine all associations. Crude models (Model 1) were adjusted for age and sex (Model 2). Differences were expressed as beta coefficients with corresponding 95% CIs. Liver sterol and triglyceride contents and liver transaminases were non-normally distributed, and therefore we used their natural logarithms in the analyses. For interpretation purposes, beta coefficients were multiplied by ln(1.1), and differences with corresponding 95% CIs were expressed per 10% relative increase in the determinant. In addition, we assessed the correlations between NMR-determined liver sterol and triglyceride data and reported Pearson's correlation coefficient.

All analyses were performed using STATA Statistical Software (Statacorp, College Station, Texas, USA), version 12.0.

Results

Population characteristics

Table 3.1 shows the characteristics of the elective bariatric surgery cohort. Body mass index (BMI) ranged from 30.6 to 73.6 kg/m^2. Mean age was 44 years, and most participants were women. Mean (SD) CETP concentration was 2.68 (0.89) μg/mL in the total population, 2.56 (0.92) μg/mL for men and 2.72 (0.88) μg/mL for women. 15% of men and 18% of women had a NAFLD activity score ≥5, which is indicative of NASH.[26] Plasma ALT concentration was comparable between men and women, and plasma AST concentration was somewhat lower in women. Liver sterol and triglyceride contents were all higher in women than in men.

Lobular inflammation and hepatocellular ballooning tend to associate with less CETP positive cells in the liver and a lower plasma CETP concentration

Associations of the separate NAFLD activity score components, i.e. steatosis, lobular inflammation and ballooning score, with the CETP-related outcomes are presented in Figure 3.1 and Supplementary table 3.A.2. The age and sex-adjusted difference in *CETP* mRNA expression in the liver for an inflammation score ≥1 compared with a score of 0 was -0.03 arbitrary units (95% CI -0.26, 0.20). Comparable small, null associations were observed for steatosis and ballooning scores. The number of CETP positive cells per mm^2 liver ranged from 11 to 140. The presence of liver lobular inflammation was associated with a lower number of CETP positive cells of -20.0 per mm^2 (95% CI -41.6, 1.9). Steatosis and ballooning scores were also negatively associated with CETP positive cell number, although confidence intervals overlapped with zero. Plasma CETP concentration was within the range of 1.45 to 5.56 μg/mL. The presence of lobular inflammation was associated with a lower plasma CETP of -0.35 μg/mL (95% CI -0.80, 0.10), although confidence intervals overlapped with zero. Hepatocellular ballooning was associated with a -0.42 μg/mL (95% CI -0.84, -0.00) μg/mL lower plasma CETP concentration, which represents approx. 15% of the mean CETP concentration. Steatosis score also showed a negative association with CETP concentration, although the confidence intervals overlapped with zero. Steatosis, inflammation and ballooning scores were not negatively associated with *VSIG4* expression (Figure 3.1, Supplementary table 3.A.2).

Table 3.1: Characteristics of the elective bariatric surgery cohort aged 17-67 years (n=93), stratified by sex.

Characteristic	Men	Women
Number of participants (% of total)	26 (28%)	67 (72%)
Age (year)	46 (11)	43 (9)
BMI (kg/m2)	50 (10)	45 (9)
Fasting plasma concentrations		
CETP (μg/mL)	2.56 (0.92)	2.72 (0.88)
Total cholesterol (mmol/L)	4.66 (0.89)	5.26 (1.15)
HDL-cholesterol (mmol/L)	0.88 (0.27)	1.02 (0.40)
LDL-cholesterol (mmol/L)	2.90 (0.86)	3.34 (1.03)
Triglycerides (mmol/L)	1.95 (0.94)	2.33 (2.27)
ALT (IU/L)	26.6 (11.8)	26.5 (17.6)
AST (IU/L)	27.1 (10.4)	23.5 (13.1)
Liver biopsies		
Total cholesterol content (nmol/mg protein)[b]	12 (9)	37 (68)
Free cholesterol content (nmol/mg protein)[b]	8 (6)	25 (44)
Cholesteryl ester content (nmol/mg protein)[b]	4 (3)	11 (26)
Triglyceride content (nmol/mg protein)[b]	90.4 (71.1)	268 (618)
Number of patients with NAFLD activity score ≥5 (% of total men or women)[c]	4 (15%)	12 (18%)

Results are presented as mean (SD) or percentage.

[a] Missing data: n=1 for BMI, n=20 for plasma CETP concentration, n=6 for total cholesterol concentration, n=7 for HDL-cholesterol, LDL-cholesterol and triglyceride concentration, n=6 for ALT and AST concentration, n=4 for NAFLD activity score, n=25 for liver content of total cholesterol, free cholesterol, cholesteryl esters and triglycerides.

[b] Measured with NMR.

[c] A NAFLD activity score≥5 is indicative of NASH.[26]

ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; IU, international unit; LDL, low-density lipoprotein; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NMR, nuclear magnetic resonance.

CETP-related outcomes are not different in patients with a NAFLD activity score ≥5

A NAFLD activity score ≥5 was associated with a higher ALT concentration of 18.2 IU/L (95% CI 10.1, 26.3) and a higher AST concentration of 13.5 IU/L (95% CI 7.2, 19.8). However, no difference in CETP-related outcomes was observed between participants with a NAFLD activity score ≥5 compared with participants with a NAFLD activity score <5 (Figure 3.2, Supplementary table 3.A.3). Plasma ALT and AST concentrations were negatively associated with liver *CETP* expression, although associations were weak (Figure 3.3, Supplementary table 3.A.4). Age- and sex-adjusted differences in *CETP* expression were -0.020 arbitrary units (95% CI -0.040, 0.000) and -0.024 arbitrary units (95% CI -0.047, -0.001) per 10% relative increase in plasma ALT and AST concentration, respectively. Associations of both liver transaminases with CETP positive cells and CETP concentration were also negative, although effect sizes were small and confidence intervals overlapped with zero (Figure 3.3, Supplementary table 3.A.4).

Associations of liver sterol and triglyceride contents with CETP-related outcomes were around the null

NMR-determined liver sterol and triglyceride data clearly show that hepatic contents of total cholesterol and free cholesterol were highly correlated (r^2=0.97, P<0.0001). In addition, the correlations between the hepatic content of total cholesterol and cholesteryl esters (r^2=0.77, P<0.001), and between total cholesterol and triglycerides (r^2=0.71, P<0.001) were high. Strikingly, all of the associations of liver triglyceride and sterol contents with CETP-related outcomes were around the null (Figure 3.4, Supplementary table 3.A.5).

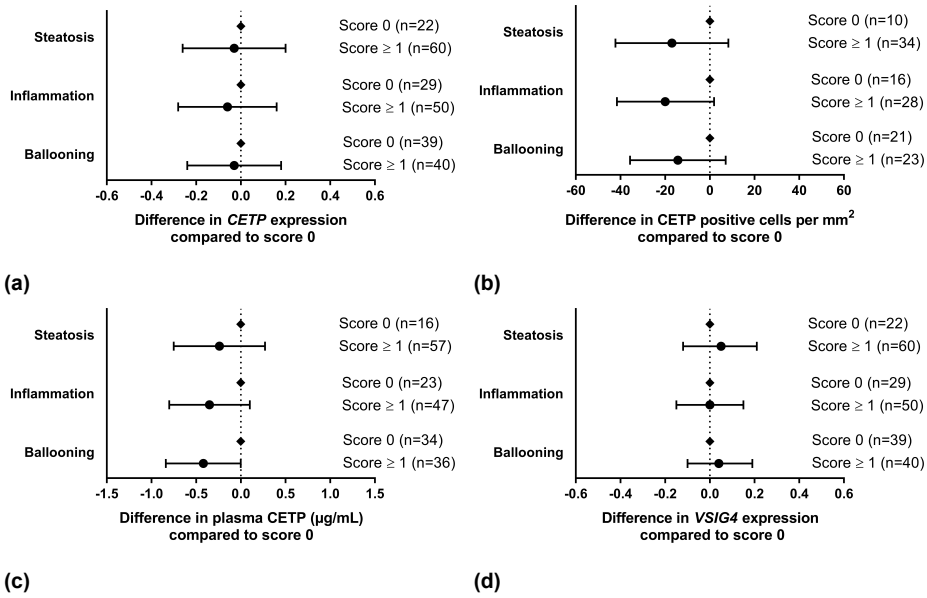


Figure 3.1: CETP-related outcomes, but not liver *VSIG4* expression, show a consistent trend for negative association with metabolic liver steatosis, inflammation and ballooning. Age and sex-adjusted differences (i.e. beta coefficients from linear regression) in (**a**) liver CETP mRNA expression, (**b**) liver CETP positive cells, (**c**) plasma CETP concentration, and (**d**) liver VSIG4 mRNA expression between absence (score 0, reference category) and presence (score ≥1) of liver steatosis, lobular inflammation and ballooning. Error bars represent 95% confidence intervals.

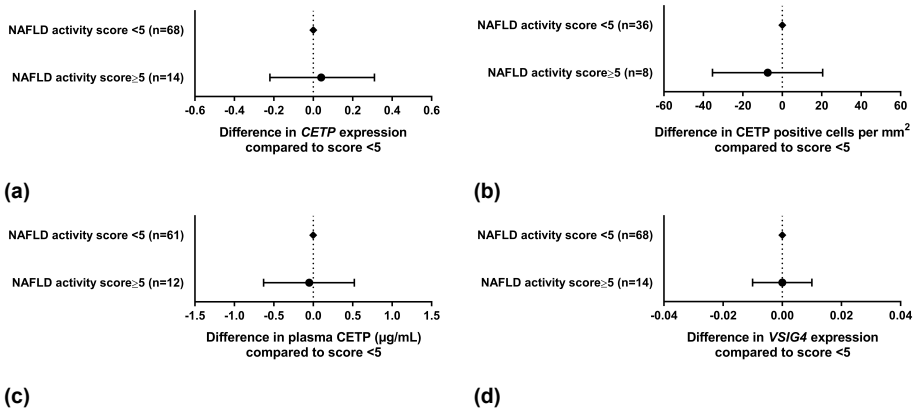


Figure 3.2: CETP-related outcomes and *VSIG4* expression are not different between participants with a non-alcoholic fatty liver disease (NAFLD) activity score <5 (reference category) and participants with a NAFLD activity score ≥5, which is indicative of non-alcoholic steatohepatitis (NASH). Age and sex-adjusted differences (i.e. beta coefficients from linear regression) in (**a**) liver CETP mRNA expression, (**b**) liver CETP positive cells, (**c**) plasma CETP concentration, and (**d**) liver VSIG4 mRNA expression between participants with a NAFLD activity score <5 (reference category) and participants with a NAFLD activity score ≥5. Error bars represent 95% confidence interval.

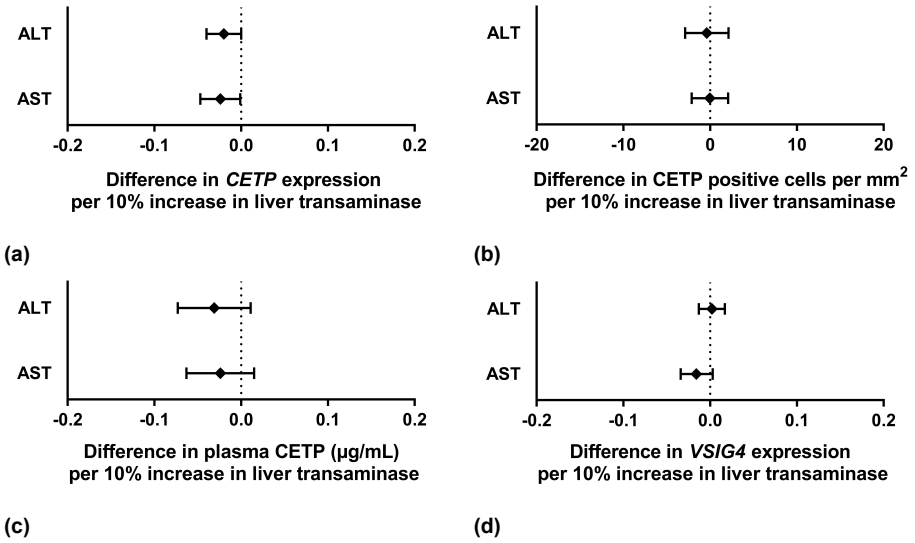


Figure 3.3: Both plasma alanine transaminase (ALT) and aspartate transaminase (AST) concentrations are negatively associated with CETP mRNA expression and plasma CETP concentration, although confidence intervals overlap with zero and effect sizes are small. Age and sex-adjusted differences[a] in (**a**) liver CETP mRNA expression, (**b**) liver CETP positive cells, (**c**) plasma CETP concentration, and (**d**) liver VSIG4 mRNA expression, per 10% relative increase in the plasma concentrations of ALT and AST. Error bars represent 95% confidence interval.

[a] (Beta coefficients from linear regression)*ln(1.1), i.e. difference per 10% relative increase in liver lipid content.

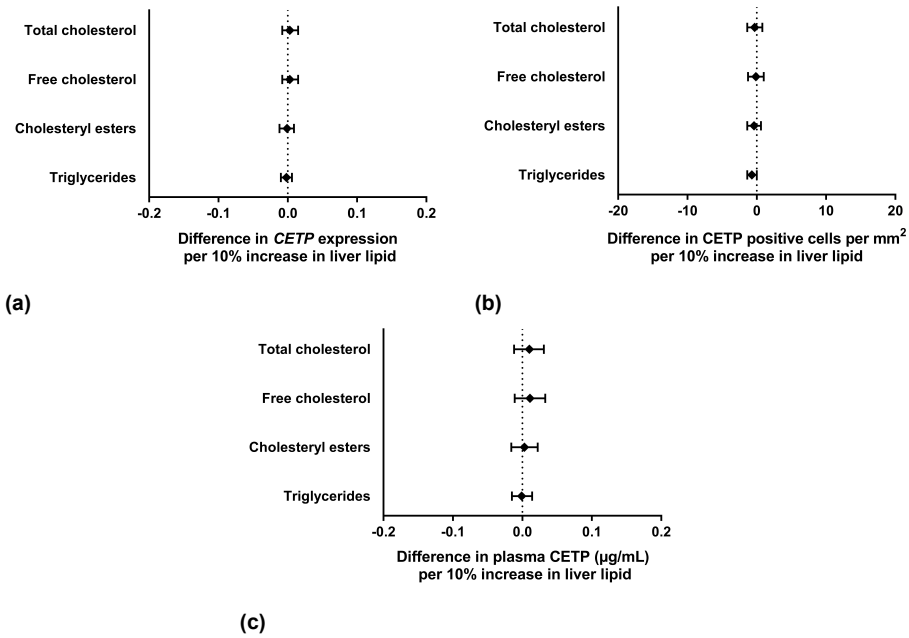


Figure 3.4: Both plasma alanine transaminase (ALT) and aspartate transaminase (AST) concentrations are negatively associated with CETP mRNA expression and plasma CETP concentration, although confidence intervals overlap with zero and effect sizes are small. Age and sex-adjusted differences[a] in (**a**) liver CETP mRNA expression, (**b**) liver CETP positive cells and (**c**) plasma CETP concentration, per 10% relative increase in the plasma concentrations of ALT and AST. Error bars represent 95% confidence interval.

[a] (Beta coefficients from linear regression)*ln(1.1), i.e. difference per 10% relative increase in liver sterol and triglyceride content.

Discussion

VSIG4-positive Kupffer cells have recently been identified as the main determinants of circulating CETP levels,[2,8] but the influence of the hepatic environment on CETP expression and CETP production is far from elucidated. Based on previous studies, showing that inflammatory stimuli downregulate CETP expression in mice and *in vitro*,[6,7,15,29,30] we hypothesized that hepatic *CETP* expression and circulating CETP concentrations are decreased in humans with metabolic liver inflammation. Therefore, we aimed to study the associations of histologically-determined liver inflammation, as a component of NAFLD, with liver *CETP* expression, CETP positive cells and circulating CETP concentrations, using liver biopsy data from an elective bariatric surgery cohort. We found no strong evidence for a negative association between liver lobular inflammation and CETP-related outcomes, as 95% confidence intervals overlapped with zero. However, we did observe consistent trends towards less CETP expression, a lower number of CETP positive cells, and a lower

circulating CETP concentration when liver inflammation was present. In line, we also observed trends for a negative association of ALT and AST concentrations, both markers for liver damage, with CETP-related outcomes.

It is interesting to speculate why only limited associations were observed between metabolic inflammation and CETP-related outcomes. Previous animal and *in vitro* studies showed a decrease in CETP expression specifically in response to infection-related inflammatory stimuli, such as LPS, IL-1β and IFN-γ.[6,7,15] In addition, we recently showed that LPS also reduces plasma CETP concentration in humans.[8] In the present study, all participants were morbidly obese, and individuals that used anti-inflammatory drugs or had acute or chronic inflammatory diseases were not able to participate. Thus, liver inflammation in the present study was assumed to be metabolically-induced. Interestingly, metabolic liver inflammation, which is a component of NAFLD, has been shown to differ from infection-induced liver inflammation.[31] In mice, LPS and IL-1β cause intrahepatic inflammation that is exclusively mediated by macrophages, while metabolic triggers (i.e. carbohydrate and cholesterol) of liver inflammation induce a NASH phenotype with mixed intrahepatic infiltrates including both macrophages and neutrophils.[31] Since CETP is primarily produced by hepatic macrophages, infection-related inflammatory stimuli may affect hepatic expression and production of CETP to a different extent than metabolic triggers. Notably, severe sepsis was previously shown in humans to largely decrease CETP concentration, i.e. by -25% after 3 days of sepsis,[32] while we only observed a trend towards a negative association between metabolic liver inflammation and CETP-related outcomes. This is in line with a previous study in CETP-transgenic mice, in which we observed that long term high-fat diet-induced obesity did not affect plasma CETP concentration.[33] The difference between metabolic versus infection-induced liver inflammation is further highlighted by our observation that *VSIG4* expression, which is a marker of resting Kupffer cells, was not lower in the presence of metabolic liver inflammation, while we previously showed that LPS injection largely reduced both *Vsig4* and *CETP* expression, at least in mice.[8] Taken together, we propose that acute and/or whole-body inflammatory responses to invading pathogens are required to induce a robust reduction in CETP production by Kupffer cells.

In the context of infection-induced *versus* metabolic liver inflammation, it is important to consider the biological relevance underlying the relationship between CETP and inflammation. CETP mediates the bidirectional exchange of triglycerides and cholesteryl esters between (V)LDL and HDL, thereby raising LDL-cholesterol and decreasing HDL-cholesterol, which is generally regarded as a biologically unwanted proatherogenic property of CETP. Since HDL is known for its inflammation-modulatory properties and beneficial role in host-defence,[34–37] we reason that the biological function of CETP in humans is modulation of

the immune response via HDL. In fact, the reduction in *CETP* expression by Kupffer cells in CETP-transgenic mice as induced by LPS is accompanied by an increase in HDL.[8] We thus postulate that inflammatory stimuli activate resting Kupffer cells to decrease CETP expression and thereby raise HDL to combat invading microorganisms. In line with this hypothesis, it seems plausible that metabolic liver inflammation will not largely reduce the expression and production of CETP.

Comparable with liver inflammation, liver steatosis score also tended to be negatively associated with the number of CETP positive cells. This negative effect direction can possibly be explained by a decrease in macrophage cell numbers per area rather than by an absolute decrease in Kupffer cell number, as the presence of steatosis results in occupation of a larger area of the microscopic field by lipid-filled hepatocytes. This is a plausible explanation, as associations between NMR-determined triglycerides content and CETP-related outcomes were all around zero. NMR triglyceride measurements were expressed per mg protein, and can therefore be interpreted as mean triglyceride content per hepatocyte. Therefore, in the light of the NMR findings, the negative association between histologically-determined steatosis and CETP-related outcomes is most likely explained by the measurement technique.

This study is one of the first studies to include NMR spectroscopy for the quantitative analysis of several lipid and sterol classes in human liver biopsies. By using NMR spectroscopy, we revealed a strong correlation between hepatic contents of total cholesterol and free cholesterol (r^2=0.97) as well as those of total cholesterol and triglycerides (r^2=0.71). The finding that none of the measured liver sterol components (i.e. total cholesterol, free cholesterol, nor cholesteryl esters) was associated with *CETP* expression in the present study, may seem counterintuitive given that *CETP* expression is under the control of LXRα.[10,11] Cholesterol derivatives activate LXRα,[12,13] which subsequently binds to an LXR-response element in the *CETP* promotor region to increase gene transcription.[10,11] It should be realized that we measured cholesterol rather than the actual LXR agonists, e.g. oxysterols and desmosterol. For future studies, mass spectrometric platforms might complement NMR analysis to measure oxysterols and desmosterol, as the commonly observed low concentrations are likely out of the measurement range of NMR spectroscopy.

Notably, compared with participants with a NAFLD activity score <5, CETP-related outcomes were not different in participants with a NAFLD activity score ≥5, which is indicative of NASH.[26] This score is composed of three summed, separate components (i.e. steatosis, lobular inflammation and ballooning scores).[26] We argue that this score may not be sufficiently specific to detect an association of specific aspects of NAFLD with CETP-related outcomes, which is the main reason that we studied the associations between the differ-

ent components of the NAFLD activity score and CETP-related outcomes separately. The same reasoning may also hold for plasma ALT and AST concentrations, which are relatively non-specific markers for liver function.[38] Associations of both liver transaminases with CETP-related outcomes were around the null.

The main strength of the present study is the availability of liver biopsies from a population of obese men and women, with a high prevalence of metabolic liver inflammation. Liver samples were extensively characterized with regard to CETP, sterol and triglyceride content, steatosis, inflammation and cell damage. Also, several limitations of the study design should be considered. Firstly, inherent to the observational cross-sectional design we cannot exclude residual confounding, or draw conclusions on causality. Secondly, this study possibly lacked power to identify statistically significant differences in CETP-related outcomes due to the relatively small study population size. Lastly, results may not be generalizable to other populations, as participants were selected from an obese cohort that underwent bariatric surgery.

In conclusion, we found no strong evidence for a negative association between metabolic liver inflammation and CETP-related outcomes, although we observed consistent trends. Given that infection-related inflammatory stimuli do significantly decrease *CETP* expression by Kupffer cells, our results suggest that metabolic liver inflammation affects the expression and production of CETP by Kupffer to a more modest extent than infection-induced liver inflammation. It is tempting to speculate that acute and/or whole-body inflammatory responses to invading pathogens are required to induce a robust reduction in CETP production by Kupffer cells. Further research into the effects of liver inflammation and host defence on hepatic CETP production is therefore eagerly warranted.

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Appendix

3.A. Supplementary tables

Table 3.A.1: Sections of the NMR spectra used to determine liver total cholesterol, free cholesterol, cholesteryl ester, and triglyceride content. In the case of free cholesterol and cholesteryl esters, due to partial overlap of the multiplet, two intervals were integrated separately, and the second interval was counted twice to account for the overlap on the other side of the multiplet.

Liver lipid	Integration interval		Integration interval 2		# of protons
	From/ppm	To/ppm	From/ppm	To/ppm	
Total cholesterol	0.659	0.696	-	-	3
Free cholesterol	3.502	3.548	3.548	3.581	1
Cholesteryl esters	4.589	4.635	4.575	4.589	1
Triglycerides	4.12	4.17	-	-	2

NMR, nuclear magnetic resonance.

Table 3.A.2: Crude (model 1) and age and sex-adjusted (model 2) differences[a] in liver *CETP* mRNA expression, liver CETP positive cells, plasma CETP concentration and liver *VSIG4* mRNA expression between absence (score 0, reference category) and presence (score ≥1) of liver steatosis, lobular inflammation and ballooning.

	Model	N	Difference in[a]	95% Confidence interval
Steatosis score				
			Liver *CETP* expression	
0	1	22	0 (reference)	-
	2	22	0 (reference)	-
≥1	1	60	-0.07	-0.30, 0.17
	2	60	-0.03	-0.26, 0.20
			CETP positive cells per mm^2	
0	1	10	0 (reference)	-
	2	10	0 (reference)	-
≥1	1	34	-15.8	-41.5, 9.8
	2	34	-17	-42.2, 8.2
			Plasma CETP concentration (μg/mL)	
0	1	16	0 (reference)	-
	2	16	0 (reference)	-
≥1	1	57	-0.23	-0.74, 0.27
	2	57	-0.24	-0.75, 0.27
			Liver *VSIG4* expression	
0	1	22	0 (reference)	-
	2	22	0 (reference)	-
≥1	1	60	0.04	-0.12, 0.20
	2	60	0.05	-0.12, 0.21
Inflammation score				
			Liver *CETP* expression	
0	1	29	0 (reference)	-
	2	29	0 (reference)	-
≥1	1	50	-0.11	-0.33, 0.11
	2	50	-0.06	-0.28, 0.16
			CETP positive cells per mm^2	
0	1	16	0 (reference)	-
	2	16	0 (reference)	-
≥1	1	28	-22.2	-43.8, -0.5
	2	28	-20	-41.6, 1.9
			Plasma CETP concentration (μg/mL)	
0	1	23	0 (reference)	-
	2	23	0 (reference)	-
≥1	1	47	-0.36	-0.80, 0.08
	2	47	-0.35	-0.80, 0.10
			Liver *VSIG4* expression	

0	1	29	0 (reference)	-
	2	29	0 (reference)	-
≥1	1	50	-0.02	-0.16, 0.13
	2	50	0	-0.15, 0.15
Ballooning score				
			Liver *CETP* expression	
0	1	39	0 (reference)	-
	2	39	0 (reference)	-
≥1	1	40	-0.07	-0.28, 0.14
	2	40	-0.03	-0.24, 0.18
			CETP positive cells per mm^2	
0	1	21	0 (reference)	-
	2	21	0 (reference)	-
≥1	1	23	-17	-38.3, 4.2
	2	23	-14.3	-35.7, 7.1
			Plasma CETP concentration (μg/mL)	
0	1	34	0 (reference)	-
	2	34	0 (reference)	-
≥1	1	36	-0.43	-0.84, -0.02
	2	36	-0.42	-0.84, -0.00
			Liver *VSIG4* expression	
0	1	39	0 (reference)	-
	2	39	0 (reference)	-
≥1	1	40	0.03	-0.11, 0.17
	2	40	0.04	-0.10, 0.19

[a] Beta coefficients from linear regression.

Table 3.A.3: Crude (model 1) and age and sex-adjusted (model 2) differences in liver *CETP* mRNA expression, liver CETP positive cells, plasma CETP concentration and liver *VSIG4* mRNA expression between participants with a non-alcoholic fatty liver disease (NAFLD) activity score <5 (reference category) and participants with a NAFLD activity score ≥5.

NAFLD activity score	Model	N	Difference in[a]	95% Confidence interval
			Liver *CETP* expression	
<5	1	68	0 (reference)	-
	2	68	0 (reference)	-
≥5	1	14	0.03	-0.25, 0.31
	2	14	0.04	-0.22, 0.31
			CETP positive cells per mm2	
<5	1	36	0 (reference)	-
	2	36	0 (reference)	-
≥5	1	8	-9.1	-37.3, 19.1
	2	8	-7.4	-35.4, 20.5
			Plasma CETP concentration (ćg/mL)	
<5	1	61	0 (reference)	-
	2	61	0 (reference)	-
≥5	1	12	-0.06	-0.63, 0.50
	2	12	-0.05	-0.63, 0.52
			Liver *VSIG4* expression	
<5	1	68	0 (reference)	-
	2	68	0 (reference)	-
≥5	1	14	0.17	-0.01, 0.36
	2	14	0	-0.01, 0.01

[a] Beta coefficients from linear regression.

Table 3.A.4: Crude (model 1) and age and sex-adjusted (model 2) differences in liver *CETP* mRNA expression, liver CETP positive cells, plasma CETP concentration and liver *VSIG4* mRNA expression, per 10% relative increase in the plasma concentrations of alanine transaminase (ALT) and aspartate transaminase (AST).

Liver transaminase	Model	N	Difference in[a]	95% Confidence interval
			Liver *CETP* expression	
ALT	1	79	-0.021	-0.041, -0.001
	2	79	-0.02	-0.040, 0.000
AST	1	79	-0.013	-0.033, 0.008
	2	79	-0.024	-0.047, -0.001
			CETP positive cells per mm^2	
ALT	1	42	-0.35	-2.89, 2.19
	2	42	-0.38	-2.88, 2.12
AST	1	42	0.28	-1.77, 2.33
	2	42	-0.03	-2.12, 2.07
			Plasma CETP concentration (μg/mL)	
ALT	1	69	-0.031	-0.073, 0.010
	2	69	-0.031	-0.073, 0.011
AST	1	69	-0.026	-0.063, 0.012
	2	69	-0.024	-0.063, 0.015
			Liver *VSIG4* expression	
ALT	1	79	0.001	-0.013, 0.015
	2	79	0.002	-0.013, 0.017
AST	1	79	0.002	-0.012, 0.015
	2	79	-0.016	-0.034, 0.003

[a] (Beta coefficients from linear regression)*ln(1.1), i.e. difference per 10% relative increase in liver sterol and triglyceride content.

Table 3.A.5: Crude (model 1) and age and sex-adjusted (model 2) differences in liver *CETP* mRNA expression, liver CETP positive cells and plasma CETP concentration, per 10% relative increase in liver total cholesterol, free cholesterol, cholesteryl ester, and triglyceride content.

Liver lipid	Model	N	Difference in[a]	95% Confidence interval
			Liver *CETP* expression	
Total cholesterol	1	63	0.003	-0.008, 0.015
	2	63	0.003	-0.008, 0.015
Free cholesterol	1	63	0.003	-0.008, 0.015
	2	63	0.003	-0.008, 0.015
Cholesteryl esters	1	63	-0.002	-0.012, 0.009
	2	63	-0.001	-0.012, 0.009
Triglycerides	1	63	-0.003	-0.010, 0.005
	2	63	-0.002	-0.010, 0.006
			CETP positive cells per mm^2	
Total cholesterol	1	41	-0.1	-1.16, 0.96
	2	41	-0.29	-1.37, 0.80
Free cholesterol	1	41	0.07	-1.04, 1.18
	2	41	-0.12	-1.26, 1.02
Cholesteryl esters	1	41	-0.25	-1.24, 0.74
	2	41	-0.39	-1.37, 0.60
Triglycerides	1	41	-0.62	-1.32, 0.08
	2	41	-0.69	-1.37, 0.00
			Plasma CETP concentration (μg/mL)	
Total cholesterol	1	64	0.012	-0.009, 0.032
	2	64	0.01	-0.012, 0.031
Free cholesterol	1	64	0.014	-0.007, 0.035
	2	64	0.011	-0.011, 0.033
Cholesteryl esters	1	64	0.005	-0.014, 0.023
	2	64	0.003	-0.016, 0.022
Triglycerides	1	64	0	-0.014, 0.014
	2	64	-0.001	-0.015, 0.014

[a] (Beta coefficients from linear regression)*ln(1.1), i.e. difference per 10% relative increase in liver lipid content.

4

CETP concentration: a genome-wide association study followed by Mendelian randomization on coronary artery disease

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Abstract

Introduction

The genetic basis of circulating cholesteryl ester transfer protein (CETP) concentration is largely unknown. We aimed to identify independent genetic determinants of circulating CETP to assess causal effects of variation in CETP concentration on circulating lipid concentrations and cardiovascular disease risk.

Methods

A genome-wide association (GWA) discovery and replication study on serum CETP concentration were embedded in the Netherlands Epidemiology of Obesity (NEO) study. Based on the independent identified variants, Mendelian randomization was conducted on serum lipids (NEO study) and coronary artery disease (CAD) (CARDIoGRAMplusC4D consortium).

Results

In the discovery analysis (N=4,248), we identified three independent variants (rs247616, rs12720922 and rs1968905; all $P<5\times10^{-8}$) that determine serum CETP concentration. These SNPs were mapped to CETP, and replicated in a separate subpopulation (N=1,458). Per-allele increase (SE) in serum CETP was 0.32 (0.02) μg/mL for rs247616-C, 0.35 (0.02) μg/mL for rs12720922-A, and 0.12 (0.02) μg/mL for rs1968905-G. Combined, these variants explained 16.4% of the total variation in CETP concentration. One μg/mL increase in genetically-determined CETP concentration strongly decreased high-density lipoprotein (HDL) cholesterol (-0.23 mmol/L; 95% CI -0.26, -0.20), moderately increased low density lipoprotein (LDL) cholesterol (0.08 mmol/L; 0.00, 0.16), and was associated with an odds ratio of 1.08 (0.94, 1.23) for CAD risk.

Conclusion

This is the first large GWAS study identifying independent variants that largely determine serum CETP concentration. While HDL-C is not a causal risk factor for CAD, it has been unequivocally demonstrated that LDL-C lowering is proportionally associated with a lower CAD risk. Therefore, the results of our study are fully consistent with the notion that CETP concentration is causally associated with CAD through LDL-C.

Introduction

Cholesteryl ester transfer protein (CETP) facilitates the net flux of cholesteryl esters from high-density lipoproteins (HDL) towards (very-) low-density lipoproteins ((V)LDL), coupled to a net flux of triglycerides from (V)LDL to HDL.[1] As such, CETP contributes to an atherogenic lipoprotein profile (i.e. high LDL-cholesterol/HDL-cholesterol ratio), as has been extensively studied in both humans and in mice transgenic for human CETP.[2,3] Therefore, inhibition of CETP has long been regarded a promising therapeutic strategy to attenuate dyslipidaemia and ultimately prevent the development of cardiovascular disease (CVD).

Of the four clinical trials that have studied the effects of pharmacological CETP inhibition on CVD risk reduction, only the fourth and most recent REVEAL trial with anacetrapib did meet its primary endpoint, i.e. a reduction in major coronary events.[4] Contrary to expectations, the clinical trials with the CETP the inhibitors torcetrapib, dalcetrapib, and evacetrapib, were terminated: torcetrapib had off-target effects on blood pressure and caused an increase in cardiovascular events,[5] and both dalcetrapib and evacetrapib lacked efficacy in reducing cardiovascular events on top of statin therapy.[6,7] All of these CETP inhibitors caused a large increase in HDL-C, and a low to moderate decrease in LDL-C.[5–8] Although high HDL-C concentration is associated with a decreased risk of CVD in epidemiological studies,[9] Voight and colleagues[10] showed in a Mendelian randomization study that genetically-determined higher HDL-C concentrations do not decrease the risk of myocardial infarction, indicating that the association between HDL-C and CVD is not causal. This may be one of the explanations for the lack of efficacy of the three initial CETP inhibitors. Although the underlying reason for success of the fourth CETP inhibitor is not yet elucidated, anacetrapib showed the largest reduction in LDL-C concentration compared to the three initial CETP inhibitors,[4] which may possibly explain its beneficial effects on CVD risk reduction.

Recent evidence shows that serum CETP is largely derived from hepatic macrophages[11], but the genetic basis of serum the CETP concentration in the general population remains to be elucidated. A large genome-wide association study (GWAS) on circulating CETP has not been performed to date. With identification of the main genetic determinants of circulating CETP, the causal effects of variation in serum CETP concentration on circulating lipid concentrations and CVD risk can be assessed using Mendelian randomization. In the past, several Mendelian randomization studies with a comparable aim have been performed,[12–14] including a recent meta-Mendelian randomization analysis by Ference et al. with data from over 100,000 participants.[15] However, these studies used candidate SNPs rather than GWAS-identified SNPs, which may be less powerful genetic instruments for assessing the causal role of CETP concentration in cardiovascular disease.[16,17]

With the present study, we aim to identify independent genetic variants that determine circulating CETP concentration, using a genome-wide rather than a candidate gene approach. In addition, we aim to use these variants as genetic instruments in Mendelian randomization to assess the causal effects of variation in CETP on serum lipids and coronary artery disease (CAD) risk, which may assist in understanding the effectiveness of pharmaceutical CETP inhibition. To this end, we performed a GWAS on serum CETP concentration, using a discovery cohort (n=4,248) and a separate replication cohort (n=1,458) from the Netherlands Epidemiology of Obesity (NEO) study. Subsequently, we used the identified SNPs in Mendelian randomization analyses on serum lipid concentrations in the NEO study population and the Global Lipids Genetics Consortium (GLGC)[18], and on CAD using publically-available data from the CARDGIoGRAMplusC4D consortium.[19]

Materials and Methods

Study design and population

The NEO study is a population-based prospective cohort study of men and women aged between 45 and 65 years. From the greater area of Leiden, The Netherlands, all inhabitants with a self-reported body mass index (BMI) of 27 kg/m or higher were eligible to participate. In addition, inhabitants from one nearby municipality (Leiderdorp, The Netherlands) in the same age group were invited to participate regardless of their BMI, forming a reference population for BMI distribution. In total, 6,671 participants were included from September 2008 until September 2012. Participants visited the NEO study center for extensive physical examination. Venous blood samples were obtained from the antecubital vein after a 10 hour overnight fast. 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 NEO study was approved by the medical ethics committee of the Leiden University Medical Center (LUMC), and all participants gave their written informed consent. Detailed information about the study design and data collection has been described elsewhere.[20]

Methods used for genotyping and biochemical analyses are described in detail in the Supplemental material 4.A.

Genome-wide association study

We conducted the GWAS on the unstandardized serum CETP concentration for all autosomal chromosomes. We divided the total NEO study population based on the graphical area of recruitment into a discovery cohort (n=4,248; greater area of Leiden, The Netherlands) and a replication cohort (n=1,458; Leiderdorp, The Netherlands). This was considered to

be valid as we recently showed, using data of the NEO study population, that serum CETP concentration was not associated with BMI nor with other measures of body fat.[21]

Additive (per-allele) linear regression analyses were conducted separately for the discovery and replication cohort in SNPTEST v2, adjusted for age, sex and the first four PCs. To identify variants that were independently associated with serum CETP concentration, we used conditional and joint analyses to perform a stepwise selection procedure using the genome-wide complex trait analysis (GCTA) tool version 1.24.4.[22] A conditioned P-value $<5\times10^{-8}$ was considered to be genome-wide significant, and a conditioned P-value $<1\times10^{-6}$ was considered a suggestive signal. Independent single nucleotide polymorphisms (SNPs) with a conditioned P-value $<1\times10^{-6}$ in the discovery analysis were validated in the replication sample. SNPs with a P-value <0.05 in the replication cohort were considered to be replicated. Upon identification of the lead SNPs, we determined whether the distribution of the coding alleles was similar in users and non-users of lipid-lowering drugs. More detailed information on this method is described in the Supplemental material 4.A.

As Taq1B (rs708272)[23] and -629C>A (rs1800775)[24] are the most studied variants in the CETP gene in literature, we specifically checked the GWAS results for their association with CETP concentration. In addition, we reported the linkage disequilibrium (NEO study) of the lead SNPs from the present GWAS with the eight CETP SNPs that were used in a recent meta-Mendelian randomization study on coronary heart disease and serum lipids by Ference et al.[15] (i.e. rs3764261, rs1800775, rs1864163, rs9929488, rs9989419, rs12708967, rs289714 and rs5880). This allows comparison between our genetic instrument composed of SNPs identified form a GWAS on serum CETP concentration and their genetic instrument composed of candidate SNPs.

The explained variance in serum CETP concentration for the independent variants was estimated in the replication cohort. For each individual SNP the explained variance was estimated as the partial R^2 from the linear regression model with the SNP as independent variable and serum CETP concentration as dependent variable. To estimate the total variance explained by all independent lead SNPs, a weighted genetic risk score (GRS) was calculated per individual. The GRS was constructed as the sum of the number of risk alleles on the lead SNPs weighted by their effect size on CETP concentration in the discovery cohort. The combined explained variance was the partial R^2 from the linear regression model, with the weighted GRS as independent variable and serum CETP concentration as dependent variable.

To quantify the genome-wide cumulative effects of independent variants influencing various phenotypes, genetic correlations of serum CETP concentration with serum lipid concentra-

tions (i.e. HDL-C, LDL-C, triglycerides and total cholesterol), and BMI were calculated (Supplemental material 4.A).

Expression quantitative trait loci (eQTLs) analysis

To investigate whether the identified lead SNPs could explain serum CETP concentration via transcriptional gene regulation, we checked if these SNPs were eQTLs for CETP using data from the genotype-tissue expression (GTEx) project portal (V7)[25] and the Blood eQTL browser.[26]

Mendelian randomization

Based on the identified independent and replicated SNPs for serum CETP concentration in our study population, we conducted Mendelian randomization analyses on serum lipid concentrations in the NEO study population, and on the risk of CAD based on publically-available summary statistics data from the CARDGIoGRAMplusC4D 1000 Genomes study.[19] A detailed description of the Mendelian randomization analyses on CAD risk using data from the CARDIoGRAMplusC4D consortium can be found in the Supplemental material 4.A. Effect estimates for CAD risk were reported as odds ratio with corresponding 95% CI. We used a publically available tool[27] to conduct a power analysis for the Mendelian randomization analysis on CAD, which was based on the findings from the GWAS on serum CETP concentration, the explained variance of the SNPs used to compose the GRS, and the sample size of the CARDIoGRAMplusC4D 1000 Genomes study (60,801 cases; 123,504 controls).

In the NEO study population, we calculated the individual weighted GRS based on the identified SNPs and determined the effect of 1 μg/mL increase in genetically-determined CETP concentration on the concentrations of total cholesterol, HDL-C, triglycerides, LDL-C, total cholesterol/HDL-C ratio, LDL-C/HDL-C ratio and apolipoprotein B (ApoB), using linear regression analysis adjusted for age and sex. Beta coefficients and 95% CIs were reported.

In addition, for replication purposes, we extracted the independent leads SNPs from publically available datasets of the GLGC.[18] We also extracted the Taq1B (rs708272) and -629C>A (rs1800775) polymorphisms from the CARDGIoGRAMplusC4D 1000 Genomes and GLGC datasets.[18]

Results

Population characteristics

Characteristics of the discovery and replication cohorts are summarized in Table 4.1. Compared with the replication cohort, there were fewer women in the discovery cohort (50.6% versus 56.1%). Also, participants in the discovery cohort had a higher BMI (30.3 kg/m versus 25.6 kg/m) and more often used lipid-lowering drugs (17.5% versus 10.4%) than participants in the replication cohort. Serum CETP and lipid concentrations were comparable between both cohorts.

Table 4.1: Characteristics of the discovery and replication cohort from the Netherlands Epidemiology of Obesity (NEO) study.

Characteristics	Discovery cohort	Replication cohort
Number of participants	4,248[a]	1,458[b]
Age (year)	56 (51, 61)	57 (51, 61)
Women	2,148 (50.6%)	818 (56.1%)
Body mass index (kg/m^2)	30.3 (28.4, 33.0)	25.6 (23.2, 28.2)
Lipid-lowering drug users	745 (17.5%)	151 (10.4%)
Fasting serum concentrations		
CETP (μg/mL)	2.50 (0.67)	2.43 (0.64)
Total cholesterol (mmol/L)	5.66 (1.08)	5.69 (1.07)
LDL-cholesterol (mmol/L)	3.58 (0.99)	3.56 (0.98)
HDL-cholesterol (mmol/L)	1.38 (0.38)	1.58 (0.46)
Triglycerides (mmol/L)	1.34 (0.95, 1.87)	1.00 (0.71, 1.45)

Results are presented as median (inter quartile range) for not normally distributed data, mean (SD) or number (percentage).

[a] Missing data: n=13 for total cholesterol concentration, n=14 for HDL-cholesterol concentration, triglycerides and LDL-cholesterol concentration.

[b] No missing data.

Table 4.2: Summary statistics of the associations of the three independent lead SNPs that reached genome-wide significance and seven suggestive signals with serum CETP concentration.

								Discovery cohort (n=4,248)				Replication cohort (n=1,458)		
Chr	SNP	Position	Location	Closest gene	Coding/ non-coding allele	Coding allele frequency[a]	Imputation quality	Effect size per allele[b](μg/mL)	SE	P-value	Conditioned P-value[c]	Effect size per allele[b](μg/mL)	SE	P-value
Lead SNPs														
16	rs247616	56989590	Intergenic	*CETP*	C/T	0.67	1	0.32	0.015	3.98×10^{-100}	1.86×10^{-64}	0.31	0.024	1.24×10^{-37}
16	rs12720922	57000885	Intron	*CETP*	A/G	0.17	0.98	0.35	0.019	3.48×10^{-74}	6.68×10^{-13}	0.36	0.03	3.27×10^{-33}
16	rs1968905	57010948	Intron	*CETP*	G/T	0.82	0.85	0.12	0.02	4.12×10^{-9}	1.66×10^{-12}	0.098	0.031	1.80×10^{-3}
Suggestive SNPs														
2	rs185550357	50249349	Intron	*NRXN1*	C/T	0.007	0.7	0.63	0.12	3.94×10^{-7}	4.57×10^{-7}	0.064	0.15	0.67
3	rs6442310	12358230	Intron	*PPARG*	A/T	0.54	0.92	0.08	0.015	5.52×10^{-8}	6.13×10^{-8}	0.0044	0.024	0.85
8	chr8:19811023:I	19811023	Intron	*LPL*	ATG/A	0.12	0.87	0.12	0.023	1.45×10^{-7}	1.59×10^{-7}	-0.018	0.038	0.63
9	rs3094377	136312119	Intron	*ADAMTS13*	T/C	0.03	0.41	0.35	0.064	7.65×10^{-8}	9.58×10^{-8}	-0.18	0.11	0.098
10	rs12253367	62003462	Intron	*ANK3*	G/A	0.17	0.94	0.1	0.019	1.08×10^{-7}	1.18×10^{-7}	0.0051	0.031	0.87
16	rs117427818	57010486	Intron	*CETP*	T/C	0.05	0.75	0.46	0.038	3.26×10^{-33}	1.02×10^{-7}	0.54	0.061	1.93×10^{-18}
20	rs150904289	18845428	Intergenic		G/A	0.11	0.79	0.13	0.026	6.31×10^{-7}	6.87×10^{-7}	-0.0084	0.042	0.84

Threshold for genome-wide significance is 5×10^{-8}, based on the conditioned P-value[c]. Threshold for the suggestive signals is 1×10^{-6}. Threshold for replication is 0.05.

[a] In the discovery cohort.

[b] Beta coefficient expressed as the difference in serum CETP concentration.

[c] P-value conditioned on the top lead SNPs using step-wise conditional analysis

Chr, chromosome

Genome-wide association analysis

The -Log(P-value) plot for the GWAS is shown in Figure 4.1. The accompanying list of SNPs that reached genome-wide significance ($P<5\times10^{-8}$) is presented in Supplementary table 4.C.1. After conditioning on the lead SNPs, three independent variants reached genome-wide significance (conditioned $P<5\times10^{-8}$; Supplementary Figure 4.B.2) and seven suggestive signals were identified (conditioned $P<1\times10^{-6}$) in the discovery cohort (Table 4.2). The three genome-wide significant variants were all mapped to the *CETP* gene. Notably, these independent variants were rs247616 ($P=1.86\times10^{-64}$), rs12720922 ($P=6.68\times10^{-13}$) and rs1968905 ($P=1.66\times10^{-12}$), which had a per-allele increase (SE) in serum CETP of 0.32 (0.02) μg/mL (rs247616-C), 0.35 (0.02) μg/mL (rs12720922-A) and 0.12 (0.02) μg/mL (rs1968905-G). These three variants were all replicated in the replication analysis ($P<0.05$). In the NEO study, these variants together explained 16.4% of the serum CETP concentration. The distributions of the effect alleles of the three lead SNPs were similar for individuals taking lipid-lowering drugs and not taking lipid-lowering drugs (Supplementary table 4.C.2). A number of SNPs were suggestively associated with serum CETP concentration, including SNPs mapped to *ADAMTS3*, *PPARG* and *LPL*.

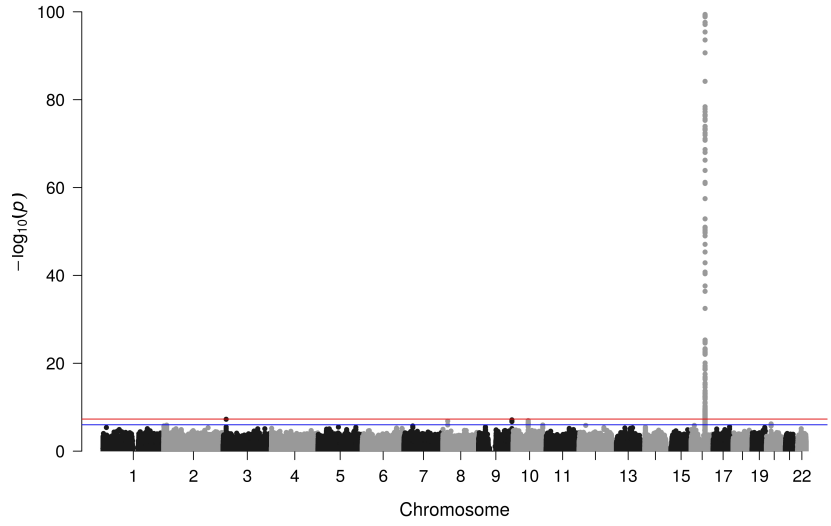


Figure 4.1: –Log(P-value) plot for the genome-wide association study in the discovery cohort (n=4,248). The *CETP* gene is located on chromosome 16. The red line represents the threshold for genome-wide significance ($P<5\times10^{-8}$). The blue line represents the threshold for suggestive signals ($P<1\times10^{-6}$).

The unconditioned per-allele effect size of the well-known Taq1B (rs708272) and -629C>A

(rs1800775) variants was 0.27 μg/mL for both SNPs (Supplementary table 4.C.1). Of the three lead SNPs, Taq1B was in high linkage disequilibrium with rs247616 (LD=0.55), as was -629C>A (LD=0.51). Taq1B and -629C>A were also in high linkage with each other (LD=0.83).

The linkage disequilibrium between the three lead SNPs and eight SNPs that were used as genetic instruments for a CETP GRS in a recent meta-Mendelian randomization analysis on coronary heart disease and serum lipids[15] are shown in Supplementary figure 4.B.3. The two strongest lead SNPs from the present GWAS, i.e. rs247616 and rs12720922, were in high linkage disequilibrium with the eight candidate SNPs of the GRS that was used in the study of Ference et al.,[15] with the highest linkage disequilibrium between rs247616 and rs3764261 (0.996), and between rs12720922 and rs1864163 (0.646).

The genetic correlation of serum CETP concentration with serum lipid concentrations and BMI is reported in Supplementary table 4.C.3. The genetic correlation of serum CETP concentration was highest with serum HDL-C concentration (0.17) and serum triglyceride concentration (-0.29), and lowest with serum total cholesterol concentration (-0.020), serum LDL-C concentration (0.074) and BMI (0.032).

eQTL analysis of the lead SNPs

Table 4.3 shows the eQTLs for the genetic variants rs247616, rs12720922 and rs1968905. The SNP that was most strongly associated with serum CETP concentration in the GWAS, i.e. rs247616, was identified as an eQTL for the *CETP* gene in several tissues (P-value range 6.3×10^{-10} to 4.1×10^{-5}). Rs12720922 was an eQTL for *NLRC5*, but not for CETP. However, we found rs1864163, which is in moderate linkage disequilibrium with rs12720922 (LD=0.65), to be an eQTL for CETP in whole blood (P=8.5×10^{-4}, effect size A-allele 3.33). The third lead SNP, i.e. rs1968905, was not identified as an eQTL for *CETP* in the studied tissues, neither were any variants in strong linkage disequilibrium with this SNP.

Table 4.3: Expression quantitative trait loci (eQTL) for the three GWAS-identified lead SNPs.

SNP	Assessed allele	Gene	Chr	P-value	Effect size	Tissue	Database
rs247616	C	*NLRC5*	16	9.5×10^{-14}	0.34	Transformed fibroblasts	GTEx
rs247616	C	*CETP*	16	6.3×10^{-10}	0.3	Lung	GTEx
rs247616	C	*CETP*	16	1.1×10^{-7}	0.41	Transverse colon	GTEx
rs247616	C	*CETP*	16	1.4×10^{-6}	0.45	Terminal Ileum	GTEx
rs247616	C	*CETP*	16	3.6×10^{-6}	0.32	Liver	GTEx
rs247616	C	*CETP*	16	7.9×10^{-6}	0.3	Esophagus (mucosa)	GTEx
rs247616	C	*CETP*	16	9.3×10^{-6}	0.41	Pancreas	GTEx
rs247616	C	*BBS2*	16	1.7×10^{-5}	0.37	Cerebellar Hemisphere	GTEx
rs247616	C	*CETP*	16	4.1×10^{-5}	4.1	Whole blood	Blood eQTL browser
rs12720922	A	*NLRC5*	16	1.9×10^{-6}	0.24	Transformed fibroblasts	GTEx
rs1968905	G	-	16	-	-	-	

Mendelian randomization

Figure 4.2 shows the results from the Mendelian randomization analyses on CAD risk and serum lipid and ApoB concentrations. We had a power of 0.90 to detect an odds ratio of 1.04 with conventional Mendelian randomization analyses (which makes use of a formal weighted genetic risk score), when taking into account an alpha of 0.05, the explained variance of the SNPs that compose the GRS (i.e. 16.4%), and the sample size of the CARDIoGRAMplusC4D 1000Genomes study. Per 1 μg/mL increase in genetically-determined serum CETP concentration the odds ratio for CAD risk was 1.08 (95% CI: 0.94, 1.23). For the lead SNPs separately, odds ratios were 1.11 (95% CI: 1.03, 1.17) for rs247616, 1.08 (95% CI: 1.01, 1.15) for rs12720922, and 0.89 (95% CI: 0.72, 1.09) for rs1968905. For Taq1B and -629C>A, odds ratios for CAD risk were 1.02 (95% CI 1.01, 1.04) and 1.03 (95% CI 1.01, 1.05), respectively (Supplementary table 4.C.5).

A 1 μg/mL increase in genetically-determined serum CETP concentration was associated with decreased total cholesterol concentration, i.e. -0.14 (95% CI: -0.22, -0.05) mmol/L, and HDL-C concentration, i.e. -0.23 (95% CI: -0.26, -0.20) mmol/L, while it was associated with increased serum LDL-C concentration, i.e. 0.08 (95% CI: 0.00, 0.16) mmol/L, and ApoB concentration, i.e. 0.02 (95% CI: 0.00, 0.03) g/L. Genetically-determined serum CETP concentration was not associated with serum triglycerides concentration, i.e. 0.02 (95% CI -0.05, 0.09) mmol/L. Supplementary table 4.C.4 shows the results from the Mendelian randomization analysis with data from GLGC. The results for total cholesterol, triglycerides and LDL-C concentrations from GLGC were comparable with the results from the NEO study. Effect sizes for HDL-C were larger in GLGC than in the NEO study.

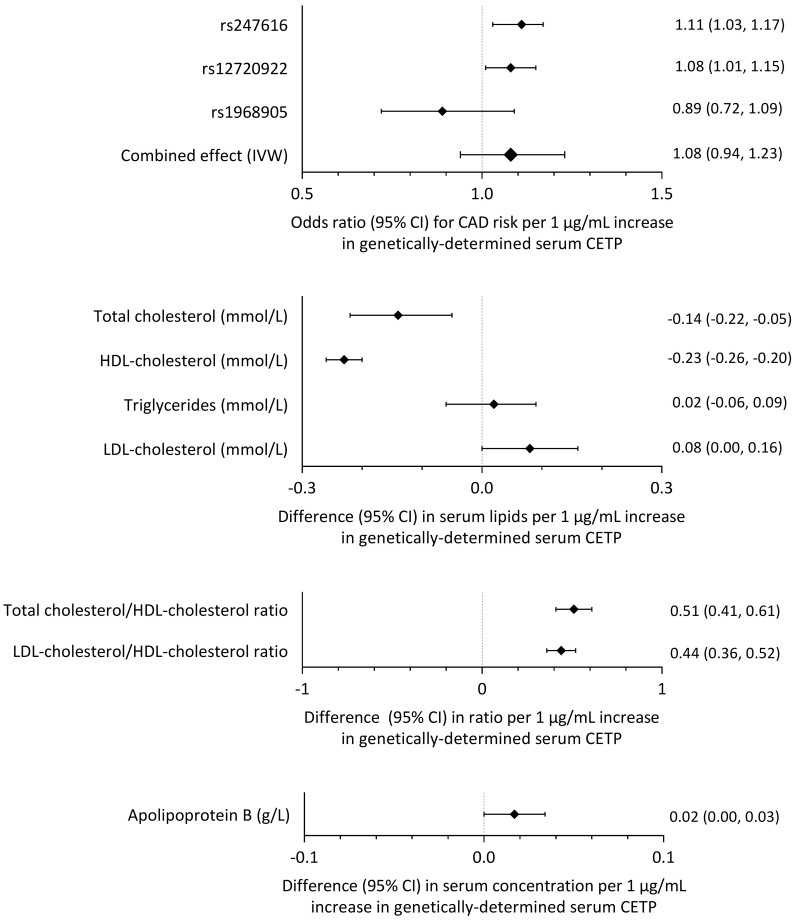


Figure 4.2: Results from the Mendelian randomization study on coronary artery disease in the CARDIoGRAMplusC4D 1000 Genomes Consortium (60,801 cases; 123,504 controls), and on serum lipid and lipoprotein B concentrations in the total Netherlands Epidemiology of Obesity (NEO) study population (n=5,706[a]).

[a] Missing data: n=13 for total cholesterol concentration, n=14 for HDL-cholesterol concentration, triglycerides and LDL-cholesterol concentration, n=41 for apolipoprotein B concentration. Results were adjusted for age and sex. CAD, coronary artery disease; CETP, cholesteryl ester transfer protein; IVW, inverse-variance weighted.

Discussion

With this first large GWAS on serum CETP concentration, we identified and replicated three independent SNPs, all mapping to the *CETP* region. These three variants, notably rs12720922, rs247616 and rs1968905, explained 16.4% of the total variation in serum CETP concentration. Effect sizes of all lead SNPs were large, with rs12720922-A having the largest effect on serum CETP: +0.35 μg/mL per additional risk allele. Also, we showed

that genetically-determined variation in circulating CETP associates with a stepwise substantial decrease in HDL-C concentration, a moderate increase in LDL-C and ApoB concentration, and a concordant 8% increase in CAD risk.

We found three independent SNPs in the *CETP* region that largely explained CETP concentration. The association of the rs12720922 variant with circulating CETP, blood lipids or risk of CAD has, to the best of our knowledge, never been described before. In the eQTL studies that we considered,[25,26] rs12720922 was not reported as an eQTL for *CETP*. However, rs1864163, which is in moderate linkage disequilibrium with rs12720922 (LD=0.65), was found to be an eQTL for *CETP* in whole blood,[26] although not in the liver.[25] Despite the absence of a direct association between rs12720922 and *CETP* mRNA levels, it is not ruled out that rs12720922 affects CETP levels via transcriptional regulation. Possibly, we were not able to identify eQTLs of rs12720922 for *CETP* due to the low sample size of eQTL studies in liver tissue, and the dilution that is introduced by considering whole liver expression, since *CETP* is specifically expressed by hepatic macrophages (i.e. Kupffer cells).[11] The second independent lead SNP, rs247616, is located in the promotor region of the *CETP* gene.[28] This SNP has also not been associated with serum CETP concentration before, but it has previously been shown that the minor allele of this variant (rs247616-T) is associated with decreased *CETP* mRNA expression in human liver and increased HDL-C concentrations[28–31], which is in line with our findings.

The third identified variant, rs1968905, was reported by one study to associate with HDL-C concentration specifically in Africans, but has not been linked to serum CETP concentration previously.[32] Of note, rs1968905 is in strong linkage disequilibrium (LD=0.89) with rs1801706 (i.e. G84A), which has been reported as a risk factor for CAD in South Indians.[33] Interestingly, rs1801706 is located in the 3' untranslated region (3' UTR) of the CETP gene, suggesting involvement in posttranscriptional regulation.[34] In addition to these lead SNPs, we found a suggestive signal (i.e. rs117427818) that could be a potential fourth hit in the *CETP* gene. Its statistical significance was, however, largely reduced upon conditional analyses. Indeed, this SNP is in linkage disequilibrium with (one of) the lead SNPs and not completely independently associated with serum CETP concentration (highest linkage disequilibrium with rs12720922; LD=0.20).

To obtain insight in the role of LDL-C and HDL-C in the causal association between serum CETP and CAD risk, we performed Mendelian randomization analyses on serum lipid concentrations. A higher CETP GRS was associated with a large decrease in HDL-C concentration and a moderate increase in LDL-C concentration. The strong association of genetically-determined serum CETP concentration with HDL-C concentration is probably partially explained by a shared genetic background of these two phenotypes, as the ge-

netic correlation between serum CETP concentration and HDL-C concentration was relatively high, which indicates pleiotropy. It should be noted, however, that HDL-C has been observationally, but not causally associated with CVD risk.[10] Thus, although a genetically-determined increase in serum CETP is causally associated with a decrease in HDL-C concentration, this likely does not explain the association between serum CETP and CAD risk. On the other hand, a causal, proportional, log-linear association between LDL-C concentration and CAD risk has been firmly established.[35] In a previously performed Mendelian randomization study using an LDL-C GRS, it was shown that the odds ratio for CAD risk was 1.68 (1.51-1.87) per 1 SD increase in LDL-C (i.e.0.98 mmol/L).[36] To compare, we showed that per 1 μg/mL increase in serum CETP, LDL-C concentration increased with 0.08 mmol/L and the odds ratio for CAD risk was 1.08. Thus, expressed per 0.98 mmol/L increase in LDL-C concentration, we observed 1.98 times increase in CAD risk using the CETP GRS, which is comparable with the effect estimate found with the LDL-C GRS.[36] Taken together, our study suggests that the causal association between CETP concentration and CAD risk may be explained by effects on LDL-C concentration. Interestingly, a recent large meta-Mendelian randomization analysis by Ference et al.,[15] indicated that ApoB concentration is an even more important causal link between CETP and CAD risk than LDL-C concentration. This implies that an increase in the absolute number of VLDL, IDL and LDL (i.e. non-HDL) particles, as reflected by ApoB concentration, due to increased circulating CETP may explain the association with CAD risk, rather than the amount of cholesterol in LDL particles.

Our findings are in line with this recent meta-Mendelian randomization analysis, including over a 100,000 participants that showed comparable effects for a CETP GRS on CAD risk, LDL-C and ApoB concentration.[15] In that meta-analysis, a CETP GRS was composed of eight candidate SNPs selected from the *CETP* gene with a forward conditional regression analysis on HDL-C concentration. In the present study, we identified three different *CETP* SNPs that independently determine circulating CETP concentration by using a hypothesis-free approach (i.e. GWAS). These GWAS-identified SNPs are therefore direct genetic instruments to study the causal effects of CETP in Mendelian randomization. Although none of the lead SNPs that we identified with GWAS were included in the GRS composed by Ference et al.[15], we observed that the two strongest lead SNPs from our GWAS were in high (rs247616) to moderate (rs12720922) linkage disequilibrium with the eight candidate SNPs of that GRS. This indicates that the GRS composed of candidate *CETP* SNPs is a reliable genetic instrument to study the causal effects of CETP, and our results therefore extend this recent meta-Mendelian randomization analysis.[15]

Although we showed a causal association between CETP concentration and CAD risk, the

three initial CETP inhibitors did not reduce the risk of cardiovascular events when given in addition to statin treatment. In fact, clinical trials with those CETP inhibitors were even terminated due to off-target effects (torcetrapib) or a lack of efficacy (dalcetrapib and evacetrapib).[5–7] Dalcetrapib had minimal effects in LDL-C concentration possibly explaining its futility.[7] Evacetrapib did significantly reduce LDL-C concentration, but did not evoke a concordant decrease in ApoB,[37] indicating unfavourable LDL particle remodelling rather than removal from the circulation.[35,38] This explanation for the failure of the evacetrapib trial is in line with a recent meta-Mendelian randomization study by Ference et al.[15] Data from that study indicate that the success of CETP inhibitors when prescribed on top of statin treatment is dependent on their capability to reduce the absolute number non-HDL particles as reflected by a reduction in ApoB concentration. A reduction in the LDL-C concentration through CETP inhibition may thus only be beneficial when a concordant reduction in ApoB concentration is achieved.[15] Indeed, anacetrapib did show a concordant reduction in non-HDL and ApoB concentration of -18%, which was accompanied by a reduced rate ratio for major coronary events of 0.91 (95% CI 0.85, 0.97).[4]

Our study may have had insufficient statistical power to identify additional variants with small effects on serum CETP concentration or with low allele frequencies. As we did not replicate our GWAS findings in additional heterogeneous populations, caution should be taken when extrapolating the results to other populations. Also, despite the similar associations between the lead SNPs and LDL-C concentrations in GLGC and the NEO study, effect sizes for HDL-C concentrations were higher in GLGC. A possible explanation might involve differences in the composition of the study populations, as GLGC is a meta-analysis of a wide variety of cohorts.

In conclusion, with a GWAS, we identified and replicated three independent SNPs mapping to the *CETP* gene that together explained 16.4% of the total variation in serum CETP concentration, which shows that serum CETP concentration is strongly genetically determined. Using Mendelian randomization, we showed that 1 μg/mL increase in serum CETP causally associates with a large decrease in HDL-C cholesterol of -0.23 mmol/L, moderate increases in LDL-C concentration of 0.08 mmol/L and ApoB concentration of 0.02 g/L, and an odds ratio of 1.08 for CAD risk. While HDL-C is not a causal risk factor for CAD, it has been unequivocally demonstrated that LDL-C lowering is proportionally associated with a lower CAD risk. Therefore, the results of our study are fully consistent with the notion that CETP concentration is causally associated with CAD through LDL-C.

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Appendix

4.A. Expanded methods

Genotyping and imputation

DNA was isolated from venous blood samples. Genotyping was performed using the Illumina HumanCoreExome-24 BeadChip (Illumina Inc., San Diego, California, United States of America). Participants were excluded (Supplementary Figure 4.B.1) in the process of quality control when 1) the sample call rate was <98%, 2) there was a sex mismatch, 3) heterozygosity rate was not within ±3 SD of mean heterozygosity rate, 4) participants widely diverged based on the first two principal components (PCs) (±3.5 SD), 5) samples were duplicates, and 6) concordance with another DNA sample was >0.25 (related individuals). Genetic variants were excluded when 1) genotype call rate was <98%, and 2) variants were not in Hardy-Weinberg equilibrium (P-value $<1\times10^{-6}$). Subsequently, genotypes were imputed to the 1000 Genome Project reference panel (v3 2011)[39] using IMPUTE (v2.2) software.[40] All genetic variants with an imputation quality below 0.4 or a minor allele frequency below 0.01 were not considered for the analyses in the present study.

Biochemical analyses

After centrifugation, aliquots of plasma and serum were stored at -80°C. From 11 April until 16 July 2014 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 (CV) 11.7%). We measured CETP concentration instead of exogenous CETP activity, both of which are highly correlated.[41–43] Participants with missing data on serum CETP concentration (n=65) and participants with a serum CETP concentration beyond four SD from the mean (n=3) were excluded for analyses (Supplementary figure 4.B.1).

Fasting serum total cholesterol and triglyceride concentrations were measured with enzymatic calorimetric assays (Roche Modular P800 Analyzer, Roche Diagnostics, Mannheim, Germany; CV <2% and CV <3%, respectively) and fasting serum HDL-C concentrations with third generation homogenous HDL-C methods (Roche Modular P800 Analyzer, Roche Diagnostics, Mannheim, Germany; CV <3%). Fasting LDL-C concentrations were calculated using the Friedewald equation.[28]

Nuclear magnetic resonance (NMR) spectroscopy

Serum apolipoprotein B (ApoB) concentration was determined with a high-throughput proton NMR metabolomics platform.[30] Details of the experimentation and applications of the NMR metabolomics platform have been described previously.[30]

Genome-wide association study

We used conditional and joint analyses to perform a stepwise selection procedure using the genome-wide complex trait analysis (GCTA) tool version 1.24.4.[8] This method uses summary statistics from the discovery phase and linkage disequilibrium correlations between SNPs estimated from the entire NEO study population. The step-wise selection of independent loci starts with the genetic variant with the lowest P-value in the summary statistics dataset, and performs association analyses to identify the next independent single nucleotide polymorphisms (SNPs) with the lowest conditioned P-value. This procedure is repeated until no more independent SNPs are identified.

Genetic correlations

To quantify the genome-wide cumulative effects of independent variants influencing various phenotypes, genetic correlations of serum CETP concentration with serum lipid concentrations (i.e. HDL-C, LDL-C, triglycerides and total cholesterol) and BMI were calculated. We used the bivariate genomic-relatedness-based restricted maximum-likelihood (GREML) approach implemented in genome-wide complex trait analysis (GCTA) to estimate the genetic correlations.[44] The genetic variance-covariance matrix between two phenotypes was estimated by the bivariate linear mixed model.[44] To take the effects of lipid-lowering drugs on blood lipid measurements into account, the total cholesterol concentration was divided by 0.8 for those individuals using lipid-lowering drugs.[45] Natural logarithmic transformation was used to obtain a normal distribution for serum triglyceride concentration.[45]

Mendelian randomization CARDIoGRAMplusC4D consortium

The genome-wide association meta-analysis of the CARDIoGRAMplusC4D consortium included 60,801 CAD cases and 123,504 controls of mainly European ancestry populations. CAD was defined as having a myocardial infarction, acute coronary syndrome, chronic stable angina or coronary stenosis of >50%.[19] The dataset contains the summary level meta-analysis data of the GWAS, comprising the additive (per-allele) beta estimates from logistic regression analyses of the SNPs on CAD risk, accompanying standard errors and effect alleles. For the Mendelian randomization on CAD, we combined the individual genetic variants for CETP concentration to estimate the causal effect of CETP concentration

on CAD risk. Analogous to pooling estimates from different observational studies with conventional meta-analysis using inverse-variance weighing, we weighted this combined effect estimate of the CETP SNPs on CAD by the inverse of the variance for each individual additive (per-allele) effect on CAD risk, and incorporated the individuals additive effects of the genetic instruments on CETP concentration. Effect estimates were reported as odds ratio with corresponding 95% CI.

4.B. Supplementary figures

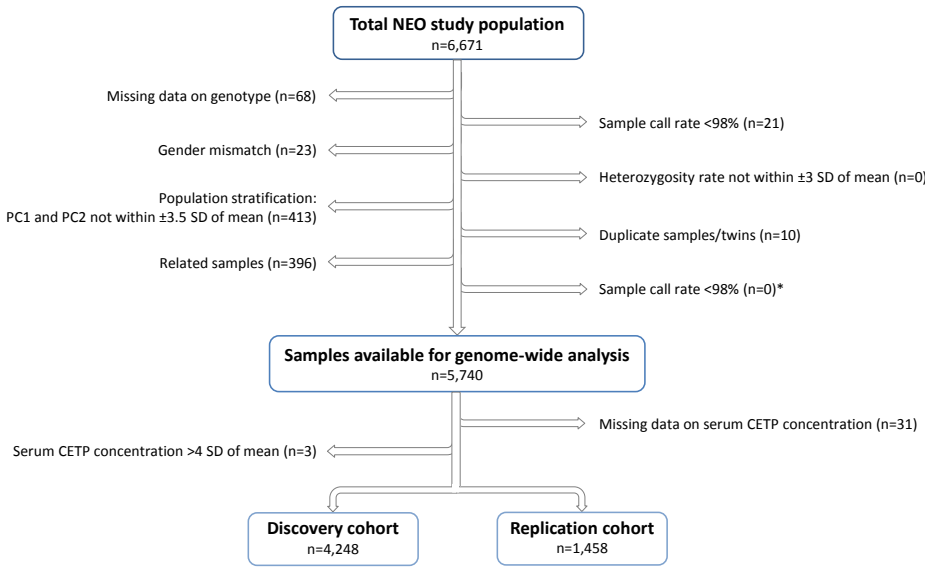


Figure 4.B.1: Quality control steps and exclusion criteria for the genome-wide association study (GWAS) on serum CETP concentration in the Netherlands Epidemiology of Obesity (NEO) study.

[a] Sample call rate was checked a second time, as it can change after removing samples on the basis of prior quality control steps.
PC, principal component.

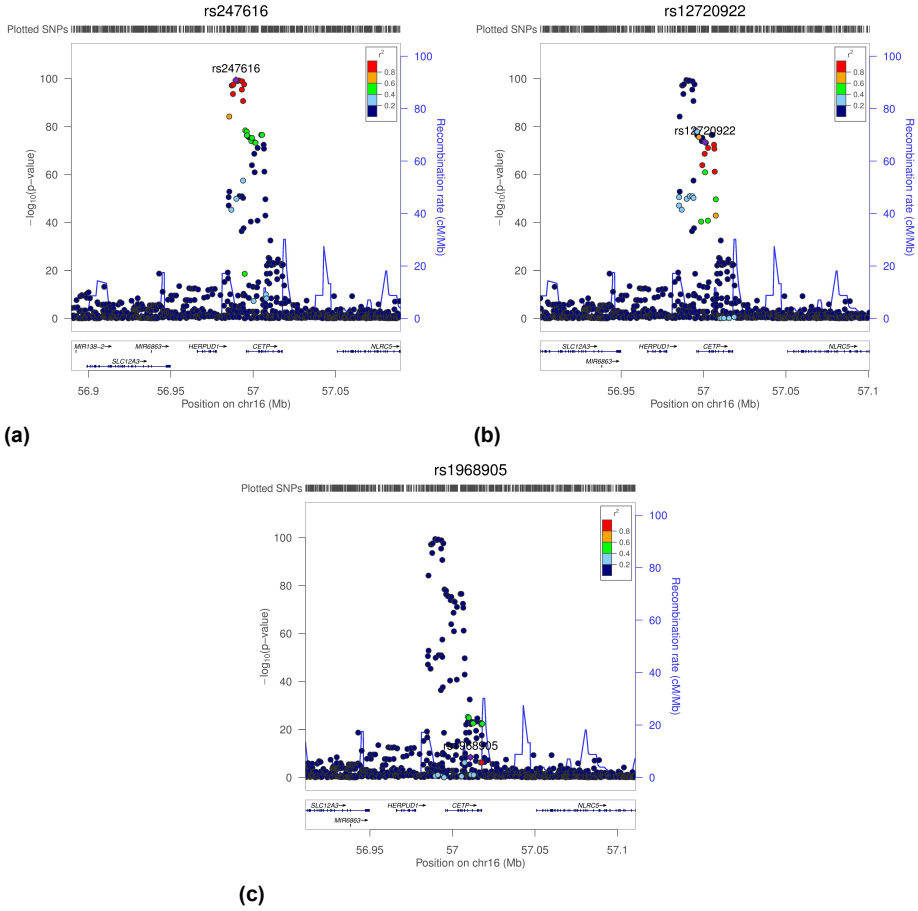


Figure 4.B.2: Regional association plots for the three independent genome-wide significant single nucleotide polymorphisms (SNPs), i.e. (**a**) rs247616, (**b**) rs12720922 and (**c**) rs1968905. The purple diamond indicates the lead SNP for the locus.

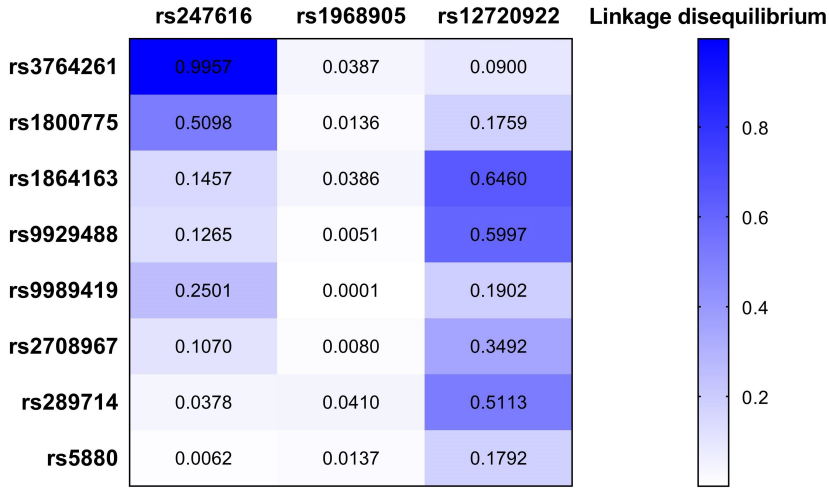


Figure 4.B.3: Linkage disequilibrium between the three GWAS-identified lead single nucleotide polymorphisms (SNPs) from the present study (i.e. rs247616, rs12720922 and rs1968905) and eight candidate SNPs that were used as genetic instruments for a CETP genetic risk score in a recent meta-Mendelian randomization analysis[a] on coronary heart disease and serum lipids (i.e. rs3764261, rs1800775, rs1864163, rs9929488, rs9989419, rs12708967, rs289714 and rs5880). Linkage disequilibrium was determined in the Netherlands Epidemiology of Obesity (NEO) study population.

[a] Ference BA, et al. Jama. 2017;318:947-956.

4.C. Supplementary tables

Table 4.C.1: Summary statistics of all single nucleotide polymorphisms (SNPs) that reached genome-wide significance in the discovery cohort, before conditioning on the independent lead SNPs.

SNP	CHR	POS	EA	NEA	EAF	BETA	SE	PVALUE
rs77712507	16	56742475	A	G	0.064	0.184	0.031	3.45E-9
rs185539847	16	56745071	C	T	0.081	-0.192	0.032	1.29E-9
rs4583235	16	56758596	A	G	0.102	0.137	0.024	1.06E-8
rs77050717	16	56767381	A	G	0.024	0.338	0.055	1.02E-9
rs76233589	16	56769902	G	A	0.103	0.134	0.024	1.71E-8
rs147726069	16	56790343	G	A	0.103	0.134	0.024	1.69E-8
rs60107605	16	56802246	G	T	0.102	0.133	0.024	2.40E-8
rs76477146	16	56814325	T	A	0.103	0.134	0.024	1.57E-8
rs8052978	16	56814847	A	G	0.376	-0.092	0.015	6.12E-10
rs77755067	16	56818259	C	A	0.103	0.134	0.024	1.58E-8
rs16962014	16	56819919	T	C	0.091	0.145	0.025	5.90E-9
rs79334440	16	56826362	C	A	0.103	0.134	0.024	1.58E-8
rs9938953	16	56826766	C	T	0.388	-0.088	0.015	1.51E-9
rs4783959	16	56834096	G	A	0.387	-0.089	0.015	1.24E-9
rs79984435	16	56834234	A	G	0.091	0.146	0.025	4.45E-9
rs79600951	16	56834254	G	C	0.091	0.146	0.025	4.45E-9
rs9929577	16	56836108	C	T	0.376	-0.092	0.015	5.32E-10
rs1803870	16	56839439	T	C	0.102	0.135	0.024	1.79E-8
rs7187512	16	56840171	G	A	0.387	-0.089	0.015	1.20E-9
chr16:56842612:D	16	56842612	T	TA	0.383	-0.107	0.016	9.78E-12
rs12444217	16	56843443	A	G	0.091	0.147	0.025	3.97E-9
rs16962399	16	56846804	T	C	0.09	0.146	0.025	4.50E-9
rs59542880	16	56848871	A	G	0.09	0.146	0.025	4.67E-9
rs1529929	16	56849496	A	G	0.389	-0.088	0.015	1.52E-9
rs9939678	16	56850602	G	A	0.389	-0.088	0.015	1.44E-9
rs1561139	16	56852822	T	G	0.386	-0.089	0.015	1.48E-9
rs80261911	16	56855192	T	C	0.09	0.146	0.025	4.57E-9
chr16:56855549:D	16	56855549	T	TC	0.09	0.146	0.025	4.58E-9
rs2895432	16	56856955	A	G	0.09	0.146	0.025	4.51E-9
rs7199480	16	56859595	A	G	0.389	-0.088	0.015	1.42E-9
rs2099536	16	56861995	G	C	0.39	-0.09	0.015	9.38E-10
chr16:56862143:D	16	56862143	G	GA	0.384	-0.086	0.015	6.00E-9
rs60310821	16	56863229	C	T	0.093	0.139	0.025	1.53E-8
rs11865000	16	56865374	A	G	0.093	0.142	0.025	8.43E-9
rs2241770	16	56866196	C	T	0.093	0.141	0.025	1.05E-8
rs3764266	16	56872824	A	G	0.093	0.14	0.025	1.29E-8
chr16:56873613:I	16	56873613	TA	T	0.093	0.14	0.025	1.27E-8
rs16962767	16	56873789	C	T	0.093	0.14	0.025	1.28E-8
rs1865830	16	56874197	A	G	0.384	-0.088	0.015	1.77E-9
rs2007432	16	56874857	T	C	0.387	-0.089	0.015	1.06E-9
chr16:56875272:I	16	56875272	AT	A	0.387	-0.089	0.015	1.06E-9
rs11860701	16	56876845	G	T	0.093	0.14	0.025	1.27E-8
rs2399562	16	56880158	G	A	0.388	-0.09	0.015	1.00E-9
rs8045306	16	56883709	C	G	0.391	-0.083	0.015	1.16E-8
rs735144	16	56883924	G	A	0.388	-0.089	0.015	9.97E-10

rs735145	16	56884081	C	G	0.094	0.14	0.025	1.37E-8
rs77850047	16	56885915	T	C	0.094	0.14	0.025	1.37E-8
rs12445993	16	56886109	G	C	0.094	0.14	0.025	1.36E-8
rs12443821	16	56886203	C	T	0.094	0.14	0.025	1.39E-8
rs80072323	16	56892769	A	G	0.096	0.137	0.025	2.37E-8
rs1436424	16	56895034	T	G	0.406	-0.09	0.015	6.16E-10
rs12599065	16	56896036	C	T	0.392	-0.093	0.015	1.79E-10
rs3829502	16	56896730	A	G	0.389	-0.091	0.014	3.20E-10
rs62035923	16	56898198	G	A	0.639	-0.096	0.015	3.23E-10
rs4784733	16	56899006	T	C	0.667	-0.098	0.015	5.33E-11
rs13306690	16	56899007	C	G	0.094	0.149	0.025	2.48E-9
rs1968493	16	56900100	G	A	0.633	-0.105	0.016	1.37E-11
rs13306673	16	56900931	T	C	0.09	0.154	0.026	4.07E-9
rs77188937	16	56909598	T	C	0.087	0.219	0.029	6.40E-14
rs13306677	16	56926195	A	G	0.08	-0.148	0.027	4.33E-8
rs9929408	16	56929944	G	A	0.475	0.091	0.015	3.88E-9
rs1138429	16	56942921	T	A	0.113	0.231	0.026	2.12E-19
rs59515242	16	56945049	A	C	0.214	0.124	0.018	7.55E-12
rs56079121	16	56948292	G	A	0.098	-0.137	0.024	1.69E-8
rs9925265	16	56950210	A	G	0.455	0.09	0.014	2.87E-10
rs718620	16	56950643	T	C	0.097	-0.137	0.024	1.79E-8
rs9921780	16	56952098	G	A	0.454	0.09	0.014	2.95E-10
rs12924331	16	56953261	G	A	0.454	0.09	0.014	2.82E-10
rs55958623	16	56955110	T	C	0.141	0.156	0.021	3.54E-14
rs9931252	16	56959019	T	C	0.098	-0.136	0.024	2.17E-8
rs75429044	16	56960147	A	G	0.097	-0.136	0.024	2.23E-8
rs247606	16	56961204	A	G	0.144	0.149	0.02	2.63E-13
rs28495885	16	56961932	T	C	0.096	-0.135	0.024	3.26E-8
rs193693	16	56962169	G	A	0.144	0.147	0.02	3.75E-13
rs247607	16	56963322	A	G	0.144	0.147	0.02	3.85E-13
rs247608	16	56963468	T	C	0.144	0.147	0.02	3.76E-13
rs2518055	16	56963643	A	T	0.144	0.147	0.02	3.93E-13
rs9932164	16	56964445	T	G	0.097	-0.136	0.024	1.99E-8
rs1366544	16	56964719	A	G	0.144	0.148	0.02	2.80E-13
rs2518058	16	56966554	C	T	0.142	0.146	0.02	8.17E-13
rs2217332	16	56969148	A	G	0.142	0.144	0.02	1.38E-12
rs72786781	16	56970210	A	T	0.026	-0.316	0.053	2.30E-9
rs9938413	16	56972250	T	C	0.108	-0.141	0.023	9.96E-10
rs2562126	16	56972713	A	G	0.144	0.144	0.02	1.42E-12
rs3903056	16	56974791	G	A	0.142	0.146	0.02	6.17E-13
chr16:56974859:I	16	56974859	GAC	G	0.109	-0.141	0.023	8.56E-10
rs952439	16	56975277	C	A	0.148	0.131	0.02	8.85E-11
rs881598	16	56976744	A	G	0.142	0.147	0.02	5.15E-13
rs112952893	16	56980135	A	G	0.113	-0.146	0.023	1.89E-10
rs193694	16	56982549	T	C	0.138	0.178	0.022	6.49E-16
rs9938160	16	56984590	C	T	0.276	-0.17	0.019	7.23E-20
rs247615	16	56984763	G	A	0.206	0.181	0.021	2.22E-17
rs9989419	16	56985139	G	A	0.627	-0.252	0.017	2.71E-51
rs193695	16	56985156	G	A	0.659	-0.252	0.017	8.39E-48
rs72786786	16	56985514	A	G	0.327	-0.312	0.016	6.69E-85
rs12448528	16	56985555	G	A	0.792	-0.308	0.02	1.34E-53

rs1122390	16	56986045	T	G	0.756	-0.113	0.017	8.50E-11
rs7203286	16	56986762	T	G	0.429	0.213	0.015	4.72E-46
rs12446515	16	56987015	T	C	0.335	-0.319	0.015	7.63E-98
rs56156922	16	56987369	C	T	0.335	-0.319	0.015	6.84E-98
rs56228609	16	56987765	T	C	0.324	-0.316	0.015	2.59E-94
rs173539	16	56988044	T	C	0.336	-0.318	0.015	2.55E-98
rs247616	16	56989590	T	C	0.333	-0.321	0.015	3.98E-100
rs12923459	16	56989830	A	G	0.421	0.22	0.015	1.33E-50
rs247617	16	56990716	A	C	0.333	-0.32	0.015	1.02E-99
rs247618	16	56990803	A	G	0.752	-0.107	0.017	3.02E-10
rs183130	16	56991363	T	C	0.333	-0.321	0.015	5.92E-100
rs28888131	16	56991624	A	G	0.177	0.298	0.02	1.06E-51
rs12934632	16	56991741	T	C	0.177	0.298	0.02	1.10E-51
rs12920974	16	56993025	T	G	0.27	0.207	0.016	4.19E-37
rs12149545	16	56993161	A	G	0.321	-0.318	0.015	3.93E-96
rs12708967	16	56993211	C	T	0.177	0.297	0.02	1.18E-51
rs3764261	16	56993324	A	C	0.333	-0.32	0.015	1.36E-99
rs821840	16	56993886	G	A	0.287	-0.335	0.017	2.17E-91
rs36229786	16	56993901	C	A	0.172	0.316	0.021	5.93E-51
rs711751	16	56993909	C	A	0.553	-0.243	0.015	3.37E-58
rs12447839	16	56993935	T	C	0.756	-0.107	0.017	2.30E-10
rs12447924	16	56994192	T	C	0.755	-0.111	0.017	5.38E-11
rs12720918	16	56994212	C	T	0.273	0.212	0.016	2.49E-38
chr16:56994244:I	16	56994244	TA	T	0.332	-0.322	0.015	8.05E-100
rs17231506	16	56994528	T	C	0.328	-0.323	0.015	2.54E-98
rs4783961	16	56994894	A	G	0.508	-0.133	0.015	2.45E-19
rs4783962	16	56995038	C	T	0.756	-0.106	0.017	2.93E-10
rs1800775	16	56995236	A	C	0.491	-0.267	0.014	4.05E-79
rs3816117	16	56996158	C	T	0.492	-0.267	0.014	1.34E-78
rs711752	16	56996211	A	G	0.446	-0.267	0.014	4.70E-77
rs708272	16	56996288	A	G	0.446	-0.267	0.014	4.83E-77
chr16:56996645:D	16	56996645	G	GCC	0.448	-0.271	0.014	5.57E-78
rs1864163	16	56997233	A	G	0.232	0.321	0.017	2.46E-76
chr16:56997349:I	16	56997349	CA	C	0.233	0.305	0.018	5.89E-67
rs4587963	16	56997369	T	A	0.755	-0.1	0.017	2.98E-9
rs4369653	16	56997551	T	C	0.698	-0.12	0.016	1.81E-14
rs9929488	16	56998572	C	G	0.252	0.223	0.017	4.21E-41
rs12720926	16	56998918	G	A	0.442	-0.263	0.014	1.19E-74
rs7203984	16	56999258	C	A	0.186	0.317	0.019	1.29E-64
rs11508026	16	56999328	T	C	0.437	-0.265	0.014	5.05E-76
chr16:56999778:D	16	56999778	C	CG	0.169	0.348	0.019	1.17E-74
rs708273	16	56999949	G	A	0.7	-0.117	0.016	6.74E-14
rs8045855	16	57000696	A	T	0.176	0.344	0.02	2.22E-69
rs12720922	16	57000885	A	G	0.168	0.348	0.019	3.48E-74
rs118146573	16	57000938	A	G	0.117	0.379	0.023	1.17E-61
rs4784741	16	57001216	T	C	0.44	-0.26	0.014	5.12E-74
chr16:57001254:I	16	57001254	TCACA	T	0.169	0.345	0.019	6.75E-74
chr16:57001274:D	16	57001274	A	AC	0.43	-0.264	0.015	1.13E-72
rs12444012	16	57001438	A	G	0.439	-0.26	0.014	6.28E-74
chr16:57001579:D	16	57001579	A	AAAAAC	0.28	-0.264	0.018	1.01E-49
chr16:57001580:D	16	57001580	A	AAAAC	0.404	-0.261	0.015	1.02E-68

chr16:57001581:D	16	57001581	A	AAAC	0.44	-0.26	0.014	7.30E-74
rs60545348	16	57001985	C	A	0.299	0.115	0.016	1.02E-13
rs72771478	16	57002118	T	G	0.02	-0.401	0.064	4.74E-10
rs72771479	16	57002121	T	A	0.02	-0.4	0.064	5.19E-10
rs12597002	16	57002404	A	C	0.299	0.115	0.016	1.06E-13
rs9926440	16	57002663	G	C	0.731	-0.215	0.016	1.72E-41
rs9939224	16	57002732	G	T	0.81	-0.321	0.018	8.31E-72
rs11076174	16	57003146	C	T	0.071	0.236	0.029	7.92E-16
rs7205804	16	57004889	A	G	0.449	-0.264	0.014	2.94E-77
rs1532625	16	57005301	T	C	0.45	-0.264	0.014	2.78E-77
rs1532624	16	57005479	A	C	0.45	-0.264	0.014	2.98E-77
rs117040820	16	57005762	T	C	0.019	-0.356	0.062	1.06E-8
rs11076175	16	57006378	G	A	0.166	0.345	0.019	3.86E-73
rs7499892	16	57006590	T	C	0.166	0.34	0.019	1.71E-71
rs289713	16	57006829	A	T	0.817	-0.312	0.019	6.44E-62
rs11076176	16	57007446	G	T	0.168	0.305	0.02	2.09E-50
rs289714	16	57007451	A	G	0.821	-0.276	0.02	1.33E-43
rs158477	16	57007610	A	G	0.509	0.092	0.015	1.35E-9
rs158478	16	57007734	C	A	0.504	0.097	0.015	9.74E-11
rs158479	16	57008048	A	G	0.516	0.087	0.015	4.82E-9
rs158480	16	57008227	A	G	0.86	0.203	0.023	2.38E-19
rs158617	16	57008287	G	A	0.848	0.217	0.022	9.14E-23
rs289715	16	57008508	T	A	0.88	0.195	0.024	3.12E-16
rs289716	16	57009376	A	T	0.692	0.164	0.016	5.45E-26
rs289717	16	57009388	A	G	0.346	0.151	0.015	6.40E-24
chr16:57009651:D	16	57009651	T	TC	0.696	0.165	0.016	4.97E-26
chr16:57009657:D	16	57009657	C	CA	0.416	0.179	0.018	7.98E-24
rs736274	16	57009769	A	T	0.114	-0.193	0.024	2.81E-15
rs289718	16	57009932	T	C	0.7	0.165	0.016	1.27E-25
rs289719	16	57009941	C	T	0.701	0.165	0.016	1.29E-25
rs56208677	16	57010232	T	C	0.066	-0.301	0.033	1.87E-19
rs117427818	16	57010486	T	C	0.05	0.462	0.038	3.26E-33
rs1968905	16	57010948	G	T	0.824	0.117	0.02	4.12E-9
rs4784744	16	57011185	A	G	0.354	0.149	0.015	6.17E-23
rs291044	16	57011452	A	G	0.345	0.152	0.015	6.28E-24
chr16:57012559:I	16	57012559	AT	A	0.246	-0.15	0.017	1.27E-17
rs12720889	16	57012563	T	A	0.285	-0.16	0.016	2.82E-23
rs291043	16	57012699	G	A	0.341	0.153	0.015	9.20E-24
rs12447620	16	57014319	G	A	0.842	0.204	0.022	3.55E-20
rs12708983	16	57014411	C	T	0.039	-0.254	0.042	2.23E-9
rs12708985	16	57014610	C	T	0.842	0.19	0.023	4.43E-17
rs4784745	16	57014875	G	A	0.342	0.155	0.015	4.90E-24
rs5880	16	57015091	C	G	0.059	0.373	0.036	2.50E-25
rs5882	16	57016092	A	G	0.685	0.143	0.015	8.48E-21
rs1800777	16	57017319	A	G	0.047	0.417	0.042	1.91E-23
rs289741	16	57017474	A	G	0.706	0.156	0.016	8.17E-23
rs289742	16	57017762	G	C	0.869	0.199	0.023	2.86E-18
rs289743	16	57017796	A	G	0.707	0.158	0.016	3.74E-23
rs289744	16	57018102	T	G	0.707	0.16	0.016	4.23E-23
rs112039804	16	57018856	A	T	0.106	-0.204	0.027	1.83E-14
rs12720917	16	57019392	C	T	0.149	-0.14	0.024	6.21E-9

rs7198642	16	57032461	G	T	0.189	-0.115	0.021	3.87E-8
rs75911530	16	57049137	A	G	0.032	0.311	0.05	5.26E-10
rs61738710	16	57060097	T	C	0.068	0.205	0.033	5.32E-10

SNP, single nucleotide polymorphism; CHR, chomosome; POS, position; (N)EA, (non) effect allele; EAF, effect allele frequency; BETA, beta coefficient; SE, standard error.

Table 4.C.2: Distribution of coding alleles of the lead single nucleotide polymorphisms (SNPs) in lipid-lowering drug users and non-lipid-lowering drug users, in the total Netherlands Epidemiology of Obesity (NEO) study population (n=5,706).

Number of coding alleles	Non-users (n, %)	Lipid lowering drug users (n, %)
rs247616-C		
0	517 (11%)	113 (13%)
1	2,165 (45%)	373 (42%)
2	2,128 (44%)	410 (46%)
rs12720922-A		
0	3,326 (69%)	605 (68%)
1	1,341 (28%)	260 (29%)
2	143 (3%)	31 (3%)
rs1968905-G		
0	145 (3%)	38 (4%)
1	1,440 (30%)	237 (26%)
2	3,225 (67%)	621 (69%)

Table 4.C.3: Genetic correlation of serum CETP concentration with serum lipid concentrations (i.e. total cholesterol, HDL-cholesterol, triglycerides and LDL-cholesterol) and body mass index, in the total Netherlands Epidemiology of Obesity (NEO) study population (n=5,706).

Serum concentration	Genetic correlation with serum CETP concentration
Total cholesterol	-0.020
HDL-C	0.17
Triglycerides	-0.29
LDL-C	0.074
Body mass index	0.032

Table 4.C.4: Effects of genetic variation in the lead single nucleotide polymorphisms (SNPs) on circulating lipid concentrations, in the total Netherlands Epidemiology of Obesity (NEO) study population and the Global Lipids Genetics Consortium.

	Difference in concentration per additional serum CETP increasing allele	95% CI	N	P-Value
	rs247616 (C allele)			
NEO study				
Total cholesterol (mmol/L)	-0.03	-0.08, 0.01	5,693	0.109
HDL-C (mmol/L)	-0.09	-0.11, -0.08	5,692	1.18E-36
Triglycerides (mmol/L)	0.01	-0.03, 0.04	5,692	0.664
LDL-C (mmol/L)	0.05	0.01, 0.09	5,692	0.009
Global Lipids Genetics Consortium				
Total cholesterol (mmol/L)	-0.05	-0.06, -0.04	185,621	4.47E-32
HDL-C (mmol/L)	-0.24	-0.24, -0.25	185,471	0
Triglycerides (mmol/L)	0.04	0.03. 0.05	176,146	1.12E-25
LDL-C (mmol/L)	0.05	0.05, 0.06	171,458	2.57E-37
	rs12720922 (A allele)			
NEO study				
Total cholesterol (mmol/L)	-0.09	-0.14, -0.04	5,693	0.001
HDL-C (mmol/L)	-0.11	-0.13, -0.09	5,692	6.30E-32
Triglycerides (mmol/L)	0.01	-0.04, 0.05	5,692	0.808
LDL-C (mmol/L)	0.02	-0.03, 0.06	5,692	0.513
Global Lipids Genetics Consortium				
Total cholesterol (mmol/L)	-0.05	-0.06, -0.04	92,615	2.14E-14
HDL-C (mmol/L)	-0.26	-0.24, -0.25	92,714	1.67E-318
Triglycerides (mmol/L)	0.04	0.03, 0.05	86,712	3.24E-11
LDL-C (mmol/L)	0.05	0.04, 0.06	83,073	1.42E-13
	rs1968905 (G allele)			
NEO study				
Total cholesterol (mmol/L)	-0.03	-0.09, 0.02	5,693	0.193
HDL-C (mmol/L)	-0.02	-0.04, -0.00	5,692	0.030
Triglycerides (mmol/L)	0.01	-0.04, 0.05	5,692	0.808
LDL-C (mmol/L)	-0.02	-0.06, 0.03	5,692	0.490
Global Lipids Genetics Consortium				
Total cholesterol (mmol/L)	N/A	N/A	N/A	N/A
HDL-C (mmol/L)	N/A	N/A	N/A	N/A
Triglycerides (mmol/L)	N/A	N/A	N/A	N/A
LDL-C (mmol/L)	N/A	N/A	N/A	N/A

N/A, not available in the Global Lipids Genetics Consortium database.

Table 4.C.5: Effects of genetic variation in the Taq1B and -629C>A CETP single nucleotide polymorphisms (SNPs) on coronary artery disease (CAD) risk and circulating lipid concentrations, in the CARDIoGRAMplusC4D 1000 Genomes Consortium and the Global Lipids Genetics Consortium.

	Difference per additional serum CETP increasing allele	95% CI	P-Value
	Taq1B (rs708272-G)		
CARDIoGRAMplusC4D 1000 Genomes Consortium			
CAD risk (odds ratio)	1.02	1.01, 1.04	0.011
Global Lipids Genetics Consortium			
Total cholesterol (mmol/L)	N/A	N/A	N/A
HDL-C (mmol/L)	N/A	N/A	N/A
Triglycerides (mmol/L)	N/A	N/A	N/A
LDL-C (mmol/L)	N/A	N/A	N/A
	-629C>A (rs1800775-C)		
CARDIoGRAMplusC4D 1000 Genomes Consortium			
CAD risk (odds ratio)	1.03	1.01, 1.05	0.0027
Global Lipids Genetics Consortium			
Total cholesterol (mmol/L)	-0.04	-0.05, -0.04	2,10E-25
HDL-C (mmol/L)	-0.20	-0.21, -0.20	0
Triglycerides (mmol/L)	0.04	0.03, 0.05	1.33E-26
LDL-C (mmol/L)	0.04	0.03, 0.05	8.5E-24

N/A, not available in the Global Lipids Genetics Consortium database

5

Exome-wide analysis for rare variants identifies *ABCA6* as determinant of circulating CETP concentration

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In preparation

Abstract

Introduction

Recently, we performed a genome-wide association analysis (GWAS) to identify common genetic determinants of circulating cholesteryl ester transfer protein (CETP), an approach by which rare variants are missed. Identification of novel coding variants may provide insight in the pathways that regulate CETP metabolism, and may contribute to our understanding of the biological function of CETP in lipid metabolism and cardiovascular disease. Therefore, in the present study, we aimed to identify rare coding variants in exonic regions that determine CETP concentration.

Methods

We performed an exome-wide gene-based aggregation analysis in 6,094 participants from the Netherlands Epidemiology of Obesity (NEO) study to identify rare coding variants (minor allele frequency <0.03) in exonic regions that are associated with serum CETP concentration.

Results

We identified the *ABCA6* gene to be exome-wide significantly associated with CETP concentration ($P=9.4\times10^{-7}$). The driving variant for the *ABCA6* genetic region was rs77542162 (MAF=0.028) with a per-allele effect of 0.19 μg/mL on circulating CETP concentration, while the mean (SD) CETP concentration in the study population was 2.48 (0.66) μg/mL.

Conclusion

The *ABCA6* gene was identified as a novel determinant of circulating CETP, which indicates that abundance or functionality of the ABCA6 protein regulates CETP concentration. Future research is warranted to elucidate the underlying biological pathways that can explain this association.

Introduction

Cholesteryl ester transfer protein (CETP) is able to transfer cholesteryl esters from high-density lipoproteins (HDL) towards apolipoprotein B (ApoB) containing triglyceride-rich lipoproteins, mainly very low-density lipoproteins (VLDL). In exchange, triglycerides are transferred from VLDL towards triglyceride-poor particles, which are both HDL and low-density lipoproteins (LDL).[1] As such, CETP contributes to an atherogenic lipoprotein profile (i.e. high LDL-cholesterol (C)/HDL-C ratio), as has been extensively studied in both humans and in mice transgenic for human CETP.[2,3] Therefore, inhibition of CETP as therapeutic strategy to attenuate dyslipidaemia and ultimately prevent the development of cardiovascular disease (CVD) has been extensively tested.

To elucidate the genetic basis of CETP, we recently performed a genome-wide association study (GWAS) on serum CETP concentration in the Netherlands Epidemiology of Obesity (NEO) study, and showed that CETP concentration is highly genetically determined by three independent loci with respectively driving single nucleotide polymorphisms (SNPs) rs247616, rs12720922 and rs1968905, which are all mapped to the *CETP* gene [Blauw LL, et al. Submitted]. This GWAS was specifically designed to identify common genetic variants in relation to CETP concentration, an approach by which rare variants that may have larger effects on serum CETP concentration than common variants are not taken into account.

Identification of novel coding gene regions that determine CETP concentration may provide insight in the pathways that regulate CETP production and clearance. Elucidation of these pathways may contribute to our understanding of the biological function of CETP in lipid metabolism and CVD. This is especially relevant in light of the recent disclosure of the results from the REVEAL trial, which showed that the CETP inhibitor anacetrapib met its primary endpoint, i.e. a reduction in major coronary events.[4] Therefore, in the present study, we aimed to identify rare coding variants (i.e. minor allele frequency (MAF) <0.03) in exonic regions that are jointly associated with serum CETP concentration by performing an exome-wide gene-based aggregation analysis.

Materials and Methods

Study design and population

The NEO study is a population-based prospective cohort study of men and women aged between 45 and 65 years. From the greater area of Leiden, The Netherlands, all inhabitants with a self-reported body mass index (BMI) of 27 kg/m^2 or higher were eligible to participate. In addition, inhabitants from one nearby municipality (Leiderdorp, The Nether-

lands) in the same age group were invited to participate regardless of their BMI, forming a reference population for BMI distribution. In total, 6,671 participants were included from September 2008 until September 2012. Participants visited the NEO study center for extensive physical examination. Venous blood samples were obtained from the antecubital vein after a 10 hour overnight fast. 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 NEO study was approved by the medical ethics committee of the Leiden University Medical Center (LUMC), and all participants gave their written informed consent. Detailed information about the study design and data collection has been described elsewhere.[5]

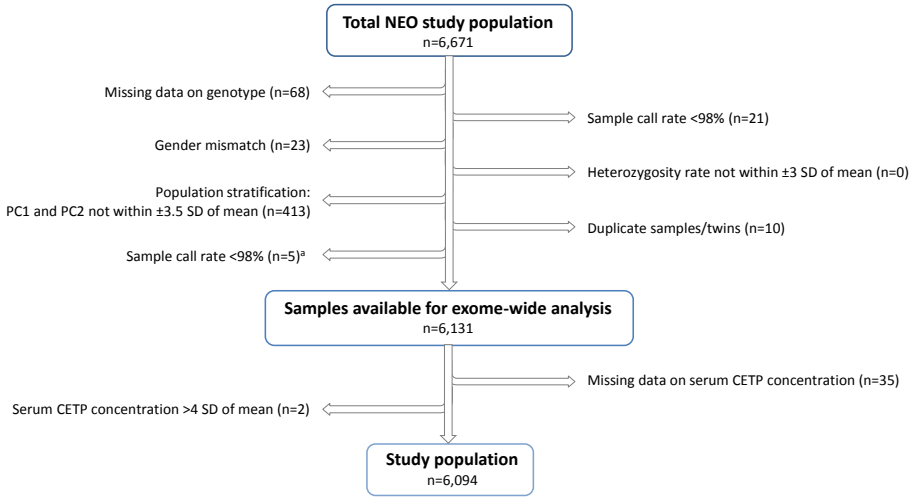


Figure 5.1: Quality control steps for the genome-wide analysis and exclusion criteria for the discovery and replication cohort from the Netherlands Epidemiology of Obesity (NEO) study.

[a] Sample call rate was checked a second time, as it can change after removing samples on the basis of prior quality control steps.
CETP, cholesteryl ester transfer protein; PC, principal component.

Biochemical analyses

After centrifugation, aliquots of plasma and serum were stored at -80°C. From 11 April until 16 July 2014 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, Alpco, Salem, USA; coefficient of variation 11.7%). Participants with missing data on serum CETP concentration (n=35) and participants with a serum CETP concentration beyond four SD from the mean (n=2)

were excluded from analyses.

Fasting serum total cholesterol and triglyceride concentrations were measured with enzymatic calorimetric assays (Roche Modular P800 Analyzer, Roche Diagnostics, Mannheim, Germany; CV <2% and CV <3%, respectively) and fasting serum HDL-C concentrations with third generation homogenous HDL-C methods (Roche Modular P800 Analyzer, Roche Diagnostics, Mannheim, Germany; CV <3%). Fasting LDL-C concentrations were calculated using the Friedewald equation.[6]

Genotyping

DNA was isolated from venous blood samples. Genotyping was performed using the Illumina HumanCoreExome-24 BeadChip (Illumina Inc., San Diego, California, United States of America), including 265,919 exome-focussed markers. Participants were excluded in the process of quality control when 1) the sample call rate was <98%, 2) there was a sex mismatch, 3) heterozygosity rate was not within ±3 SD of mean heterozygosity rate, 4) participants widely diverged based on the first two principal components (±3.5 SD), and 5) samples were duplicates. The present study population consists of 6,094 individuals (Figure 5.1).

Genetic variants were excluded when genotype call rate was <98%, and when variants were not in Hardy-Weinberg equilibrium (P-value $<1\times10^{-6}$). In the present study, we restricted to genetic variants, which were located in exonic regions, which had a MAF<0.03, and which had deleterious effects (i.e. splice donor, splice acceptor, stop gained, stop lost, initiator codon or missense).

Gene-based aggregation analysis

We performed a gene-based aggregation analysis to identify rare coding variants (i.e. MAF <0.03) that are jointly associated with serum CETP concentration. With this approach, a gain in statistical power is achieved by evaluating the cumulative effects of multiple genetic variants in a gene region.[7] Two different gene-based aggregation methods were applied to test for different assumptions about the underlying genetic model. The first method, i.e. the combined multivariate and collapsing (CMC) method,[8] is a Burden test, which assumes that 1) the variants in a genetic region are causal and 2) that effects of these variants on the outcome all have the same direction and magnitude. The second method, i.e. the sequence kernel association test (SKAT),[9] is a variance-component test. This test assumes that the effects of the variants on the outcome can have opposite directions (i.e. both positive and negative), and does not make assumptions about causality. We used RAREMETAL version 4.13.9[10] to perform the analyses with the CMC method and SKAT.

To account for the multiple testing of 12,157 genes, a Bonferroni-corrected threshold of $P<4.1\times10^{-6}$ was regarded exome-wide significant.

To identify the driving variants within the identified gene regions, conditional analysis was used. The variant with the lowest P-value was selected, and subsequently the association analysis was adjusted for this variant. If the joint association thereafter showed no statistical significant result anymore, the variant that was adjusted for was proven to be the driving variant within the gene region. Otherwise, the analysis was additionally adjusted for the next variant with the lowest P-value. This procedure was repeated until no more exome-wide significant variants were identified.

We reported for the CMC test, both the joint effect size for the identified gene region and the single effect size of the driving variant. For SKAT only the single effect size of the driving variant was reported, as a joined effect size cannot be generated form this method.

In the case that a genetic region was identified in near proximity of the *CETP* gene, we tested whether the association between the driving variant of this genetic region and serum CETP concentration was explained by one of the common CETP SNPs that determine CETP concentration (i.e. rs247616, rs12720922 and rs1968905, as previously identified with GWAS [Blauw LL, Li-Gao R, et al. Under review]). This was done by adding the three common *CETP* SNPs as covariates to a linear regression model for the association between the rare driving variant and CETP concentration. If the association between the identified rare variant and CETP concentration lost statistical significance after adjustment for the three common *CETP* variants, we regarded the rare variant a proxy for the common CETP variants, rather than an independent determinant of circulating CETP concentration.

Results

The characteristics of the 6,094 study participants are described in Table 5.1. Median age was 56 years and 52% of participants were women. The mean (SD) CETP concentration was 2.48 (0.66) μg/mL. With the gene-based aggregation analysis, we identified regions in the *ABCA6* and *NLRC5* genes to be exome-wide significantly associated with serum CETP concentration (Table 5.2).

Table 5.1: Characteristics of the study population (n=6,094).

Characteristics	
Age (year)	56 (51, 61)
Women (n, %)	3,168 (52.0%)
Body mass index (kg/m^2)	29.5 (27.3, 32.3)
Lipid-lowering drug users	963 (15.8%)
Fasting serum concentrations	
CETP (μg/mL)	2.48 (0.66)
Total cholesterol (mmol/L)	5.66 (1.07)
LDL-C (mmol/L)	3.56 (0.98)
HDL-C (mmol/L)	1.43 (0.41)
Triglycerides (mmol/L)	1.25 (0.88, 1.76)

Results are presented as median (inter quartile range) for not normally distributed data, mean (SD) or number (percentage).
[a] Missing data: n=14 for total cholesterol, LDL-C, HDL-C and triglycerides concentrations.
CETP, cholesteryl ester transfer protein; C, cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Twelve variants that mapped to the *ABCA6* gene on chromosome 17 were jointly associated with a 0.16 μg/mL higher CETP concentration (joint effect P=9.4×10^{-7}), using the CMC method. The analyses performed with SKAT confirmed this association (joint effect P=3.2×10^{-7}). Of the 12 different genetic variants, the rs77542162 polymorphism was the driving variant of this region with a per-allele increase in CETP concentration of 0.19 μg/mL. This driving variant had a MAF of 0.028.

Twenty variants that mapped to the *NLRC5* gene were jointly associated with a higher CETP concentration, when tested with SKAT only (joint effect P=2.8×10^{-9}). The driving variant of this genetic region was the rs1672867 polymorphism (MAF=0.017). This variant was associated with a per-allele increase in CETP concentration of 0.26 μg/mL. Analyses performed with the CMC method showed no joint association for the *NLRC5* region (joint effect P=0.79). NLRC5 is located directly downstream of the *CETP* gene on chromosome 16. When the association between the *NLRC5* driving variant rs1672867 and serum CETP concentration was adjusted for three common *CETP* variants that determine CETP concentration, the association between rs1672867 and CETP concentration lost statistical significance (P=0.089).

Table 5.2: Summary statistics of the rare variants that were associated with serum CETP concentration, as identified with exome-wide gene-based aggregation analysis using either the combined multivariate and collapsing (CMC) method or the sequence kernel association test (SKAT) (n=6,094).

				CMC method[a]				SKAT[b]		
Chr	Gene	Number of aggregated variants	Amino acid change (minor allele count)	Joint effect size (μg/mL)	P-value	Conditioned P-value[c]	Effect size driving variant[d](μg/mL)	P-value	Conditioned P-value[c]	Effect size driving variant[d](μg/mL)
17	*ABCA6*	12	p.Thr1581Ile (2), p.Leu1556Ile (1), p.Ile1494Thr (4), p.Thr1441Met (1), Ala1379Thr (62), p.Cys1359Arg (336), p.Gly1222Val (1), p.Cys1216Gly (3), p.Cys1059Tyr (9), p.Met686Ile (6), p.Arg660His (19), p.Asn565Ser (3) p.Arg152Gln (1), p.Ala155Thr (7), p.Ser210Leu (316), p.Ala382Ser (1), p.Thr470Ile (1), p.Tyr540Cys (5), p.Gly835Cys (11), p.His857Arg (78), p.Asn907Asp (210), p.Ala1033Thr (5), p.Val1049Met (1), p.Val1217Met (10), p.Asp1228Asn (280), p.Lys1297Asn (337), p.Gly1317Arg (15), p.Val1367Ile (5), p.Arg1401His (72), p.Pro1667Gln (23), p.Arg1705His (26), p.Ser1817Gly (1)	0.16	9.4?10-7	0.58	0.19	3.2?10-7	0.84	0.19
16	*NLRC5*	20		0.005	0.79	0.05	0.26	2.8?10-9	0.01	0.26

The driving variant is underlined. A P-value <4.1×10^{-6} is regarded exome-wide significant.

[a] The CMC method assumes that variants are causal and have the same effect direction.

[b] SKAT assumes that variants can have different effect directions. A joint effect size can therefore not be generated from the model, only a P-value.

[c] P-value conditioned on the driving variant.

[d] Per-allele effect.

CETP, cholesteryl ester transfer protein; Chr, chromosome.

Discussion

In this study, we identified *ABCA6* as a novel determinant of circulating CETP concentration in a Dutch population-based cohort of 6,094 participants. In an exome-wide gene-based aggregation analysis for rare variants, 12 variants mapped to *ABCA6* were jointly associated with CETP concentration. The per-allele effect of the driving variant for the *ABCA6* region on CETP concentration was relatively large (i.e. 0.19 μg/mL) compared to the mean CETP concentration of 2.48 (0.66) μg/mL, although not as large as the effect size that we recently found for a GWAS-identified common genetic determinant of CETP concentration (i.e. 0.35 μg/mL) [Blauw LL, Li-Gao R, et al. Under review]. Notably, GWAS-identified common variants that determine CETP concentration were all mapped to the *CETP* gene, while we identified no rare *CETP* variants in the present study. This may indicate that within the *CETP* gene mainly common variation determines CETP expression.

In addition to *ABCA6*, the *NLRC5* gene, which is located directly downstream of *CETP* on chromosome 16, was identified as a determinant of circulating CETP concentration. However, this association lost statistical significance after adjustment for the three common *CETP* SNPs that strongly determine circulating CETP concentrations, as we previously identified with a GWAS [Blauw LL, Li-Gao R, et al. Under review]. Since the three common *CETP* SNPs were very strongly associated with serum CETP concentration (lowest P-value 3.98×10^{-100}), it is presumable that even variants in low linkage disequilibrium with these common variants may show significant effects on CETP concentration. The loss of the association between *NLRC5* and CETP concentration after adjustment indicates that *NLRC5* is not an independent determinant of circulating CETP, but rather a proxy variant for the common CETP SNPs.

We identified 12 variants mapped to *ABCA6* that were jointly associated with CETP concentration. Since we used two distinct tests (i.e. CMC method and SKAT) with different underlying assumptions about causality and effect directions of the aggregated variants in the gene region,[7] we were able to compare the results of these two tests to shed light on the underlying genetic model of the identified joint variants. For the *ABCA6* variants, both tests showed comparable results, which suggests that all variants in the identified *ABCA6* region have unidirectional effects on CETP concentration.[11] Of the 12 jointly identified *ABCA6* variants , rs77542162 was shown to be the driving variant of this gene region. Interestingly, in 2015, this same *ABCA6* exonic variant was identified to determine LDL-C and total cholesterol concentrations in the Dutch population independent of other loci,[12] which was thereafter replicated in an independent population.[13] The cysteine to arginine change at position 1359 induced by the rs77542162 missense variant is predicted by PolyPhen-2[14] to be ‘probably damaging’ with a probabilistic score of 0.999. This implies that this

missense mutation is very likely to damage the structure and reduce the function of the ABCA6 protein.[12,14] Taken together, the results of the present study indicate that abundance or functionality of the ABCA6 protein influence circulating CETP concentrations.

It is interesting to speculate on how ABCA6 may influence the circulating CETP concentration. In 2001, *ABCA6* was cloned and sequenced from human macrophages and identified as a novel member of the ATP binding cassette (ABC) A transporter subfamily,[15] of which the members are involved in cellular transmembrane transport of sterols.[16] Although a relation between ABCA6 and CETP has not been described before, striking analogies exist between the two proteins. First, expressions of both *ABCA6* and *CETP* in humans are highest in liver tissue.[14,15] For CETP, specifically resident liver macrophages (i.e. Kupffer cells) were identified as the main determinants of circulating CETP concentration.[14] Although the main hepatic cell type expressing *ABCA6* is currently unknown, *ABCA6* is also expressed by macrophages.[17] Second, ABCA6[17] and CETP[18] are both upregulated during monocyte-to-macrophage differentiation, which suggests that they may be involved in macrophage polarization and immunity. Third, expression of both genes is regulated in response to sterols, albeit that *ABCA6* is downregulated[17] and *CETP* is upregulated.[19] Although the function of ABCA6 is far from completely understood, ABCA6 has been proposed to be involved in cellular lipid and sterol transport.[15,20,21] As *CETP* expression is regulated by sterol derivatives via a liver X receptor α (LXRα) responsive element in its promoter region,[19,22] it is tempting to speculate that ABCA6 determines CETP production via intracellular regulation of lipid and sterol trafficking.

The MAF of the driving variant of the *ABCA6* region (i.e. rs77542162) was 0.028 in the present Dutch study population. Previously, it has been shown that the minor allele of rs77542162 is enriched in the Dutch population (i.e. MAF=0.030; Genome of the Netherlands (GoNL) population) by 3.65-fold, compared to the world population (i.e. MAF=0.008; 1000 Genomes Project (1KG) population), which implies that there has been genetic drift for this locus in the Netherlands.[12] Due to the enrichment of this variant in the study population, we benefited from an improved power to identify this rare variant as a determinant of CETP concentration. Although we did not replicate the present findings in an independent non-Dutch population, it is interesting to note that the results of a previous study, which used the Dutch population to identify the rs77542162 as a determinant of LDL-C and total cholesterol concentration, were robustly replicated in independent international populations.[12,13] This suggests that results for rs77542162 from a specific homogeneous population may well-translate to a worldwide scale.[12]

In conclusion, in the present study we used exome-wide analysis to show that rare variants mapped to *ABCA6* are associated with circulating CETP concentration. This finding is

completely novel, as the *ABCA6* gene has never been described before in relation to CETP. Future research is warranted to elucidate the underlying biological pathways that explain this novel association. Hypothetically, abundance or functionality of the ABCA6 protein may determine CETP production via the regulation of intracellular lipid and sterol trafficking.

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6

Circulating CETP causally decreases large HDL and increases small VLDL without affecting LDL

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Submitted

Abstract

Introduction

According to current dogma, cholesteryl ester transfer protein (CETP) decreases high-density lipoprotein (HDL)-cholesterol (C) and increases low-density lipoprotein (LDL)-C. HDL-C is measured directly, whereas LDL-C is commonly inferred from the Friedewald formula without direct measurement of specific lipoprotein subclasses. In depth insight in the causal effects of CETP on the lipoprotein profile may assist in understanding outcomes of recent CETP inhibitor trials. Therefore, we aimed to assess associations between CETP and the detailed lipoprotein profile, using Mendelian randomization and observational analyses.

Methods

Using nuclear magnetic resonance spectroscopy, we quantified 159 metabolic measures in 5,672 participants of the Netherlands Epidemiology of Obesity (NEO) study. We used three previously identified SNPs (rs247616, rs12720922 and rs1968905) that determine CETP concentrations as genetic instruments to estimate the causal effects on the metabolic measures, and reported effect estimates as the Ln-transformed SD difference in metabolic measure per unit (μg/mL) increase in CETP. Findings were replicated in an independent dataset (MAGNETIC NMR GWAS; n≈20,000). Additionally, observational associations between ELISA-measured CETP concentration and the metabolic measures were assessed.

Results

Higher CETP concentrations were casually associated with less large HDL components (largest effect XL-HDL-C, $P=6\times10^{-22}$), and with more small VLDL components (largest effect S-VLDL cholesteryl esters, $P=6\times10^{-6}$), while no association was observed with any LDL subclasses or constituents. This pattern of lipidomic associations was replicated. In contrast, observationally, measured CETP concentration predominantly associated with more VLDL, IDL and LDL components.

Conclusion

Based on Mendelian randomization, we conclude that CETP is an important determinant of HDL and VLDL concentration and composition. Since HDL does not causally affect cardiovascular disease risk, we speculate that the CETP inhibitor anacetrapib decreased cardiovascular disease risk through specific reduction of small VLDL rather than LDL. We propose that the contrast between genetic and observational associations may be explained by the higher capacity of VLDL, IDL and LDL subclasses to carry CETP, thereby concealing causal associations with HDL.

Introduction

Cholesteryl ester transfer protein (CETP) is able to transfer cholesteryl esters from high-density lipoproteins (HDL) to apolipoprotein (Apo) B containing triglyceride-rich lipoproteins, mainly very-low-density lipoproteins (VLDL). In exchange, triglycerides are transferred from VLDL to triglyceride-poor particles, which are both HDL and low-density lipoprotein (LDL).[1] As such, CETP facilitates an atherogenic lipoprotein profile, as has been extensively studied in both humans and in mice transgenic for human CETP.[2,3]

Despite the promising results of preclinical studies,[4,5] the clinical trials with the initial CETP inhibitors torcetrapib, dalcetrapib, and evacetrapib, were terminated: torcetrapib had off-target effects on blood pressure and caused an increase in cardiovascular events,[6] and both dalcetrapib and evacetrapib lacked efficacy in reducing cardiovascular events on top of statin therapy.[7,8] All these CETP inhibitors caused a large increase in HDL-C, accompanied by a modest or no decrease in LDL-C. Although a high HDL-C concentration was previously proposed to decrease the risk of cardiovascular disease (CVD) based on observational studies,[9] Mendelian randomization showed that higher HDL-C concentrations do not lower the risk of myocardial infarction,[10–13] which implies that the association between HDL-C and CVD is not causal. This may provide one of the explanations for the lack of efficacy of the three initial CETP inhibitors.[6–8]

Interestingly, the fourth clinical trial with the CETP inhibitor anacetrapib (REVEAL) did meet its primary endpoint by showing a 9% relative risk reduction in major coronary events.[14] The reduction in coronary events by anacetrapib, albeit limited, was attributed to a reduction in LDL-C and a corresponding reduction in ApoB-containing lipoprotein particles.[14,15] However, the specific lipoprotein subclasses affected by CETP have not been determined. Specific lipoprotein fractions have previously been associated with increased CVD risk,[16] and more in-depth insight in the causal effects of CETP on the circulating lipoprotein profile may therefore assist in understanding CETP inhibitor trial outcomes. This is also of specific importance in the light of the dal-GenE randomized phase III clinical trial (NCT number: NCT02525939), which is currently being performed to confirm whether dalcetrapib is able to reduce CVD risk in patients with the AA genotype of the rs1967309 polymorphism in the *ADCY9* gene.[7,17]

We recently performed a genome-wide association study (GWAS) on serum CETP concentration in the Netherlands Epidemiology of Obesity (NEO) study, and showed that CETP concentration has a strong genetic component.[18] Notably, three independent single nucleotide polymorphisms (SNPs), all mapped to the *CETP* gene, together explained 16.4% of the total variation in serum CETP concentration. With the use of these SNPs as ge-

netic instruments in Mendelian randomization, the causal effects of circulating CETP on lipoprotein subclasses can be determined. Besides, by using a Mendelian randomization approach, the effects of these genetic variants can mimic CETP inhibition, and we may infer about the mechanisms underlying the outcomes of CETP inhibitor trials.[19,20]

In the present study, we aimed to assess the causal effects of CETP concentration on 159 circulating metabolic measures, primarily lipoprotein subclasses, using a Mendelian randomization approach in a cohort of the Dutch general population.[21] We also compared the causal effect estimates with observational associations between serum CETP concentration and these measures of lipid metabolism.

Materials and Methods

Study design and population

The present study is embedded in the Netherlands Epidemiology of Obesity (NEO) study, a population-based prospective cohort study of men and women aged 45 to 65 years. From the greater area of Leiden, The Netherlands, all inhabitants with a self-reported body mass index (BMI) of 27 kg/m^2 or higher were eligible to participate. In addition, inhabitants from one nearby municipality (Leiderdorp, The Netherlands) in the same age group were invited to participate regardless of their BMI, forming a reference population for BMI distribution. In total, 6,671 participants were included from September 2008 until September 2012. Participants visited the NEO study center for extensive physical examination. Venous blood samples were obtained from the antecubital vein after a 10 hour overnight fast. 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. For the present study, we excluded participants with missing data on serum CETP concentration, metabolic profiling or genotype. Therefore, the present study population consists of 5,672 individuals. The NEO study was approved by the medical ethics committee of the Leiden University Medical Center (LUMC), and all participants gave their written informed consent. Detailed information about the study design and data collection has been described elsewhere.[22]

Genotyping and imputation

DNA was isolated from venous blood samples. Genotyping was performed in participants form European ancestry, 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)[23] using IMPUTE (v2.2) software.[24] From the whole-genome data, we extracted the three independent ge-

netic variants that have been previously identified in relationship to CETP concentration in the NEO study population,[18] notably rs247616 (directly genotyped; coding allele (C) frequency 0.67), rs12720922 (imputation quality 0.98; coding allele frequency (A) 0.17) and rs1968905 (imputation quality 0.85; coding allele frequency (G) 0.82). The CETP-increasing alleles are rs247616-C, rs12720922-A and rs1968905-G. Based on these three polymorphisms, we calculated a weighted genetic score per participant. The genetic score was constructed as the sum of the number of CETP-increasing alleles weighted by their effect size on CETP concentration, as previously described.[18]

Serum CETP concentration and routine-lipid profile

After centrifugation, aliquots of serum were stored at -80°C. From 11 April until 16 July 2014 CETP concentrations were measured with enzyme-linked immune sorbent assay (ELISA) kits according to the manufacturer's instructions (DAIICHI CETP ELISA, Alpco, Salem, USA; coefficient of variation (CV) 11.7%) in serum that had undergone one previous freeze-thaw cycle. Fasting serum total cholesterol and triglycerides 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). Fasting LDL-C concentrations were calculated using the Friedewald equation.[2]

NMR-based metabolic biomarker profiling

A high-throughput proton NMR metabolomics platform[21] (Nightingale Health Ltd., Helsinki, Finland) was used to quantify 159 lipid and metabolite measures. The NMR spectroscopy was conducted at the Medical Research Council Integrative Epidemiology Unit (MRC IEU) at the University of Bristol, Bristol, United Kingdom, and processed by Nightingale's biomarker quantification algorithms (version 2014). This method provides quantification of lipoprotein subclass profiling with lipid concentrations within 14 lipoprotein subclasses. The 14 subclass sizes were defined as follows: extremely large VLDL with particle diameters from 75 nm upwards and a possible contribution of chylomicrons, five VLDL subclasses (average particle diameters of 64.0 nm, 53.6 nm, 44.5 nm, 36.8 nm, and 31.3 nm), IDL (28.6 nm), three LDL subclasses (25.5 nm, 23.0 nm, and 18.7 nm), and four HDL subclasses (14.3 nm, 12.1 nm, 10.9 nm, and 8.7 nm). Within the lipoprotein subclasses the following components were quantified: total cholesterol, total lipids, phospholipids, free cholesterol, cholesteryl esters, and triglycerides. The mean size for VLDL, LDL and HDL particles was calculated by weighting the corresponding subclass diameters with their particle concentrations. Furthermore, 58 metabolic measures were determined that belong to

classes of apolipoproteins, cholesterol, fatty acids, glycerides, phospholipids, amino acids, fluid balance, glycolysis-related metabolites, inflammation, and ketone bodies. Details of the experimentation and applications of the NMR metabolomics platform have been described previously,[21] as well as CVs for the metabolic biomarkers.[25] A full list of the measured biomarkers is included in Supplementary table 6.C.1.

Statistical analyses

For all analyses, metabolic measures were natural-log transformed to obtain normal distributions. For comparison of the strengths of the associations between the different metabolic measures, standardized z-scores were composed. Consequently, the outcome variable of all analyses was the natural-log transformed SD difference in metabolic measure. When the concentration of a metabolic measure for an individual was below the detection limit, a value of half of the minimum concentration of that metabolic measure in the total population was imputed.

First, we performed a Mendelian randomization analysis to determine the causal associations between serum CETP concentration and the 159 metabolic measures. The genetic score was used as determinant in a linear regression analysis to assess the effect of a one unit (μg/mL) increase in serum CETP concentration on the metabolic measures. Second, the observational associations between serum CETP concentration and the 159 metabolic measures were determined with linear regression analyses. Participants with a serum CETP concentration beyond four SD from the mean were excluded (n=1). All linear regression analyses were adjusted for age and sex. Beta coefficients, SE and P-values from linear regression analyses were reported. In addition, a linear regression analysis adjusted for age and sex was performed to determine the associations of serum CETP concentration with routinely measured HDL-C concentration and calculated Friedewald LDL-C concentration.

The metabolic biomarkers used for the present study are correlated with each other, and therefore, conventional correction for multiple testing (e.g. Bonferroni) is too stringent. To obtain the number of independent metabolic biomarkers, we used the method as described by Li et al.,[26] which takes the correlation between the different metabolic biomarkers into account. Based on this method, we found 37 independent metabolic markers. For this reason, associations were considered to be statistically significant in case the P-value was below 0.00134 (i.e. 0.05/37).

All results were based on analyses weighted towards the reference BMI distribution of the general Dutch population, and therefore apply to a population-based study without oversampling of individuals with overweight or obesity (see Supplement 6.A). Analyses

were performed using STATA Statistical Software version 12.0 (Statacorp, College Station, Texas, USA) and R version 3.4.0 (The R Project, https://www.r-project.org/). Figures were designed with Python version 2.7.6 (Python Software Foundation, https://www.python.org/).

Replication

We aimed to replicate the findings from the Mendelian randomization analyses in the NEO study in an independent population. For that purpose, we used publically-available summary statistics from the MAGNETIC NMR GWAS dataset,[25] which comprises the additive (per-allele) beta coefficients with accompanying standard errors of the associations between genome-wide SNPs and 123 metabolic measures, of which 111 overlapped with the 159 metabolic measures that were quantified in the NEO study. This GWAS meta-analysis included data of approximately 20,000 individuals from 14 datasets that were derived from cohorts of European ancestry. The 123 metabolic measures were quantified by a prior version of the same high-throughput proton NMR metabolomics platform[21] (Nightingale Health Ltd., Helsinki, Finland) as used in the NEO study. The 123 metabolic measures were quantified by a prior version of the same high-throughput proton NMR metabolomics platform[21] (Nightingale Health Ltd., Helsinki, Finland) as used in the NEO study. In these metabolomics data, 22 principal components were identified,[25] and therefore associations were considered to be statistically significant in case the P-value was below 0.00227 (i.e. 0.05/22).

For the Mendelian randomization analyses, we combined the individual genetic variants for CETP concentration to estimate the causal effect of CETP concentration on the metabolic measures. Analogous to pooling estimates from different observational studies with conventional meta-analysis using inverse-variance weighting, we weighted this combined effect estimate of the *CETP* SNPs on the metabolic measures by the inverse of the variance for each individual additive (per-allele) effect on the metabolic measures, and incorporated the individuals additive effects of the genetic instruments on CETP concentration. Additionally, we determined the correlation (R2) between the effect estimates from the NEO study and the MAGNETIC NMR GWAS dataset. A strong correlation indicates high consistency in the overall association profile of higher CETP concentrations with the lipid and metabolite measures.

Results

Population characteristics

Characteristics of the total study population are summarized in Table 6.1. The mean (SD) age was 56 (6) years. Mean (SD) concentration of CETP was 2.47 (0.65) μg/mL, of LDL-C 3.56 (0.96) mmol/L, and of and HDL-C 1.57 (0.46) mmol/L.

Table 6.1: Characteristics of the discovery and replication cohort from the Netherlands Epidemiology of Obesity (NEO) study.

Characteristics	
Age (year)	56 (6)
Women (%)	56
Educational level (% high[a])	47
Body mass index (kg/m^2)	26.3 (4.4)
Tobacco smoking (% current and former smokers)	62
Cardiovascular disease (%)	5
Lipid-lowering drug users (%)	11
Fasting serum concentrations	
CETP (μg/mL)	2.47 (0.65)
Total cholesterol (mmol/L)	5.70 (1.05)
LDL-cholesterol (mmol/L)	3.56 (0.96)
HDL-cholesterol (mmol/L)	1.57 (0.46)
Triglycerides (mmol/L)	1.23 (0.85)

n=5,672[b]. Results are based on analyses weighted towards the reference BMI distribution of the general Dutch population, and presented as mean (SD) or percentage.

[a] High educational level: higher secondary education (according to Dutch educational system), higher vocational education, university, PhD.

[b] Missing data: n=4 for ethnicity, n=56 for educational level, n=4 for smoking, n=21 for cardiovascular disease, n=8 for total cholesterol concentration, n=10 for LDL-cholesterol concentration, n=9 for HDL-cholesterol concentration and n=9 for triglycerides concentration.

CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Causally, CETP is negatively associated with large HDL components and positively associated with small VLDL components

Figure 6.1 and Supplementary table 6.C.2 show the results from the Mendelian randomization analyses of CETP and 159 circulating metabolic measures. The association with the CETP genetic score was statistically significant for 46 metabolic measures (P<0.00134). CETP concentration most strongly affected very large, large and medium HDL subclasses. With a 1 μg/mL increase in CETP, all components of these lipoprotein subclasses de-

creased. The only exception was the triglyceride content in medium HDL, for which the effect size was positive (effect (SE): 0.272 (0.055), $P=9\times10^{-7}$). In line with this, triglycerides in small HDL also showed a positive beta (effect (SE): 0.293 (0.055), $P=1\times10^{-9}$). In accordance with the decrease in larger HDL subclasses with higher CETP concentration, higher CETP was also associated with a smaller HDL diameter and less ApoA1. Overall, the largest effects was found for cholesterol in very large HDL (effect (SE): -0.517 (0.053), $P=6\times10^{-22}$). When comparing the lipoprotein components within the very large, large and medium HDL subclasses, the cholesterol components, i.e. cholesteryl esters and free cholesterol, consistently showed the largest effect sizes.

Remarkably, CETP concentration did not associate with any of the LDL subclass components, while a higher CETP concentration associated with more small and very small VLDL. The largest increasing effect was found for cholesteryl esters in small VLDL (effect (SE): 0.276 (0.061), $P=6\times10^{-6}$). There were no pronounced differences in the effect sizes between the various components within VLDL subclasses.

The results from the independent replication dataset can be found in Supplementary figure 6.B.1 and Supplementary table 6.C.3. The pattern of lipidomic associations was consistent in the replication dataset (Figure 6.2), also when evaluated for the three SNPs of the genetic score separately (Supplementary figures 6.B.2, 6.B.3 and 6.B.4).

Observationally, CETP concentration is predominantly positively associated with very small VLDL, IDL and LDL components

Figure 6.3 and Supplementary table 6.C.4 show the observational associations between measured serum CETP concentration and the 159 circulating metabolic measures. A higher CETP concentration was positively associated with all VLDL, IDL and LDL subclasses, of which very small VLDL, IDL, and all three LDL subclasses showed the strongest associations. This is in line with the associations between serum CETP concentration and the routine-lipid measurements (age- and sex- adjusted), since we observed a large effect on Friedewald LDL-C concentration per unit increase in CETP concentration (effect size 0.46 mmol/L; 95% CI 0.40, 0.52), compared to the relatively small effect on HDL-C concentration (effect size -0.05 mmol/L; 95% CI -0.07, -0.02). Crude associations are shown in Supplementary figure 6.B.5.

The largest effect sizes for observational associations were found for free cholesterol in very small VLDL (effect (SE): 0.476 (0.030), $P=2\times10^{-55}$) and phospholipids in very small VLDL (effect (SE): 0.472 (0.030), $P=2\times10^{-56}$). When comparing the lipoprotein components (i.e. cholesteryl esters, free cholesterol, phospholipids and triglycerides) within the very small VLDL, IDL and LDL subclasses, the shell components, i.e. phospholipids and

free cholesterol, quite consistently (apart from small LDL) showed the largest effect sizes, whereas the effect for the core component triglycerides was, consistently, the smallest. Concerning the HDL subclasses, higher CETP concentration was associated with a higher concentration of small HDL particles, whereas no associations were found with concentrations of very large, large and medium HDL particles.

Overall, observational and genetic associations were hardly consistent. Only for VLDL effect directions were similar (i.e. positive) between genetic and observational associations.

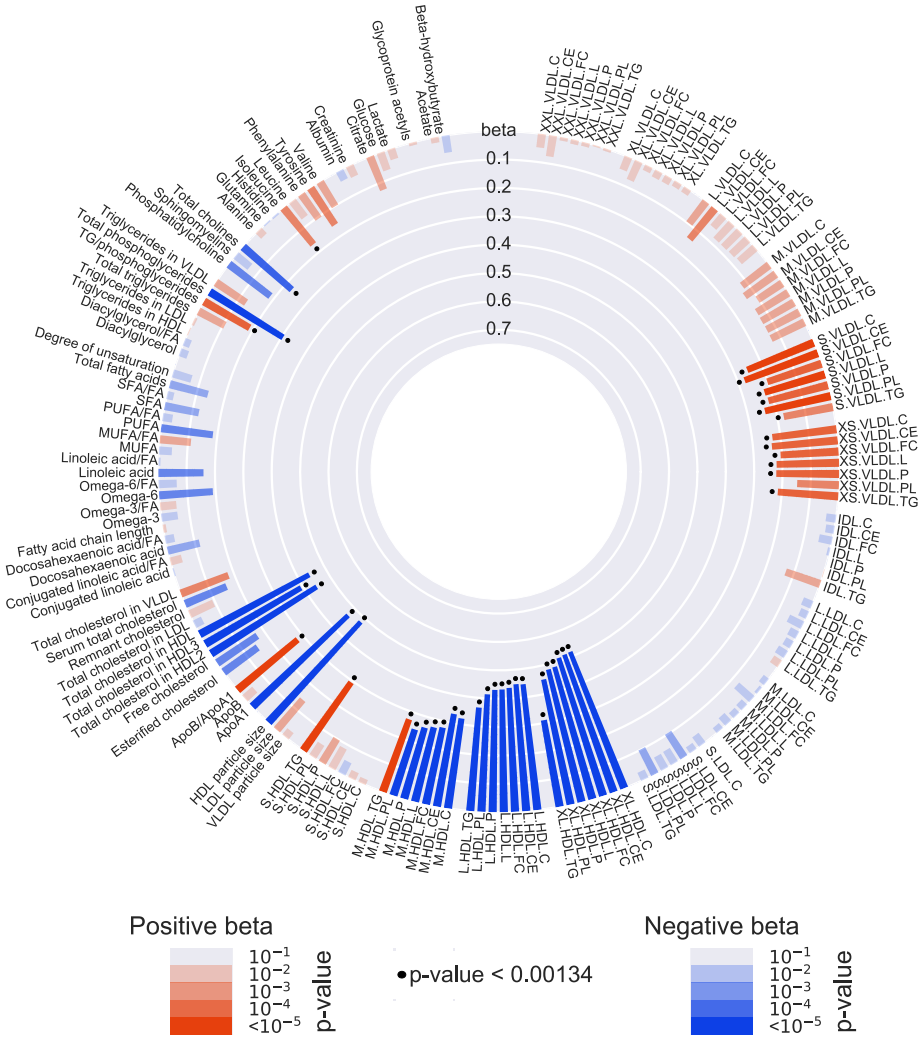


Figure 6.1: Causal associations between CETP concentration and 159 circulating metabolic measures, which were assessed with a Mendelian randomization approach based on a CETP genetic score, in the Netherlands Epidemiology of Obesity (NEO) study (n=5,672). The genetic score for Mendelian randomization is based on the CETP SNPs rs247616, rs12720922 and rs1968905, as previously determined with a genome-wide association study on serum CETP concentration.[18] Bar heights represent the magnitude of the beta coefficient from linear regression, which is expressed as the SD difference in metabolic measure per 1 μg/mL increase in CETP concentration. Red bars indicate positive betas and blue bars indicate negative betas. The transparency of the bars indicates the level of statistical significance. A P-value<0.00134 is regarded statistical significant, as represented by the black dots. Full names and descriptions of metabolic measures are listed in Supplementary table 6.C.1. Results are based on analyses weighted towards the reference BMI distribution of the general Dutch population.

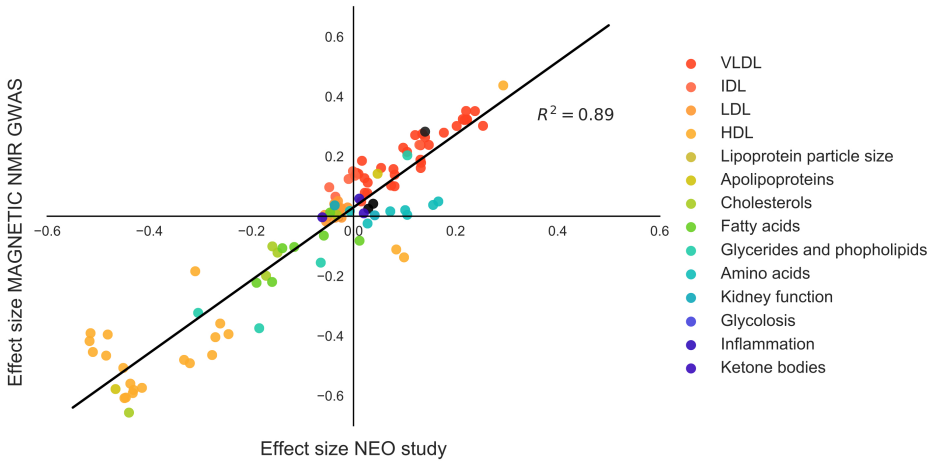


Figure 6.2: The effects sizes of the causal associations between CETP concentration and metabolic measures in the Netherlands Epidemiology of Obesity (NEO) study are strongly replicated in independent European populations (MAGNETIC NMR GWAS dataset), as shown by the high correlation between the beta coefficients from both cohorts. Beta coefficient from linear regression are expressed as the SD difference in metabolic measure per 1 μg/mL increase in CETP concentration. NEO study results are based on analyses weighted towards the reference BMI distribution of the general Dutch population.

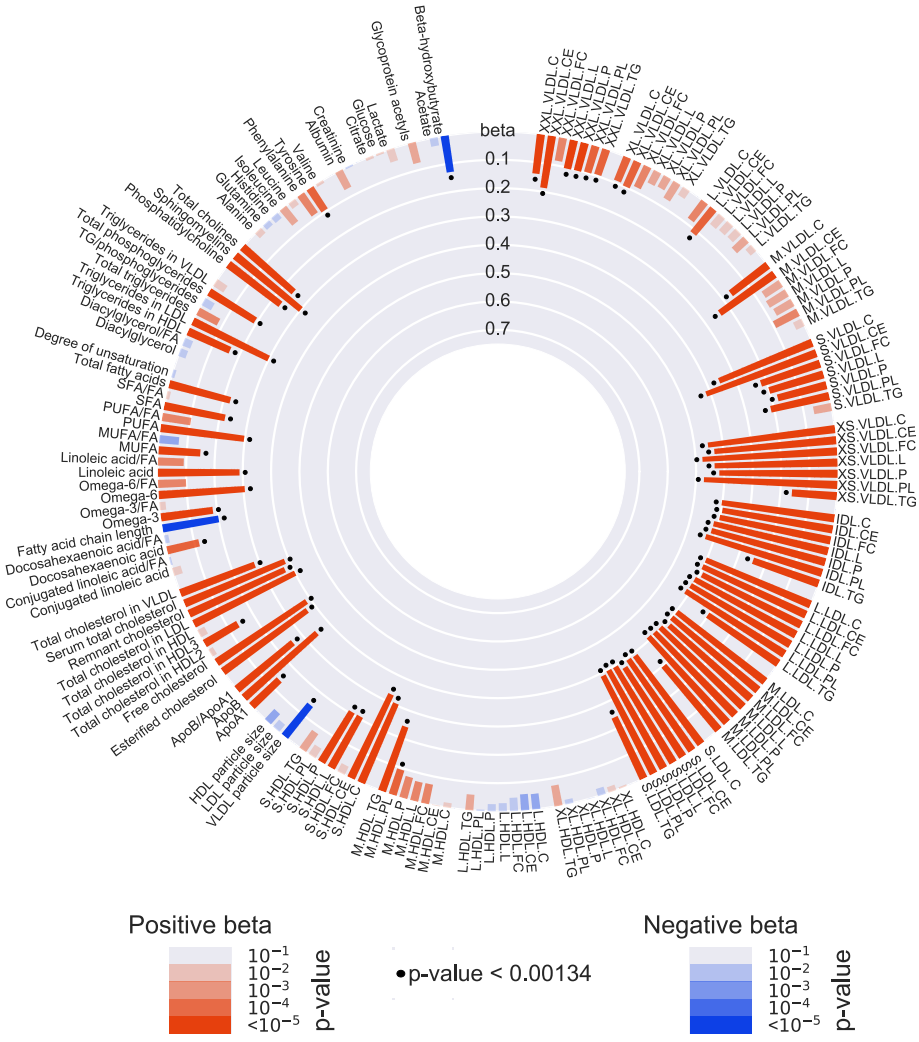


Figure 6.3: Observational associations between serum CETP concentration and 159 circulating metabolic measures, in the Netherlands Epidemiology of Obesity (NEO) study (n=5,672). Bar heights represent the magnitude of the beta coefficient from linear regression, which is expressed as the SD difference in metabolic measure per 1 μg/mL increase in serum CETP. Red bars indicate positive betas and blue bars indicate negative betas. The transparency of the bars indicates the level of statistical significance. A P-value<0.00134 is regarded statistical significant, as represented by the black dots. Full names and descriptions of metabolic measures are listed in Supplementary table 6.C.1. Results are based on analyses weighted towards the reference BMI distribution of the general Dutch population.

Discussion

In this study, we determined the causal effects of serum CETP on the circulating lipoprotein profile, and contrasted the results to observational associations. We used three recently identified CETP SNPs (i.e. rs247616, rs12720922 and rs1968905) that together explain 16.4% of the total variation in serum CETP concentration as genetic instruments in Mendelian randomization, to enable inferences of causality on 159 circulating metabolic measures, and mimic the effects of CETP inhibition. Higher circulating CETP concentrations were causally most strongly associated with lower concentrations of very large, large and medium-sized HDL components, a smaller overall HDL diameter, less ApoA1, and more small VLDL components, while there was no association with LDL components. In contrast, observationally, measured serum CETP concentration predominantly associated with more VLDL, IDL and LDL components. We propose that this contrast between genetic and observational associations may be explained by the higher capacity of VLDL, IDL and LDL subclasses to carry CETP, thereby concealing causal associations with HDL.

Based on the principle of Mendelian randomization, the association between the CETP genetic score and lipoprotein subclasses can be interpreted as causal. The results from our Mendelian randomization analyses are fully consistent with the mechanism by which CETP transfers cholesteryl esters from HDL towards ApoB containing triglyceride-rich lipoproteins, mainly VLDL, and mediates the transfer of triglycerides from VLDL towards triglyceride-poor particles, such as HDL.[1,7,27] CETP accelerates the clearance of HDL from blood by enrichment of HDL with triglycerides, which makes this HDL a preferred substrate for hepatic triglyceride lipase.[2] Catabolism of triglyceride-rich HDL by hepatic triglyceride lipase leads to the formation of very small remnant HDL that is cleared by the liver and kidneys.[2,13,28–31] Indeed, in the present study, the CETP genetic score was associated with a smaller HDL diameter, and with a higher triglyceride content specifically in the medium and small HDL particles. Also, our results show that the CETP mainly affects the HDL pool, which is in accordance with the lipoprotein profile observed in CETP-deficient individuals, who have markedly increased amounts of large HDL particles,[32–34] while effects on LDL and VLDL subclasses are less pronounced.[34]

Based on the principle of Mendelian randomization, our results may also shed light on the mechanisms that underlie the effectiveness of CETP inhibition.[19,20] Therefore, it is highly interesting to discuss the present study results in light of the results from the REVEAL trial with the CETP inhibitor anacetrapib, which showed a reduction in major coronary events.[35] Our findings implicate that CETP inhibition causes a relatively large increase in HDL components, which is predominantly caused by an increase in large and medium-sized HDL particles, in addition to a more modest reduction in VLDL components, which

is mainly caused by a decrease in small and extra small VLDL particles. Of note, LDL concentration and composition were not affected by CETP, which was unexpected based on the current dogma that CETP increases LDL-C. However, it should be realized that LDL-C is generally not measured directly but calculated from the Friedewald formula,[2] which may well misclassify cholesterol contained within small VLDL subclasses as LDL-C. It was previously reported that small and very small VLDL particle concentrations (reflecting triglyceride-rich lipoprotein remnants) and cholesterol in VLDL (reflecting remnant cholesterol) are among the lipoprotein components that most strongly associate with an increased CVD risk.[16,36,37] Thus, the effects of CETP on small VLDL particles may well explain the positive outcome of the latest CETP inhibition trial with anacetrapib.[14]

We compared the genetic associations with non-causal observational associations between serum CETP concentration measured with ELISA and all 159 metabolic measures. Observationally, associations were markedly different, as CETP concentration was predominantly positively associated with very small VLDL, IDL and LDL components, whereas no negative associations with HDL components were observed. This indicates that confounding factors conceal the true causal association. Although we showed previously that CETP concentration is strongly genetically determined,[18] still the majority of the total variation in CETP concentration between individuals is caused by environmental factors. It should be realized that, just like apolipoproteins, CETP is an amphiphilic protein that binds to lipid surfaces of lipoproteins. It is thus conceivable that the capacity of lipoproteins to carry CETP is one of the factors that may explain the marked differences between the observational and causal associations.

The distribution of CETP over circulating lipoproteins has been extensively studied in the past, however, with diverging conclusions. The earliest studies proposed that CETP is predominantly bound to HDL particles.[24,38,39] However, thereafter, it was shown that CETP also avidly binds to LDL and VLDL, although the binding to these particles might be more labile.[40] It should be noted that the distribution of CETP over the various lipoprotein subclasses has commonly been determined after separating lipoproteins with ultracentrifugation, thereby presumably disrupting the binding within the least stable CETP-lipoprotein complexes. In addition, storage of plasma samples may also alter the distribution of CETP over lipoproteins. Indeed, marked effects of storage on the lipoprotein distribution have been observed for ApoC3 and ApoE following fast protein liquid chromatography.[41] Results from previous studies may therefore not accurately represent the distribution of CETP over lipoproteins within the circulation. Remarkably, in the present study, observational serum CETP concentration showed the strongest associations with the shell components (i.e. phospholipids and free cholesterol) of very small VLDL, IDL, and LDL subclasses. This

may indicate that CETP is mostly carried on the surface of these particles in the circulation, although future studies are needed to substantiate this hypothesis.

The main strength of the present study is the use of a strong genetic instrument in Mendelian randomization to draw conclusions on the causal effects of CETP concentration on the circulating lipoprotein profile. In addition, sufficient statistical power for the analyses was provided, as we had genetic data and NMR-metabolomic profiles available of 5,672 individuals, which we were able to replicate in an independent dataset (n≈20,000). However, the study populations were from European ancestry and results may therefore not be generalizable to other populations. Interestingly, a recent study in a Chinese population, which used a different CETP genetic score (i.e. rs3764261, rs1800775, rs708272, rs9939224, and rs2303790), showed comparable results.[42] This indicates consistency of the results among different ancestries.

Based on Mendelian randomization, we conclude that CETP is an important determinant of HDL concentration and composition, without affecting LDL concentration and composition. Our finding challenges the current dogma that CETP increases LDL-C, mainly based on indirect LDL-C estimation using the Friedewald formula.[2] Instead, by directly assessing the lipoprotein profile by NMR, we now show that CETP specifically increases small VLDL fractions that likely represent VLDL remnants. Therefore, we speculate that the CETP inhibitor anacetrapib attenuated cardiovascular disease risk through specific reduction of remnant cholesterol rather than LDL-C.

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Conflict of Interest: S. Soidinsalo and P. Würtz are employees of Nightingale Health Ltd, a company offering NMR-based metabolite profiling. P. Würtz is shareholder of Nightingale Health Ltd.

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Appendix

6.A. Expanded methods

BMI-weighted analyses

In the NEO study, individuals with a BMI of 27 kg/m^2 or higher were oversampled. Men and women aged between 45 and 65 years with a self-reported BMI of 27 kg/m^2 or higher were eligible to participate. From one nearby municipality (Leiderdorp, The Netherlands) all inhabitants aged between 45 and 65 years were invited to participate regardless of their BMI, in order to obtain a reference distribution for BMI. To correctly represent baseline associations in the general population [43], adjustments for the oversampling of individuals with a BMI ≥27 kg/m^2 were made in the analyses. This was done by weighting all participants towards the BMI distribution of participants from the Leiderdorp municipality [44], whose BMI distribution was similar to the BMI distribution of the general Dutch population in the age range of 45-65 years [8]. In practice, this means that participants with a lower BMI were assigned larger weights than participants with a higher BMI in the analyses. All results are based on weighted analyses, and therefore apply to a population-based study without oversampling of individuals with overweight or obesity. As a consequence, the weighted characteristics of the population are expressed in proportions instead of absolute numbers.

6.B. Supplementary figures

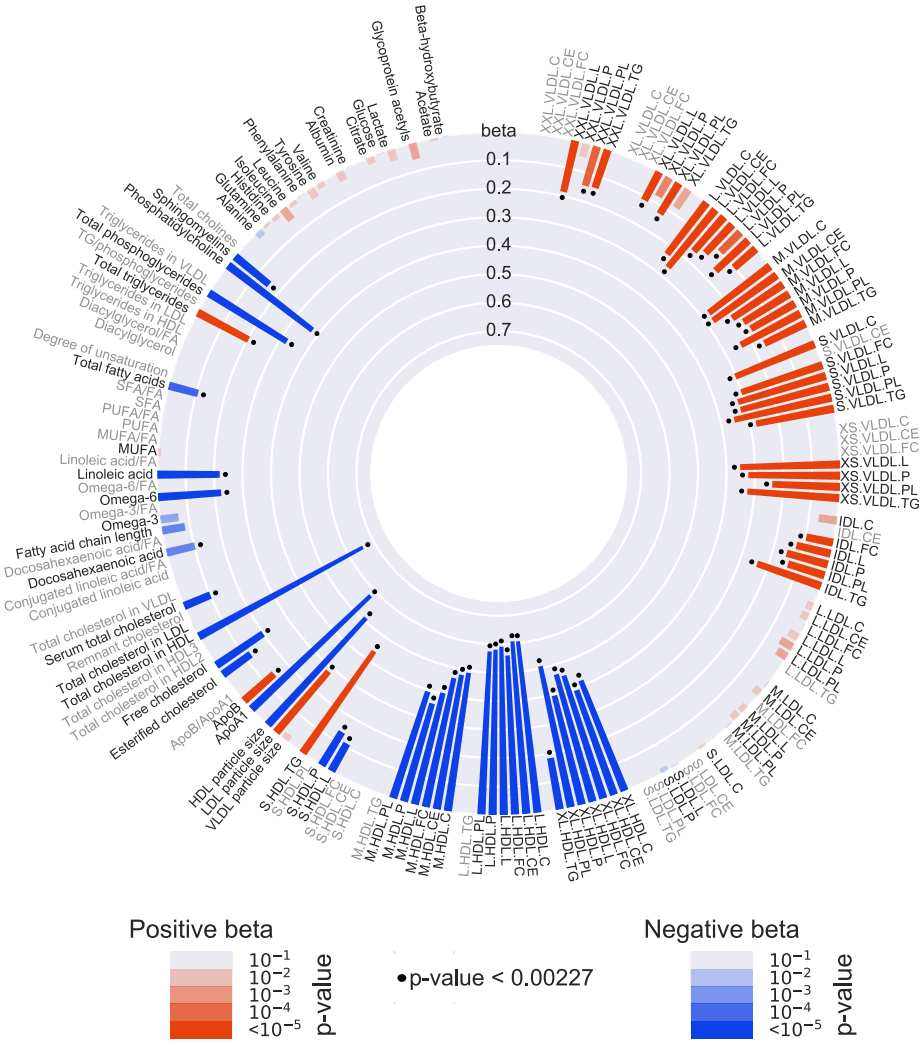


Figure 6.B.1: Replication of the causal associations between serum CETP concentration and 111 circulating metabolic measures, which were assessed with a Mendelian randomization approach based on a CETP genetic score, using summary statistics from the MAGNETIC NMR GWAS dataset (n≈20,000). The genetic score for Mendelian randomization is based on the CETP SNPs rs247616, rs12720922 and rs1968905, as previously determined with a genome-wide association study on serum CETP concentration. [18] Bar heights represent the magnitude of the beta coefficient from linear regression, which is expressed as the SD difference in metabolic measure per 1 µg/mL increase in CETP concentration. Red bars indicate positive betas and blue bars indicate negative betas. The transparency of the bars indicates the level of statistical significance. Names of unmeasured metabolic measures are grey. A P-value<0.00227 is regarded statistical significant, as represented by the black dots. Full names and descriptions of metabolic measures are listed in Supplementary table 6.C.1.

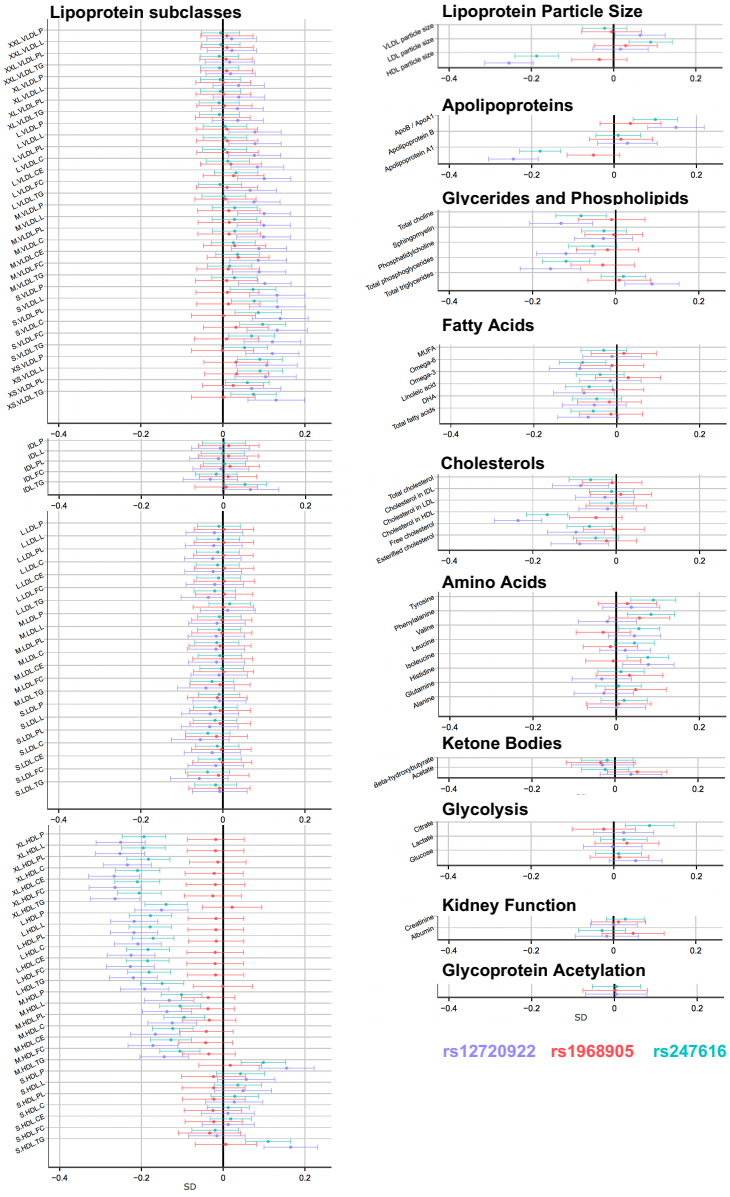


Figure 6.B.2: Causal associations between the CETP SNPs rs247616, rs12720922 and rs1968905 and 159 circulating metabolic measures, which were assessed with a Mendelian randomization approach, in the Netherlands Epidemiology of Obesity (NEO) study (n=5,672). Dots represent the beta coefficient from linear regression, which is expressed as the SD difference in metabolic measure per additional CETP-increasing allele. Bars represent 95% confidence intervals. Alleles that are associated with an increase in serum CETP concentration are rs247616-C, rs12720922-A and rs1968905-G. Full names and descriptions of metabolic measures are listed in Supplementary table 6.C.1.

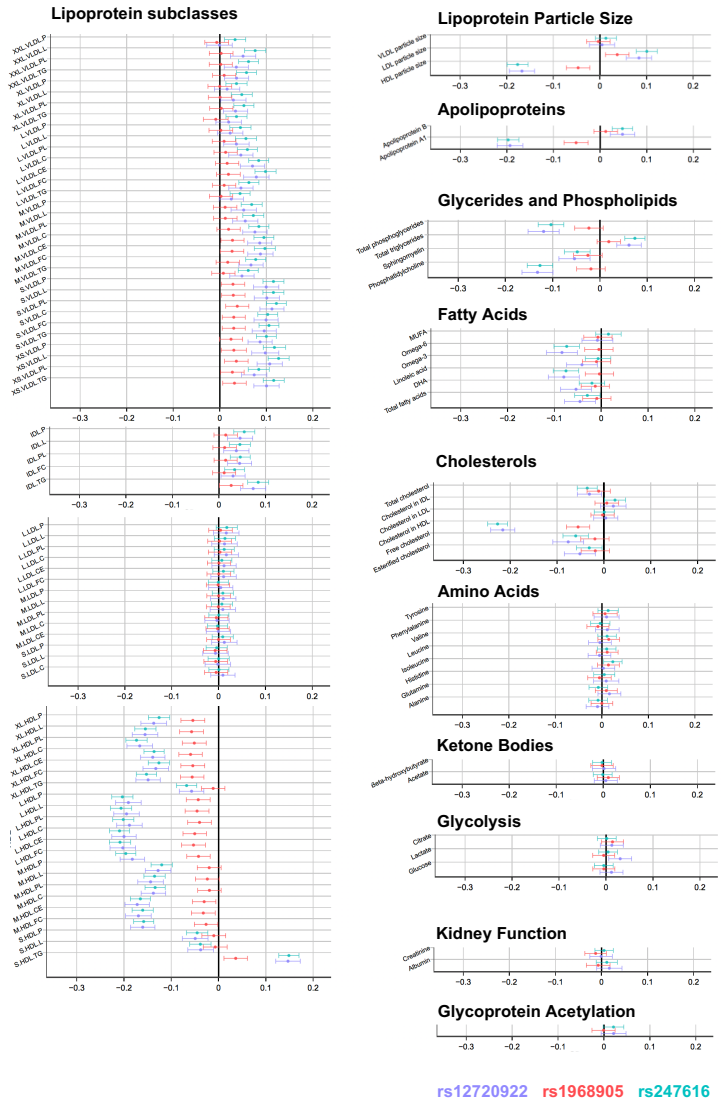


Figure 6.B.3: Replication of the causal associations between the CETP SNPs rs247616, rs12720922 and rs1968905 and 112 circulating metabolic measures, which were assessed with a Mendelian randomization approach, using summary statistics from the MAGNETIC NMR GWAS dataset (n≈20,000). Dots represent the beta coefficient from linear regression, which is expressed as the SD difference in metabolic measure per additional CETP-increasing allele. Bars represent 95% confidence intervals. Alleles that are associated with an increase in serum CETP concentration are rs247616-C, rs12720922-A and rs1968905-G. Full names and descriptions of metabolic measures are listed in Supplementary table 6.C.1.

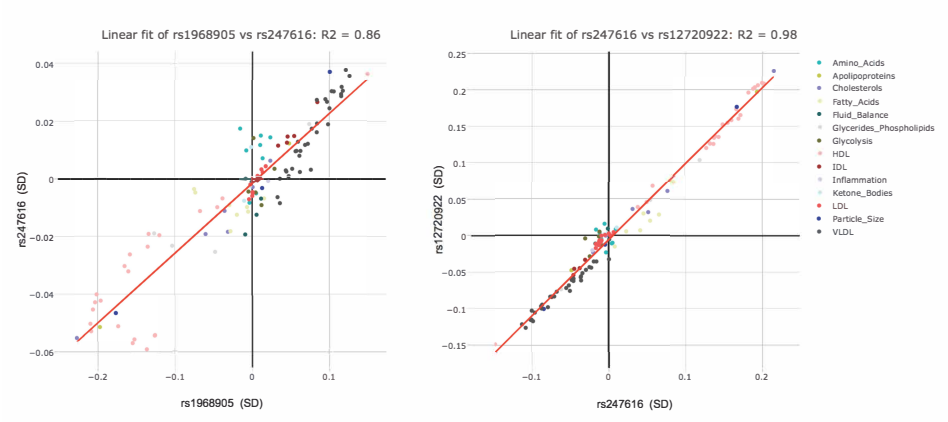
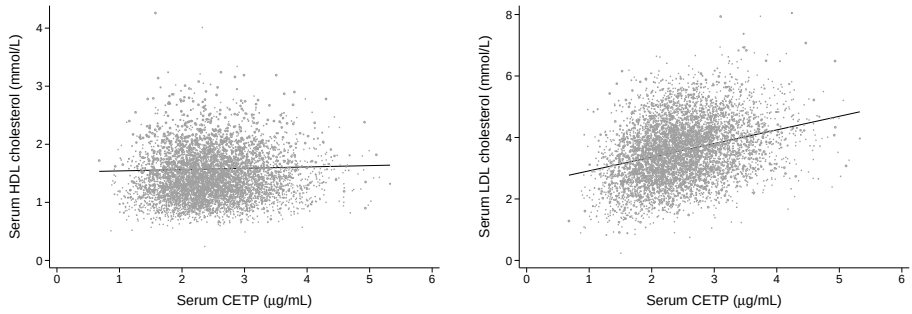


Figure 6.B.4: Correlation between the effect sizes (i.e. beta coefficients) for the different CETP genetic variants included in the genetic score, i.e. (**a**) rs247616 vs rs12720922 and (**b**) rs1968905 vs rs247616, in the MAGNETIC NMR GWAS dataset (n≈20,000). Beta coefficient from linear regression are expressed as the SD difference in metabolic measure per 1 μg/mL increase in CETP concentration.



(a) **(b)**

Figure 6.B.5: Crude correlations of serum CETP concentration with (**a**) calculated Friedewald LDL cholesterol concentration and (**b**) routinely measured HDL cholesterol concentration (clinical chemistry assay), in the Netherlands Epidemiology of Obesity (NEO) study (n=5,672). Results are based on analyses weighted towards the reference BMI distribution of the general Dutch population.

6.C. Supplementary tables

Table 6.C.1: Description of 159 metabolic measures that were measured with a high-throughput proton nuclear magnetic resonance metabolomics platform (Nightingale Health Ltd., Helsinki, Finland).

Short name	Full name	Unit
Very-low-density lipoproteins (VLDL)		
XXL.VLDL.C	Total cholesterol in chylomicrons and extremely large VLDL	mmol/L
XXL.VLDL.CE	Cholesterol esters in chylomicrons and extremely large VLDL	mmol/L
XXL.VLDL.FC	Free cholesterol in chylomicrons and extremely large VLDL	mmol/L
XXL.VLDL.L	Total lipids in chylomicrons and extremely large VLDL	mmol/L
XXL.VLDL.P	Concentration of chylomicrons and extremely large VLDL particles	mol/L
XXL.VLDL.PL	Phospholipids in chylomicrons and extremely large VLDL	mmol/L
XXL.VLDL.TG	Triglycerides in chylomicrons and extremely large VLDL	mmol/L
XL.VLDL.C	Total cholesterol in very large VLDL	mmol/L
XL.VLDL.CE	Cholesterol esters in very large VLDL	mmol/L
XL.VLDL.FC	Free cholesterol in very large VLDL	mmol/L
XL.VLDL.L	Total lipids in very large VLDL	mmol/L
XL.VLDL.P	Concentration of large VLDL particles	mol/L
XL.VLDL.PL	Phospholipids in large VLDL	mmol/L
XL.VLDL.TG	Triglycerides in large VLDL	mmol/L
L.VLDL.C	Total cholesterol in large VLDL	mmol/L
L.VLDL.CE	Cholesterol esters in large VLDL	mmol/L
L.VLDL.FC	Free cholesterol in large VLDL	mmol/L
L.VLDL.L	Total lipids in large VLDL	mmol/L
L.VLDL.P	Concentration of large VLDL particles	mol/L
L.VLDL.PL	Phospholipids in medium VLDL	mmol/L
L.VLDL.TG	Triglycerides in medium VLDL	mmol/L
M.VLDL.C	Total cholesterol in medium VLDL	mmol/L
M.VLDL.CE	Cholesterol esters in medium VLDL	mmol/L
M.VLDL.FC	Free cholesterol in medium VLDL	mmol/L
M.VLDL.L	Total lipids in medium VLDL	mmol/L
M.VLDL.P	Concentration of medium VLDL particles	mol/L
M.VLDL.PL	Phospholipids in medium VLDL	mmol/L
M.VLDL.TG	Triglycerides in medium VLDL	mmol/L
S.VLDL.C	Total cholesterol in small VLDL	mmol/L
S.VLDL.CE	Cholesterol esters in small VLDL	mmol/L
S.VLDL.FC	Free cholesterol in small VLDL	mmol/L
S.VLDL.L	Total lipids in small VLDL	mmol/L
S.VLDL.P	Concentration of small VLDL particles	mol/L
S.VLDL.PL	Phospholipids in small VLDL	mmol/L
S.VLDL.TG	Triglycerides in small VLDL	mmol/L
XS.VLDL.C	Total cholesterol in very small VLDL	mmol/L
XS.VLDL.CE	Cholesterol esters in very small VLDL	mmol/L
XS.VLDL.FC	Free cholesterol in very small VLDL	mmol/L
XS.VLDL.L	Total lipids in very small VLDL	mmol/L
XS.VLDL.P	Concentration of very small VLDL particles	mol/L
XS.VLDL.PL	Phospholipids in very small VLDL	mmol/L
XS.VLDL.TG	Triglycerides in very small VLDL	mmol/L

Intermediate-density lipoproteins (IDL)		
IDL.C	Total cholesterol in IDL	mmol/L
IDL.CE	Cholesterol esters in IDL	mmol/L
IDL.FC	Free cholesterol in IDL	mmol/L
IDL.L	Total lipids in IDL	mmol/L
IDL.P	Concentration of IDL particles	mol/L
IDL.PL	Phospholipids in IDL	mmol/L
IDL.TG	Triglycerides in IDL	mmol/L
Low-density lipoproteins (LDL)		
L.LDL.C	Total cholesterol in large LDL	mmol/L
L.LDL.CE	Cholesterol esters in large LDL	mmol/L
L.LDL.FC	Free cholesterol in large LDL	mmol/L
L.LDL.L	Total lipids in large LDL	mmol/L
L.LDL.P	Concentration of large LDL particles	mol/L
L.LDL.PL	Phospholipids in medium LDL	mmol/L
L.LDL.TG	Triglycerides in medium LDL	mmol/L
M.LDL.C	Total cholesterol in medium LDL	mmol/L
M.LDL.CE	Cholesterol esters in medium LDL	mmol/L
M.LDL.FC	Free cholesterol in medium LDL	mmol/L
M.LDL.L	Total lipids in medium LDL	mmol/L
M.LDL.P	Concentration of medium LDL particles	mol/L
M.LDL.PL	Phospholipids in medium LDL	mmol/L
M.LDL.TG	Triglycerides in medium LDL	mmol/L
S.LDL.C	Total cholesterol in small LDL	mmol/L
S.LDL.CE	Cholesterol esters in small LDL	mmol/L
S.LDL.FC	Free cholesterol in small LDL	mmol/L
S.LDL.L	Total lipids in small LDL	mmol/L
S.LDL.P	Concentration of small LDL particles	mol/L
S.LDL.PL	Phospholipids in small LDL	mmol/L
S.LDL.TG	Triglycerides in small LDL	mmol/L
High-density lipoproteins (HDL)		
XL.HDL.C	Total cholesterol in very large HDL	mmol/L
XL.HDL.CE	Cholesterol esters in very large HDL	mmol/L
XL.HDL.FC	Free cholesterol in very large HDL	mmol/L
XL.HDL.L	Total lipids in very large HDL	mmol/L
XL.HDL.P	Concentration of large HDL particles	mol/L
XL.HDL.PL	Phospholipids in large HDL	mmol/L
XL.HDL.TG	Triglycerides in large HDL	mmol/L
L.HDL.C	Total cholesterol in large HDL	mmol/L
L.HDL.CE	Cholesterol esters in large HDL	mmol/L
L.HDL.FC	Free cholesterol in large HDL	mmol/L
L.HDL.L	Total lipids in large HDL	mmol/L
L.HDL.P	Concentration of large HDL particles	mol/L
L.HDL.PL	Phospholipids in medium HDL	mmol/L
L.HDL.TG	Triglycerides in medium HDL	mmol/L
M.HDL.C	Total cholesterol in medium HDL	mmol/L
M.HDL.CE	Cholesterol esters in medium HDL	mmol/L
M.HDL.FC	Free cholesterol in medium HDL	mmol/L
M.HDL.L	Total lipids in medium HDL	mmol/L
M.HDL.P	Concentration of medium HDL particles	mol/L
M.HDL.PL	Phospholipids in medium HDL	mmol/L

M.HDL.TG	Triglycerides in medium HDL	mmol/L
S.HDL.C	Total cholesterol in small HDL	mmol/L
S.HDL.CE	Cholesterol esters in small HDL	mmol/L
S.HDL.FC	Free cholesterol in small HDL	mmol/L
S.HDL.L	Total lipids in small HDL	mmol/L
S.HDL.P	Concentration of small HDL particles	mol/L
S.HDL.PL	Phospholipids in small HDL	mmol/L
S.HDL.TG	Triglycerides in small HDL	mmol/L
Lipoprotein particle size		
VLDL particle size	Mean diameter for VLDL particles	nm
LDL particle size	Mean diameter for LDL particles	nm
HDL particle size	Mean diameter for HDL particles	nm
Apolipoproteins		
ApoA1	Apolipoprotein A1	g/L
ApoB	Apolipoprotein B	g/L
ApoB/ApoA1	Ratio of apolipoprotein B to apolipoprotein A1	
Cholesterols		
Esterified cholesterol	Esterified cholesterol	mmol/L
Free cholesterol	Free cholesterol	mmol/L
Total cholesterol in HDL2	Total cholesterol in HDL2[a]	mmol/L
Total cholesterol in HDL3	Total cholesterol in HDL3[a]	mmol/L
Total cholesterol in HDL	Total cholesterol in HDL	mmol/L
Total cholesterol in LDL	Total cholesterol in HDL	mmol/L
Remnant cholesterol	Remnant cholesterol[b]	mmol/L
Serum total cholesterol	Serum total cholesterol	mmol/L
Total cholesterol in VLDL	Total cholesterol in VLDL	mmol/L
Fatty acids		
Conjugated linoleic acid	Conjugated linoleic acid	mmol/L
Conjugated linoleic acid/FA	Ratio of conjugated linoleic acid to total fatty acids	%
Docosahexaenoic acid	22:6 docosahexaenoic acid	mmol/L
Docosahexaenoic acid/FA	Ratio of 22:6 docosahexaenoic acid to total fatty acids	%
Fatty acid chain length	Estimated fatty acid chain length	
Omega-3	Omega-3 fatty acids	mmol/L
Omega-3/FA	Ratio of omega-3 fatty acids to total fatty acids	%
Omega-6	Omega-6 fatty acids	mmol/L
Omega-6/FA	Ratio of omega-6 fatty acids to total fatty acids	%
Linoleic acid	18:2 linoleic acid	mmol/L
Linoleic acid/FA	Ratio of 18:2 linoleic acid to total fatty acids	%
MUFA	Monounsaturated fatty acids	mmol/L
MUFA/FA	Ratio of monounsaturated fatty acids to total fatty acids	%
PUFA	Polyunsaturated fatty acids	mmol/L
PUFA/FA	Ratio of polyunsaturated fatty acids to total fatty acids	%
SFA	Saturated fatty acids	mmol/L
SFA/FA	Ratio of saturated fatty acids to total fatty acids	%
Total fatty acids	Total fatty acids	mmol/L
Degree of unsaturation	Estimated degree of unsaturation	
Glycerides		
Diacylglycerol	Diacylglycerol	mmol/L
Diacylglycerol/FA	Ratio of diacylglycerol to triglycerides	
Triglycerides in HDL	Triglycerides in HDL	mmol/L
Triglycerides in LDL	Triglycerides in LDL	mmol/L

Total triglycerides	Serum total triglycerides	mmol/L
TG/phosphoglycerides	Ratio of triglycerides to phosphoglycerides	
Total phosphoglycerides	Total phosphoglycerides	mmol/L
Triglycerides in VLDL	Triglycerides in VLDL	mmol/L
Phospholipids		
Phosphatidylcholine	Phosphatidylcholine and other cholines	mmol/L
Sphingomyelins	Sphingomyelins	mmol/L
Total cholines	Total cholines	mmol/L
Amino acids		
Alanine	Alanine	mmol/L
Glutamine	Glutamine	mmol/L
Histidine	Histidine	mmol/L
Isoleucine	Isoleucine	mmol/L
Leucine	Leucine	mmol/L
Phenylalanine	Phenylalanine	mmol/L
Tyrosine	Tyrosine	mmol/L
Valine	Valine	mmol/L
Kidney function		
Albumin	Albumin	mmol/L
Creatinine	Creatinine	mmol/L
Glycolysis		
Citrate	Citrate	mmol/L
Glucose	Glucose	mmol/L
Lactate	Lactate	mmol/L
Inflammation		
Glycoprotein acetyls	Glycoprotein acetyls, mainly alpha-1-acid glycoprotein	mmol/L
Ketone bodies		
Acetate	Acetate	mmol/L
Beta-hydroxybutyrate	Beta-hydroxybutyrate	mmol/L

The genetic score for Mendelian randomization is based on the CETP SNPs rs247616, rs12720922 and rs1968905. A P-value<0.00134 is regarded statistical significant. Full names and descriptions of metabolic measures are listed in Supplementary table 6.C.1. Results are based on analyses weighted towards the reference BMI distribution of the general Dutch population.

[a] The HDL2 and HDL3 fractions are defined as those HDL particles within the density range of 1.063-1.125 g/mL and 1.125-1.210 g/mL, respectively.

[b] Remnant cholesterol is defined as non-HDL and non-LDL-cholesterol.

Table 6.C.2: Causal associations between CETP concentration and 159 circulating metabolic measures, which were assessed with a Mendelian randomization approach based on a CETP genetic score, in the Netherlands Epidemiology of Obesity (NEO) study (n=5,672).

Metabolic measure	Beta coefficient[a]	SE	P-value
Very-low-density lipoproteins (VLDL)			
XXL.VLDL.C	0.049	0.050	0.3331
XXL.VLDL.CE	0.075	0.051	0.1440
XXL.VLDL.FC	0.013	0.049	0.7929
XXL.VLDL.L	0.017	0.049	0.7365
XXL.VLDL.P	0.015	0.049	0.7592
XXL.VLDL.PL	0.006	0.049	0.9025

XXL.VLDL.TG	0.010	0.049	0.8424
XL.VLDL.C	0.050	0.051	0.3296
XL.VLDL.CE	0.074	0.052	0.1523
XL.VLDL.FC	0.021	0.051	0.6766
XL.VLDL.L	0.028	0.051	0.5851
XL.VLDL.P	0.027	0.051	0.5953
XL.VLDL.PL	0.022	0.051	0.6737
XL.VLDL.TG	0.022	0.051	0.6643
L.VLDL.C	0.097	0.053	0.0679
L.VLDL.CE	0.140	0.054	0.0089
L.VLDL.FC	0.054	0.053	0.3098
L.VLDL.L	0.081	0.053	0.1288
L.VLDL.P	0.080	0.053	0.1296
L.VLDL.PL	0.078	0.053	0.1414
L.VLDL.TG	0.074	0.053	0.1644
M.VLDL.C	0.121	0.056	0.0297
M.VLDL.CE	0.135	0.056	0.0170
M.VLDL.FC	0.105	0.054	0.0538
M.VLDL.L	0.131	0.055	0.0163
M.VLDL.P	0.132	0.054	0.0151
M.VLDL.PL	0.131	0.055	0.0165
M.VLDL.TG	0.132	0.054	0.0150
S.VLDL.C	0.254	0.060	2E-5
S.VLDL.CE	0.276	0.061	6E-6
S.VLDL.FC	0.202	0.057	0.0004
S.VLDL.L	0.223	0.057	0.0001
S.VLDL.P	0.218	0.056	0.0001
S.VLDL.PL	0.238	0.057	3E-5
S.VLDL.TG	0.177	0.055	0.0013
XS.VLDL.C	0.228	0.061	0.0002
XS.VLDL.CE	0.233	0.061	0.0001
XS.VLDL.FC	0.205	0.059	0.0005
XS.VLDL.L	0.221	0.060	0.0002
XS.VLDL.P	0.222	0.060	0.0002
XS.VLDL.PL	0.148	0.057	0.0099
XS.VLDL.TG	0.214	0.056	0.0001
Intermediate-density lipoproteins (IDL)			
IDL.C	-0.035	0.056	0.5294
IDL.CE	-0.031	0.057	0.5903
IDL.FC	-0.048	0.053	0.3694
IDL.L	-0.010	0.056	0.8634
IDL.P	-0.001	0.056	0.9837
IDL.PL	0.003	0.054	0.9568
IDL.TG	0.129	0.056	0.0207
Low-density lipoproteins (LDL)			
L.LDL.C	-0.037	0.055	0.5018
L.LDL.CE	-0.030	0.055	0.5843
L.LDL.FC	-0.056	0.053	0.2857
L.LDL.L	-0.034	0.055	0.5398
L.LDL.P	-0.030	0.055	0.5801
L.LDL.PL	-0.038	0.055	0.4916

L.LDL.TG	0.033	0.054	0.5435
M.LDL.C	-0.023	0.055	0.6688
M.LDL.CE	-0.012	0.055	0.8323
M.LDL.FC	-0.073	0.055	0.1834
M.LDL.L	-0.027	0.055	0.6247
M.LDL.P	-0.024	0.055	0.6568
M.LDL.PL	-0.036	0.056	0.5222
M.LDL.TG	-0.023	0.053	0.6644
S.LDL.C	-0.042	0.054	0.4413
S.LDL.CE	-0.026	0.054	0.6331
S.LDL.FC	-0.103	0.056	0.0632
S.LDL.L	-0.055	0.055	0.3136
S.LDL.P	-0.054	0.055	0.3305
S.LDL.PL	-0.101	0.056	0.0712
S.LDL.TG	-0.032	0.054	0.5546
High-density lipoproteins (HDL)			
XL.HDL.C	-0.517	0.053	6E-22
XL.HDL.CE	-0.515	0.054	2E-21
XL.HDL.FC	-0.511	0.052	3E-22
XL.HDL.L	-0.485	0.052	2E-20
XL.HDL.P	-0.482	0.052	3E-20
XL.HDL.PL	-0.451	0.052	3E-18
XL.HDL.TG	-0.310	0.054	1E-8
L.HDL.C	-0.446	0.051	5E-18
L.HDL.CE	-0.449	0.051	4E-18
L.HDL.FC	-0.437	0.051	2E-17
L.HDL.L	-0.432	0.051	2?10-17
L.HDL.P	-0.431	0.051	3E-17
L.HDL.PL	-0.414	0.050	2E-16
L.HDL.TG	-0.366	0.053	4E-12
M.HDL.C	-0.321	0.051	3E-10
M.HDL.CE	-0.332	0.051	8E-11
M.HDL.FC	-0.277	0.050	3E-8
M.HDL.L	-0.271	0.051	1E-7
M.HDL.P	-0.261	0.051	3E-7
M.HDL.PL	-0.245	0.051	1E-6
M.HDL.TG	0.272	0.055	9E-7
S.HDL.C	0.019	0.052	0.7092
S.HDL.CE	0.028	0.051	0.5807
S.HDL.FC	-0.049	0.057	0.3941
S.HDL.L	0.084	0.058	0.1459
S.HDL.P	0.099	0.058	0.0886
S.HDL.PL	0.055	0.058	0.3420
S.HDL.TG	0.293	0.055	1E-9
Lipoprotein particle size			
VLDL particle size	0.029	0.052	0.5786
LDL particle size	0.140	0.055	0.0111
HDL particle size	-0.481	0.052	2E-20
Apolipoproteins			
ApoA1	-0.466	0.051	5E-20
ApoB	0.047	0.058	0.4184

ApoB/ApoA1	0.277	0.056	1E-8
Cholesterols			
Esterified cholesterol	-0.149	0.056	0.0084
Free cholesterol	-0.171	0.056	0.0023
Total cholesterol in HDL2	-0.441	0.049	4E-19
Total cholesterol in HDL3	-0.392	0.050	5E-15
Total cholesterol in HDL	-0.440	0.049	4E-19
Total cholesterol in LDL	-0.033	0.055	0.5499
Remnant cholesterol	0.093	0.059	0.1144
Serum total cholesterol	-0.159	0.054	0.0035
Total cholesterol in VLDL	0.180	0.058	0.0020
Fatty acids			
Conjugated linoleic acid	-0.005	0.053	0.9249
Conjugated linoleic acid/FA	0.042	0.056	0.4513
Docosahexaenoic acid	-0.116	0.063	0.0652
Docosahexaenoic acid/FA	-0.033	0.061	0.5873
Fatty acid chain length	0.012	0.054	0.8270
Omega-3	-0.058	0.062	0.3468
Omega-3/FA	0.058	0.059	0.3249
Omega-6	-0.190	0.060	0.0016
Omega-6/FA	-0.064	0.061	0.2915
Linoleic acid	-0.159	0.060	0.0084
Linoleic acid/FA	-0.007	0.064	0.9097
MUFA	-0.046	0.058	0.4310
MUFA/FA	0.110	0.058	0.0570
PUFA	-0.184	0.061	0.0024
PUFA/FA	-0.035	0.060	0.5614
SFA	-0.123	0.060	0.0394
SFA/FA	-0.024	0.063	0.7027
Total fatty acids	-0.139	0.059	0.0189
Degree of unsaturation	-0.069	0.058	0.2391
Glycerides			
Diacylglycerol	-0.029	0.051	0.5654
Diacylglycerol/FA	-0.024	0.059	0.6816
Triglycerides in HDL	0.004	0.052	0.9387
Triglycerides in LDL	0.010	0.054	0.8589
Total triglycerides	0.106	0.054	0.0505
TG/phosphoglycerides	0.186	0.055	0.0008
Total phosphoglycerides	-0.304	0.060	5E-7
Triglycerides in VLDL	0.128	0.054	0.0178
Phospholipids			
Phosphatidylcholine	-0.185	0.059	0.0018
Sphingomyelins	-0.064	0.057	0.2567
Total cholines	-0.229	0.063	0.0003
Amino acids			
Alanine	0.028	0.058	0.6327
Glutamine	-0.006	0.057	0.9202
Histidine	-0.007	0.059	0.9033
Isoleucine	0.166	0.052	0.0013
Leucine	0.072	0.051	0.1598
Phenylalanine	0.105	0.060	0.0809

Tyrosine	0.156	0.057	0.0065
Valine	0.101	0.053	0.0559
Kidney function			
Albumin	-0.037	0.062	0.5546
Creatinine	0.042	0.046	0.3708
Glycolysis			
Citrate	0.127	0.060	0.0352
Glucose	0.067	0.055	0.2276
Lactate	0.039	0.057	0.4997
Inflammation			
Glycoprotein acetyls	0.011	0.061	0.8540
Ketone bodies			
Acetate	0.020	0.061	0.7400
Beta-hydroxybutyrate	-0.061	0.064	0.3374

The genetic score for Mendelian randomization is based on the CETP SNPs rs247616, rs12720922 and rs1968905. A P-value<0.00134 is regarded statistical significant. Full names and descriptions of metabolic measures are listed in Supplementary table 6.C.1. Results are based on analyses weighted towards the reference BMI distribution of the general Dutch population.

[a] The beta coefficient is the natural-log transformed SD difference in metabolic measure per 1 μg/mL increase in CETP concentration.

Table 6.C.3: Replication of the causal associations between CETP concentration and 111 circulating metabolic measures, which were assessed with a Mendelian randomization approach based on a CETP genetic score, using summary statistics from the MAGNETIC NMR GWAS dataset (n 20,000).

Metabolic measure	Beta coefficient[a]	SE	P-value
Very-low-density lipoproteins (VLDL)			
XXL.VLDL.C	N/A	N/A	N/A
XXL.VLDL.CE	N/A	N/A	N/A
XXL.VLDL.FC	N/A	N/A	N/A
XXL.VLDL.L	0.185	0.043	1.50E-5
XXL.VLDL.P	0.049	0.040	0.2163
XXL.VLDL.PL	0.145	0.038	0.0001
XXL.VLDL.TG	0.142	0.028	5.14E-7
XL.VLDL.C	N/A	N/A	N/A
XL.VLDL.CE	N/A	N/A	N/A
XL.VLDL.FC	N/A	N/A	N/A
XL.VLDL.L	0.112	0.029	9.02E-5
XL.VLDL.P	0.077	0.027	0.0037
XL.VLDL.PL	0.127	0.028	5.52E-6
XL.VLDL.TG	0.078	0.032	0.0143
L.VLDL.C	0.229	0.026	3.13E-18
L.VLDL.CE	0.263	0.034	9.09E-15
L.VLDL.FC	0.162	0.026	1.02E-9
L.VLDL.L	0.139	0.027	3.70E-7
L.VLDL.P	0.100	0.029	0.0006
L.VLDL.PL	0.158	0.025	1.96E-10
L.VLDL.TG	0.102	0.026	8.72E-5
M.VLDL.C	0.271	0.025	2.79E-28

M.VLDL.CE	0.276	0.026	1.25E-26
M.VLDL.FC	0.215	0.025	3.63E-18
M.VLDL.L	0.189	0.028	2.33E-11
M.VLDL.P	0.179	0.027	4.68E-11
M.VLDL.PL	0.237	0.025	1.01E-21
M.VLDL.TG	0.161	0.025	9.07E-11
S.VLDL.C	0.302	0.025	9.12E-35
S.VLDL.CE	N/A	N/A	N/A
S.VLDL.FC	0.302	0.025	1.02E-34
S.VLDL.L	0.323	0.028	1.17E-30
S.VLDL.P	0.321	0.030	3.00E-27
S.VLDL.PL	0.351	0.025	1.90E-46
S.VLDL.TG	0.279	0.026	5.68E-27
XS.VLDL.C	N/A	N/A	N/A
XS.VLDL.CE	N/A	N/A	N/A
XS.VLDL.FC	N/A	N/A	N/A
XS.VLDL.L	0.352	0.032	1.30E-28
XS.VLDL.P	0.323	0.032	1.60E-23
XS.VLDL.PL	0.238	0.026	2.92E-20
XS.VLDL.TG	0.325	0.028	2.29E-31
Intermediate-density lipoproteins (IDL)			
IDL.C	0.065	0.026	0.0115
IDL.CE	N/A	N/A	N/A
IDL.FC	0.097	0.025	8.31E-5
IDL.L	0.124	0.026	1.53E-6
IDL.P	0.150	0.026	5.98E-9
IDL.PL	0.135	0.025	3.90E-8
IDL.TG	0.239	0.026	2.52E-20
Low-density lipoproteins (LDL)			
L.LDL.C	0.026	0.025	0.2887
L.LDL.CE	0.030	0.026	0.2450
L.LDL.FC	0.005	0.025	0.8495
L.LDL.L	0.039	0.026	0.1356
L.LDL.P	0.051	0.026	0.0503
L.LDL.PL	0.041	0.025	0.0937
L.LDL.TG	N/A	N/A	N/A
M.LDL.C	-0.005	0.025	0.8405
M.LDL.CE	0.029	0.026	0.2549
M.LDL.FC	N/A	N/A	N/A
M.LDL.L	0.022	0.026	0.3877
M.LDL.P	0.027	0.026	0.2947
M.LDL.PL	-0.005	0.025	0.8380
M.LDL.TG	N/A	N/A	N/A
S.LDL.C	0.010	0.025	0.6776
S.LDL.CE	N/A	N/A	N/A
S.LDL.FC	N/A	N/A	N/A
S.LDL.L	-0.004	0.026	0.8617
S.LDL.P	-0.018	0.026	0.4914
S.LDL.PL	N/A	N/A	N/A
S.LDL.TG	N/A	N/A	N/A
High-density lipoproteins (HDL)			

XL.HDL.C	-0.417	0.025	7.49E-65
XL.HDL.CE	-0.391	0.026	6.29E-52
XL.HDL.FC	-0.454	0.025	2.45E-76
XL.HDL.L	-0.466	0.026	2.76E-73
XL.HDL.P	-0.396	0.026	3.64E-53
XL.HDL.PL	-0.507	0.027	4.38E-81
XL.HDL.TG	-0.184	0.025	7.72E-14
L.HDL.C	-0.606	0.042	3.42E-46
L.HDL.CE	-0.608	0.038	1.09E-56
L.HDL.FC	-0.560	0.047	1.22E-32
L.HDL.L	-0.591	0.048	5.39E-35
L.HDL.P	-0.581	0.050	2.39E-31
L.HDL.PL	-0.574	0.052	3.29E-28
L.HDL.TG	N/A	N/A	N/A
M.HDL.C	-0.491	0.041	1.00E-32
M.HDL.CE	-0.480	0.038	1.92E-37
M.HDL.FC	-0.464	0.043	8.54E-27
M.HDL.L	-0.404	0.037	2.00E-28
M.HDL.P	-0.359	0.034	1.44E-25
M.HDL.PL	-0.394	0.041	2.64E-22
M.HDL.TG	N/A	N/A	N/A
S.HDL.C	N/A	N/A	N/A
S.HDL.CE	N/A	N/A	N/A
S.HDL.FC	N/A	N/A	N/A
S.HDL.L	-0.111	0.026	1.65E-5
S.HDL.P	-0.138	0.026	9.86E-8
S.HDL.PL	N/A	N/A	N/A
S.HDL.TG	0.437	0.027	4.10E-57
Lipoprotein particle size			
VLDL particle size	0.025	0.026	0.3348
LDL particle size	0.283	0.026	3.21E-27
HDL particle size	-0.511	0.033	7.99E-54
Apolipoproteins			
ApoA1	-0.578	0.034	4.52E-65
ApoB	0.142	0.025	1.53E-8
ApoB/ApoA1	N/A	N/A	N/A
Cholesterols			
Esterified cholesterol	-0.122	0.031	9.83E-5
Free cholesterol	-0.199	0.031	1.72E-10
Total cholesterol in HDL2	N/A	N/A	N/A
Total cholesterol in HDL3	N/A	N/A	N/A
Total cholesterol in HDL	-0.657	0.046	1.42E-46
Total cholesterol in LDL	0.004	0.025	0.8563
Remnant cholesterol	N/A	N/A	N/A
Serum total cholesterol	-0.101	0.025	4.31E-5
Total cholesterol in VLDL	N/A	N/A	N/A
Fatty acids			
Conjugated linoleic acid	N/A	N/A	N/A
Conjugated linoleic acid/FA	N/A	N/A	N/A
Docosahexaenoic acid	-0.103	0.031	0.0010
Docosahexaenoic acid/FA	N/A	N/A	N/A

Fatty acid chain length	-0.082	0.031	0.0087
Omega-3	-0.065	0.033	0.0500
Omega-3/FA	N/A	N/A	N/A
Omega-6	-0.223	0.032	6.30E-12
Omega-6/FA	N/A	N/A	N/A
Linoleic acid	-0.220	0.034	5.88E-11
Linoleic acid/FA	N/A	N/A	N/A
MUFA	0.012	0.031	0.6946
MUFA/FA	N/A	N/A	N/A
PUFA	N/A	N/A	N/A
PUFA/FA	N/A	N/A	N/A
SFA	N/A	N/A	N/A
SFA/FA	N/A	N/A	N/A
Total fatty acids	-0.107	0.031	0.0006
Degree of unsaturation	N/A	N/A	N/A
Glycerides			
Diacylglycerol	N/A	N/A	N/A
Diacylglycerol/FA	N/A	N/A	N/A
Triglycerides in HDL	N/A	N/A	N/A
Triglycerides in LDL	N/A	N/A	N/A
Total triglycerides	0.204	0.025	1.25E-16
TG/phosphoglycerides	N/A	N/A	N/A
Total phosphoglycerides	-0.323	0.031	3.05E-25
Triglycerides in VLDL	N/A	N/A	N/A
Phospholipids			
Phosphatidylcholine	-0.374	0.039	4.23E-22
Sphingomyelins	-0.155	0.031	6.61E-7
Total cholines	N/A	N/A	N/A
Amino acids			
Alanine	-0.024	0.023	0.2902
Glutamine	0.011	0.027	0.6694
Histidine	0.017	0.026	0.5001
Isoleucine	0.049	0.025	0.0469
Leucine	0.016	0.023	0.4783
Phenylalanine	0.005	0.024	0.8504
Tyrosine	0.038	0.025	0.1376
Valine	0.020	0.024	0.3883
Kidney function			
Albumin	0.036	0.026	0.1660
Creatinine	0.003	0.023	0.8975
Glycolysis			
Citrate	0.024	0.023	0.2951
Glucose	0.004	0.023	0.8502
Lactate	0.042	0.028	0.1419
Inflammation			
Glycoprotein acetyls	0.059	0.026	0.0230
Ketone bodies			
Acetate	0.010	0.023	0.6778
Beta-hydroxybutyrate	-0.004	0.023	0.8800

The genetic score for Mendelian randomization is based on the CETP SNPs rs247616, rs12720922 and rs1968905. A P-value<0.00227 is regarded statistical significant. Full names and descriptions of metabolic measures are listed in Supplementary table 6.C.1. N/A, not quantified.

[a] The beta coefficient is the natural-log transformed SD difference in metabolic measure per 1 μg/mL increase in CETP concentration.

Table 6.C.4: Observational associations between CETP concentration and 159 circulating metabolic measures, in the Netherlands Epidemiology of Obesity (NEO) study (n=5,672).

Metabolic measure	Beta coefficient[a]	SE	P-value
Very-low-density lipoproteins (VLDL)			
XXL.VLDL.C	0.140	0.027	2E-07
XXL.VLDL.CE	0.186	0.027	8E-12
XXL.VLDL.FC	0.084	0.026	0.0014
XXL.VLDL.L	0.104	0.026	0.0001
XXL.VLDL.P	0.102	0.026	0.0001
XXL.VLDL.PL	0.098	0.026	0.0002
XXL.VLDL.TG	0.095	0.026	0.0003
XL.VLDL.C	0.091	0.028	0.0010
XL.VLDL.CE	0.098	0.028	0.0004
XL.VLDL.FC	0.084	0.028	0.0024
XL.VLDL.L	0.051	0.027	0.0640
XL.VLDL.P	0.047	0.027	0.0873
XL.VLDL.PL	0.070	0.028	0.0112
XL.VLDL.TG	0.034	0.027	0.2096
L.VLDL.C	0.081	0.030	0.0074
L.VLDL.CE	0.113	0.030	0.0002
L.VLDL.FC	0.049	0.030	0.1032
L.VLDL.L	0.044	0.030	0.1457
L.VLDL.P	0.041	0.030	0.1769
L.VLDL.PL	0.057	0.030	0.0597
L.VLDL.TG	0.027	0.030	0.3828
M.VLDL.C	0.165	0.030	5E-08
M.VLDL.CE	0.237	0.030	1E-15
M.VLDL.FC	0.073	0.031	0.0167
M.VLDL.L	0.079	0.031	0.0103
M.VLDL.P	0.072	0.031	0.0203
M.VLDL.PL	0.094	0.031	0.0023
M.VLDL.TG	0.034	0.031	0.2804
S.VLDL.C	0.356	0.032	3E-28
S.VLDL.CE	0.415	0.032	2E-38
S.VLDL.FC	0.220	0.032	6E-12
S.VLDL.L	0.200	0.032	4E-10
S.VLDL.P	0.178	0.032	3E-08
S.VLDL.PL	0.211	0.032	8E-11
S.VLDL.TG	0.064	0.032	0.0421
XS.VLDL.C	0.452	0.030	1E-49
XS.VLDL.CE	0.432	0.030	5E-45
XS.VLDL.FC	0.476	0.030	2E-55

XS.VLDL.L	0.434	0.031	1E-44
XS.VLDL.P	0.418	0.031	7E-41
XS.VLDL.PL	0.472	0.030	5E-56
XS.VLDL.TG	0.159	0.032	6E-07
Intermediate-density lipoproteins (IDL)			
IDL.C	0.402	0.029	4E-43
IDL.CE	0.396	0.029	1E-40
IDL.FC	0.400	0.027	1E-47
IDL.L	0.416	0.029	8E-47
IDL.P	0.418	0.029	7E-47
IDL.PL	0.431	0.028	5E-52
IDL.TG	0.246	0.030	7E-16
Low-density lipoproteins (LDL)			
L.LDL.C	0.402	0.029	2E-43
L.LDL.CE	0.403	0.029	2E-42
L.LDL.FC	0.392	0.028	4E-45
L.LDL.L	0.408	0.029	3E-44
L.LDL.P	0.409	0.029	3E-44
L.LDL.PL	0.414	0.029	1E-45
L.LDL.TG	0.302	0.029	2E-25
M.LDL.C	0.397	0.029	9E-43
M.LDL.CE	0.398	0.029	1E-42
M.LDL.FC	0.387	0.028	3E-42
M.LDL.L	0.405	0.029	6E-44
M.LDL.P	0.407	0.029	3E-44
M.LDL.PL	0.410	0.030	2E-42
M.LDL.TG	0.298	0.028	2E-25
S.LDL.C	0.389	0.028	1E-42
S.LDL.CE	0.392	0.028	1E-42
S.LDL.FC	0.376	0.028	1E-39
S.LDL.L	0.395	0.029	1E-42
S.LDL.P	0.397	0.029	2E-42
S.LDL.PL	0.389	0.029	2E-40
S.LDL.TG	0.239	0.029	4E-16
High-density lipoproteins (HDL)			
XL.HDL.C	0.007	0.030	0.8267
XL.HDL.CE	0.021	0.030	0.4962
XL.HDL.FC	-0.027	0.030	0.3643
XL.HDL.L	-0.002	0.029	0.9558
XL.HDL.P	0.000	0.029	0.9962
XL.HDL.PL	-0.017	0.029	0.5503
XL.HDL.TG	0.074	0.029	0.0108
L.HDL.C	-0.055	0.029	0.0582
L.HDL.CE	-0.057	0.029	0.0488
L.HDL.FC	-0.047	0.029	0.1025
L.HDL.L	-0.029	0.029	0.3064
L.HDL.P	-0.026	0.029	0.3664
L.HDL.PL	-0.005	0.028	0.8531
L.HDL.TG	0.056	0.029	0.0565
M.HDL.C	0.018	0.028	0.5210
M.HDL.CE	0.003	0.028	0.9215

M.HDL.FC	0.071	0.027	0.0077
M.HDL.L	0.070	0.027	0.0093
M.HDL.P	0.078	0.027	0.0039
M.HDL.PL	0.094	0.026	0.0004
M.HDL.TG	0.242	0.030	1E-15
S.HDL.C	0.306	0.028	2E-27
S.HDL.CE	0.328	0.028	7E-32
S.HDL.FC	0.033	0.030	0.2781
S.HDL.L	0.209	0.030	7E-12
S.HDL.P	0.207	0.031	2E-11
S.HDL.PL	0.040	0.031	0.1879
S.HDL.TG	0.077	0.031	0.0123
Lipoprotein particle sizes			
VLDL particle size	-0.143	0.029	9E-07
LDL particle size	-0.033	0.030	0.2686
HDL particle size	-0.049	0.029	0.0854
Apolipoproteins			
ApoA1	0.134	0.027	7E-07
ApoB	0.344	0.030	2E-29
ApoB/ApoA1	0.258	0.030	3E-17
Cholesterols			
Esterified cholesterol	0.368	0.029	2E-35
Free cholesterol	0.384	0.028	1E-42
Total cholesterol in HDL2	0.018	0.027	0.5005
Total cholesterol in HDL3	0.136	0.027	3E-07
Total cholesterol in HDL	0.028	0.027	0.3003
Total cholesterol in LDL	0.400	0.029	2E-43
Remnant cholesterol	0.373	0.030	4E-34
Serum total cholesterol	0.384	0.028	1E-41
Total cholesterol in VLDL	0.281	0.031	6E-20
Fatty acids			
Conjugated linoleic acid	0.032	0.027	0.2384
Conjugated linoleic acid/FA	-0.007	0.028	0.8035
Docosahexaenoic acid	0.116	0.032	0.0002
Docosahexaenoic acid/FA	-0.016	0.031	0.6130
Fatty acid chain length	-0.201	0.028	2E-12
Omega-3	0.185	0.031	2E-09
Omega-3/FA	0.022	0.031	0.4684
Omega-6	0.305	0.030	1E-24
Omega-6/FA	0.099	0.032	0.0018
Linoleic acid	0.289	0.030	4E-22
Linoleic acid/FA	0.091	0.032	0.0049
MUFA	0.148	0.030	7E-07
MUFA/FA	-0.070	0.031	0.0238
PUFA	0.297	0.031	4E-22
PUFA/FA	0.102	0.032	0.0014
SFA	0.219	0.030	2E-13
SFA/FA	0.013	0.032	0.6880
Total fatty acids	0.223	0.030	1E-13
Degree of unsaturation	-0.012	0.030	0.6964
Glycerides			

Diacylglycerol	-0.028	0.025	0.2789
Diacylglycerol/FA	-0.031	0.030	0.2975
Triglycerides in HDL	0.163	0.029	1E-08
Triglycerides in LDL	0.298	0.029	8E-25
Total triglycerides	0.082	0.031	0.0074
TG/phosphoglycerides	-0.039	0.031	0.2093
Total phosphoglycerides	0.192	0.030	2E-10
Triglycerides in VLDL	0.043	0.031	0.1659
Phospholipids			
Phosphatidylcholine	0.232	0.032	7E-13
Sphingomyelins	0.300	0.028	4E-26
Total cholines	0.241	0.033	2E-13
Amino acids			
Alanine	0.024	0.030	0.4102
Glutamine	-0.021	0.031	0.4946
Histidine	-0.024	0.031	0.4351
Isoleucine	0.069	0.028	0.0138
Leucine	0.024	0.026	0.3593
Phenylalanine	0.086	0.029	0.0030
Tyrosine	0.094	0.028	0.0006
Valine	0.011	0.027	0.6816
Kidney function			
Albumin	0.070	0.030	0.0219
Creatinine	-0.007	0.024	0.7841
Glycolysis			
Citrate	0.011	0.031	0.7329
Glucose	0.008	0.028	0.7779
Lactate	0.046	0.029	0.1145
Inflammation			
Glycoprotein acetyls	0.075	0.032	0.0181
Ketone bodies			
Acetate	-0.030	0.031	0.3284
Beta-hydroxybutyrate	-0.130	0.032	0.0001

A P-value<0.00134 is regarded statistical significant. Full names and descriptions of metabolic measures are listed in Supplementary table 6.C.1. Results are based on analyses weighted towards the reference BMI distribution of the general Dutch population.

[a] The beta coefficient is the natural-log transformed SD difference in metabolic measure per 1 μg/mL increase in CETP concentration.

7

Diabetes incidence and glucose intolerance prevalence increase with higher outdoor temperature

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In memoriam (17 January 2016) Anton J. de Craen

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Abstract

Introduction

Rising global temperatures might contribute to the current worldwide diabetes epidemic, as higher ambient temperature can negatively impact glucose metabolism via a reduction in brown adipose tissue activity. Therefore, we examined the association between outdoor temperature and diabetes incidence in the United States of America (USA) as well as the prevalence of glucose intolerance worldwide.

Methods

Using meta-regression, we determined the association between mean annual temperature and diabetes incidence during 1996-2009 for each USA state separately. Subsequently results were pooled in a meta-analysis. On a global scale, we performed a meta-regression analysis to assess the association between mean annual temperature and the prevalence of glucose intolerance.

Results

We demonstrated that on average per 1°C increase in temperature age-adjusted diabetes incidence increased with 0.314 (95% CI 0.194, 0.434) per 1000. Similarly, the worldwide prevalence of glucose intolerance increased by 0.170% (95% CI 0.107, 0.234) per 1°C rise in temperature. These associations persisted after adjustment for obesity.

Conclusion

Our findings indicate that diabetes incidence rate in the USA and prevalence of glucose intolerance worldwide increase with higher outdoor temperature.

Introduction

The worldwide prevalence of type 2 diabetes is rapidly increasing. In 2015, 415 million adults globally were suffering from diabetes, and expectations are that the prevalence will rise by almost 55%, up to 642 million cases by 2040.[1] In high-income countries, 91% of adults affected by diabetes have type 2 diabetes.[1] The type 2 diabetes epidemic accompanies the increasing prevalence of obesity.[2]

With increasing body mass index (BMI), glucose and lipids are initially stored in expanding (subcutaneous) adipose tissue compartments. However, according to the lipid overflow hypothesis, when the storage capacity of adipose tissue is exceeded, lipids can accumulate in organs (steatosis) including the pancreas, liver, heart and skeletal muscle, resulting in insulin resistance of those organs.[3] The variability in the degree of steatosis and the heterogeneity of body fat distribution over subcutaneous and visceral fat depots probably explains the only modest association between measures of overall body fat and insulin resistance.[3–5] For example, South Asians are prone to develop type 2 diabetes at a relatively low BMI, presumably because of the limited lipid storage capacity of their adipose tissue depots.[6] Interestingly, a very low-calorie diet can rapidly diminish steatosis and insulin resistance independent of weight loss[7], indicating dissociation between insulin resistance and obesity per se.

Recently, brown adipose tissue (BAT) has emerged as an organ that is capable of combusting large amounts of lipids to generate heat.[8] Physiologically, BAT is activated by cold exposure.[9?] Indeed, prolonged cold acclimatization recruits BAT activity[10] and is able to induce modest weight loss.[11] A recent landmark paper showed that acclimatization of patients with type 2 diabetes to moderate cold for only ten days already improved insulin sensitivity as determined by a markedly higher glucose infusion rate during a hyperinsulinemic-euglycemic clamp, while body weight was unaltered.[12] It is conceivable that an increased flux of fatty acids towards BAT will result in a compensatory increased flux of glucose to other metabolically active tissues, thereby improving systemic insulin sensitivity.[13]

It has previously been shown that BAT activity is negatively associated with outdoor temperature and is highest in winter.[14–16] Considering the putative role of BAT in the control of insulin action, combined with the effect of ambient temperature on BAT activity, we hypothesized that the global increase in temperature contributes to the current type 2 diabetes epidemic. Recently, a positive association was found between outdoor temperature and glycated haemoglobin (HbA1c),[17] indicating that systemic glucose homeostasis is influenced by environmental temperature. However, the importance of this association, especially in relation to the increasing diabetes burden, has never been studied on the

population level. Therefore, in the present study we aimed to assess the association between outdoor temperature and glucose metabolism on a country-wide as well as a global scale. We specifically hypothesized that diabetes incidence and prevalence of glucose intolerance increase with rising outdoor temperatures.

Materials and Methods

Diabetes incidence rate in the USA

Data on diabetes incidence in 50 states of the United States of America (USA) and three territories (i.e. Guam, Puerto Rico and Virgin Islands) were available for the years 1996-2013. Via the National Diabetes Surveillance System of the Centers for Disease Control and Prevention (CDC) age-adjusted diabetes incidence rates per 1000, along with the corresponding 95% CI, were available for all states and territories per year.[18] The diabetes incidence rate was self-reported via the Behavioral Risk Factor Surveillance System (BRFSS) and defined as the rate of adults (18-76 years) who reported to be told by a health professional in the last year they had diabetes (type 1 or 2). Data were age-adjusted to the 2000 USA standard population using age strata of 18-44, 45-64 and 65-76. Detailed information on survey methods can be found elsewhere.[19] We restricted our analysis to the 1996-2009 period as major changes in the survey methods were implemented between 2010 and 2011. Data on mean the annual temperature per state were collected through the National Centers for Environmental Information.[20] Obesity prevalence (i.e. BMI ≥30) per state or territory per year, expressed as a percentage with corresponding 95% CI, was also obtained via the BRFSS of the CDC.[19,21]

Prevalence of raised fasting blood glucose worldwide

Data on country-wise prevalence rates of raised fasting blood glucose and obesity (i.e. BMI ≥30) were available and obtained through the World Health Organization's Global Health Observatory online data repository system, which provides various health-related statistics for its member states.[22] In this database, the prevalence of raised fasting blood glucose is defined as the percentage of the population with fasting blood glucose ≥7.0 mmol/L or on medication for raised blood glucose.[23] We used the most recent age-adjusted and sex-adjusted estimates available which were based on data from 2014. Country-wise mean annual temperature data for 2014 were obtained via the Climatic Research Unit, University of East Anglia, using the 3.23 release of its data set.[24,25]

Statistical analyses

First, for each state or territory of the USA separately, we performed a weighted meta-regression analysis to estimate the association between mean annual temperatures (°C)

and age-adjusted diabetes incidence rates, both measured yearly during the period 1996-2009. Age-adjusted diabetes incidence rate was included as the dependent variable and mean annual temperature was included as the independent variable. Moreover, in order to adjust for the underlying secular trends in temperature and diabetes incidence, all meta-regression models were adjusted for the effect of time passage by including the variable year as an additional independent variable in the models. For Guam, Illinois and the Virgin Islands, there were insufficient data to perform a meta-regression analysis, and therefore these were excluded from further analyses.

Second, a meta-analysis was performed to integrate the results of the meta-regression analyses into an overall effect estimate, representing the mean strength of the association between mean annual temperature and diabetes incidence rate in the USA during the period 1996-2009. For the worldwide data, we performed a meta-regression analysis with age-adjusted and sex-adjusted country-wise prevalence of raised fasting blood glucose as the dependent variable and mean annual temperature as well as the World Bank income group (i.e. low-, lower-middle-, upper-middle- and high-income)[22] as the independent variables. For both meta-regression analysis and meta-analysis, the DerSimonian and Laird random-effects model was used.[26,27] Weights were calculated as $\frac{1}{\sigma_i^2+\tau^2}$ with σ_i^2 being the standard error of the age-adjusted incidence rate in year i and τ^2 the estimated between-study variance.[28] Standard errors of the age-adjusted incidence rates were unknown and therefore estimated as $\frac{0.5(UL-LL)}{1.96}$ with UL the upper limit and LL the lower limit of the 95% CI of the age-adjusted incidence rate.

To establish whether the association between mean annual temperature and diabetes incidence rate was mediated through effects on body weight, we first calculated an overall effect estimate for the association between mean annual temperature and obesity prevalence in the USA using a similar approach as described above. Since this overall effect size significantly differed from zero, we added obesity prevalence as an independent variable in the meta-regression models relating mean annual temperature and diabetes incidence rate. Subsequently, we again performed a meta-analysis to integrate the estimates of the obesity prevalence corrected meta-regression analyses into an overall effect estimate for the association between mean annual temperature and diabetes incidence rate adjusted for obesity prevalence in the USA during the period 1996-2009. For the global data we performed a similar analysis by considering obesity prevalence as a mediator variable in the meta-regression models.

We also composed a map of the USA showing the mean annual temperature over the period 1996-2009 and beta coefficients from the obesity-adjusted meta-regression analyses per state. This map provides insight into potential geographically determined confounding

factors (e.g. sea influences, biomes or altitudes), which could be apparent from a non-random distribution of beta coefficients overlapping with geographical patterns.

Programming was performed in STATA Statistical Software version 12.0 (Statacorp, College Station, Texas, USA)) and R version 3.1.0 using the 'metafor' package.[29] Maps were computed using MATLAB version R2015a (The MathWorks Inc., Natick, Massachusetts, USA).

Results

National level: USA

Table 7.1 shows data on annual temperature, diabetes incidence rate and obesity prevalence in the period 1996-2009 for each included USA state or territory. During these fourteen years, the age-adjusted diabetes incidence rate was highest in Puerto Rico, West Virginia and South Carolina, and lowest in Minnesota, Massachusetts and Colorado. The mean change (Δ) in annual temperature between two consecutive years ranged from -0.11°C (Minnesota) to +0.09°C (Hawaii).

Both diabetes incidence rate (Figure 7.1) and obesity prevalence (Supplemental figure 7.A.1) increased with higher mean annual temperature. The overall effect estimate for the association between mean annual temperature and diabetes incidence rate was 0.314 (95% CI 0.194, 0.434), indicating a 0.314 increase in the diabetes incidence rate per 1000 for each degree Celsius increase in mean annual temperature. Similarly, obesity prevalence increased by 0.173% (95% CI 0.050%, 0.296%) for each degree Celsius increase in mean annual temperature. After adjustment for obesity prevalence the positive association between mean annual temperature and diabetes incidence rate attenuated only slightly (Supplemental figure 7.A.2). Per degree Celsius increase in mean annual temperature the obesity-adjusted diabetes incidence rate increased by 0.290 (95% CI 0.164, 0.416) per 1000.

Figure 7.2 shows a map of the USA, illustrating the relation between the mean annual temperature per state over the period 1996-2009 and the magnitude of the corresponding change in obesity-adjusted diabetes incidence rate. As can be appreciated from this figure, the diabetes incidence rate increases in most states with higher mean annual temperature, while there is no apparent geographical pattern which could explain this association.

Global level

Prevalence data on raised fasting blood glucose and obesity were available for 190 countries. The worldwide aggregated age-adjusted and sex-adjusted prevalences of raised

fasting blood glucose levels and obesity were 9.65% (95% CI 9.11%, 10.13%) and 19.51% (95% CI 17.94%, 21.07%), respectively. There was a positive association between mean annual temperature and country-wise age-adjusted, sex-adjusted and income-adjusted prevalence of raised fasting blood glucose. The prevalence increased by 0.170% for each degree Celsius rise in temperature (95% CI 0.107, 0.234).

There was also a positive association between mean annual temperature and country-wise age-adjusted, sex-adjusted and income-adjusted obesity prevalence. For each degree Celsius rise in temperature, the obesity prevalence increased by 0.295% (95% CI 0.137%, 0.454%). Interestingly, the increase in obesity prevalence for each degree Celsius rise in average temperature was larger in women (0.503%) as compared with men (0.111%; mean difference 0.391%, 95% CI 0.160%, 0.622%). After additional adjustment for obesity prevalence, the association between mean annual temperature and country-wise age-adjusted, sex-adjusted and income-adjusted prevalence of raised fasting glucose only slightly attenuated (0.106% increase for each degree Celsius rise in temperature, 95% CI 0.057%, 0.155%) (Figure 7.3).

Given the extremely high prevalence of raised fasting glucose in some relatively small countries (Figure 7.3), we performed a sensitivity analysis excluding all countries (n=29) with a prevalence of raised fasting blood glucose higher than 15%. Under this restrictive scenario the association between mean annual temperature and country-wise age-adjusted, sex-adjusted, income-adjusted and obesity-adjusted prevalence of raised fasting blood glucose remained present: 0.051% increase in prevalence for each degree Celsius rise in temperature (95% CI 0.003%, 0.099%; see Supplementary figure 7.A.3).

Table 7.1: Temperature characteristics, mean diabetes incidence and mean obesity prevalence in the United States of America over the period 1996-2009.

	Mean annual temperature (°C)	ΔTemperature per year (°C)[b]	Age adjusted diabetes incidence rate per 1000	Obesity prevalence (%)
Alabama	17.46	-0.03	9.7	26.3
Alaska	-2.48	-0.07	6.5	23.5
Arizona	16.13	0.03	7.7	19.3
Arkansas	16.11	-0.02	8.6	24.6
California	14.85	0.03	8.6	20.9
Colorado	7.86	0.03	5.2	15.9
Connecticut	9.78	-0.02	6.5	18.3
Delaware	13.27	-0.06	8.2	22.1
Florida	21.72	-0.05	8.4	20.8
Georgia	17.74	-0.03	9.2	23.0
Hawaii	23.32	0.09	6.4	17.9
Idaho	6.54	0.01	7.3	21.1
Indiana	11.26	-0.05	8.3	24.4
Iowa	9.12	-0.07	6.6	23.4
Kansas	12.81	-0.02	7.2	22.2
Kentucky	13.46	-0.04	8.1	25.3
Louisiana	19.48	-0.03	9.5	25.7
Maine	5.37	0.00	7.9	21.1
Maryland	12.87	-0.06	8.1	22.1
Massachusetts	9.16	-0.02	5.1	17.8
Michigan	7.37	-0.05	8.5	24.9
Minnesota	5.48	-0.11	4.6	20.9
Mississippi	17.82	-0.03	9.1	27.7
Missouri	12.88	-0.04	7.5	24.3
Montana	5.91	-0.08	5.6	18.9
Nebraska	9.76	-0.04	6.1	22.8
Nevada	10.50	0.05	7.7	20.3
New Hampshire	6.59	0.00	6.4	19.9
New Jersey	11.84	-0.05	7.0	19.8
New Mexico	12.42	0.02	7.6	20.2
New York	7.73	-0.02	8.2	20.4
North Carolina	15.17	-0.05	8.8	23.9
North Dakota	5.04	-0.09	5.9	23.1
Ohio	10.84	-0.04	8.4	23.9
Oklahoma	15.71	-0.02	8.9	23.9
Oregon	8.75	0.02	6.7	21.7
Pennsylvania	9.61	-0.04	7.5	23.2
Puerto Rico	26.94	-0.07	10.9	22.7
Rhode Island	10.20	-0.03	7.6	18.8
South Carolina	17.28	-0.04	10.2	24.6
South Dakota	7.71	-0.08	5.9	22.3
Tennessee	14.63	-0.04	9.6	24.9
Texas	18.74	-0.01	9.0	24.4
Utah	9.55	0.00	6.4	19.4
Vermont	6.07	-0.01	5.9	19.0
Virginia	13.20	-0.05	7.7	21.8
Washington	8.36	-0.03	8.1	21.2
West Virginia	11.34	-0.04	10.7	26.8
Wisconsin	6.68	-0.08	7.0	22.3
Wyoming	5.70	0.00	6.9	20.2

Results are presented as mean values over the period 1996-2009.

[a] 49 states and one territory (Puerto Rico): insufficient data were available for Guam, Illinois and the Virgin Islands.

[b] Mean change (Δ) over the period 1996-2009 in mean annual temperature between two consecutive years.

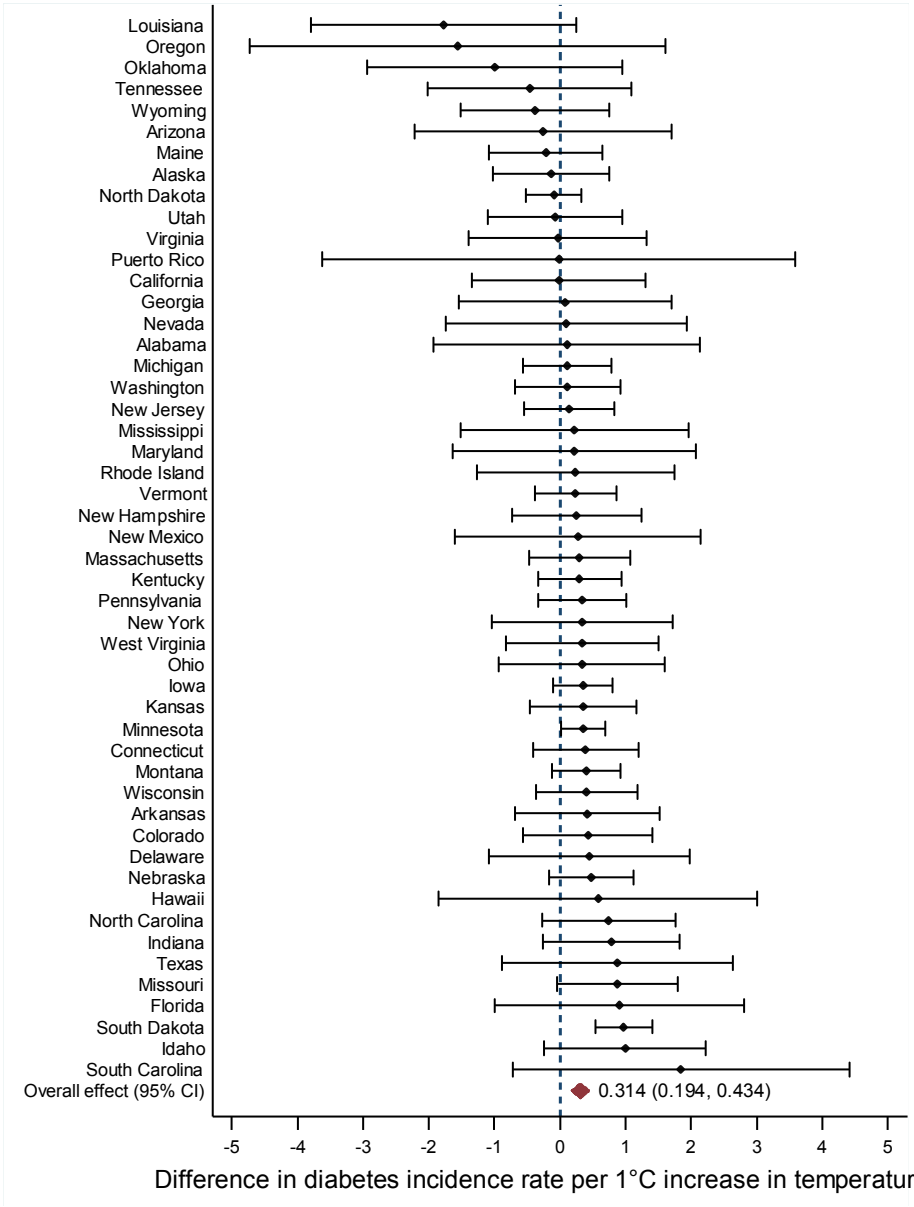


Figure 7.1: The association between mean annual temperature and diabetes incidence in the United States of America over the period 1996-2009. The forest plot represents the difference[a,b] in diabetes incidence rate[c] per 1°C increase in temperature.

[a] Beta coefficient from meta-regression analysis. Error bars represent 95% CI.
[b] Adjusted for the effect of time passage.
[c] Diabetes incidence rate is the age-adjusted diabetes incidence rate per 1000.

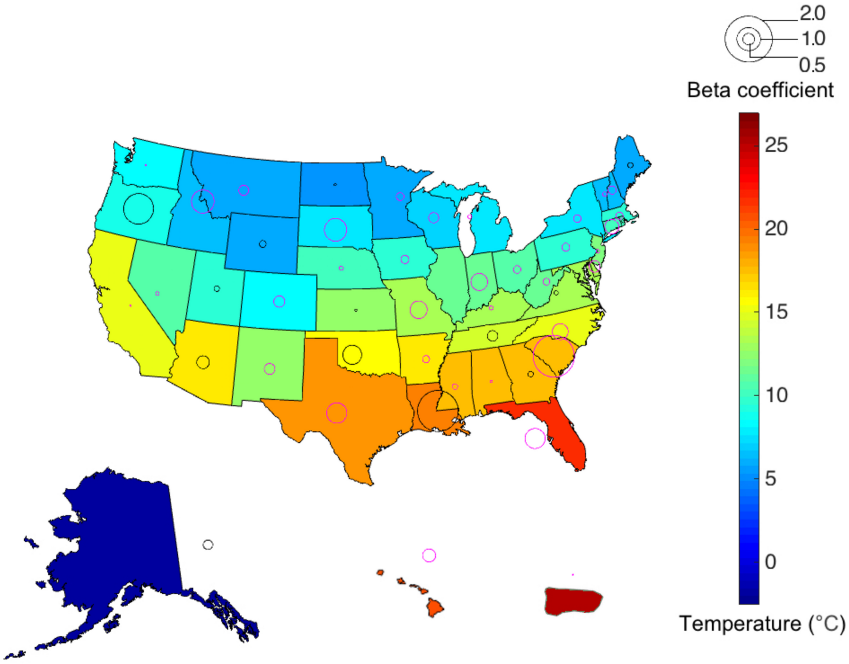


Figure 7.2: Map of the Unites States of America (including Alaska, Hawaii and Puerto Rico), showing the mean annual temperature and the magnitude of the beta coefficients[a] from the obesity-adjusted meta-regression analysis[b], per state or territory over the period 1996-2009.

[a] Beta coefficient from meta-regression analysis, representing the difference in diabetes incidence rate[c] per 1°C increase in temperature; red circles indicate a positive beta coefficient, blue circles indicate a negative beta coefficient.
[b] Adjusted for the effect of time passage.
[c] Diabetes incidence rate is the age-adjusted diabetes incidence rate per 1000.

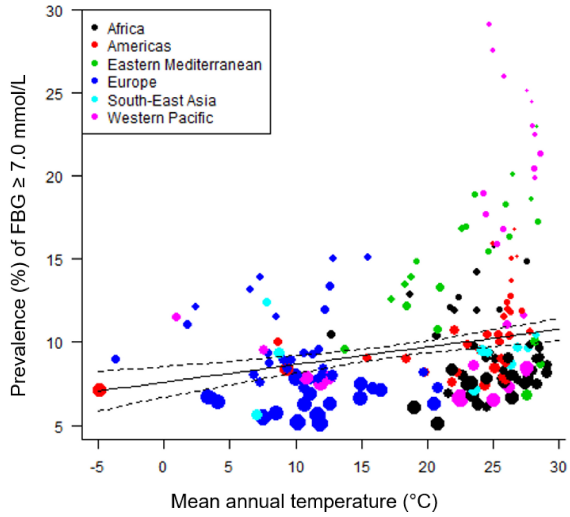


Figure 7.3: The worldwide association between mean annual temperature and age-, sex-, income- and obesity-adjusted prevalence of raised fasting blood glucose (FBG), for 190 countries in 2014. Colours indicate the six World Health Organization regions.[30] The size of each circle is inversely proportional to the standard error of the estimate of the prevalence of raised fasting blood glucose.

Discussion

The association between outdoor temperature and fasting blood glucose concentration has rarely been examined in the past, and studies showed discordant results.[31,32] These studies were performed in selected study populations from specific cities and the conflicting results indicate that the results are not generalizable to other places. To the best of our knowledge, the present study is the first to assess the association of outdoor temperature with diabetes incidence and the prevalence of raised fasting blood glucose on both a national and global level. Using fourteen-year longitudinal state level data from the USA, we show that the overall diabetes incidence rate is higher in warmer years. Per 1°C increase in temperature, we found an overall increase in age-adjusted diabetes incidence of 0.314 per 1000.

Importantly, we expect confounding in this association to be minimal, as temperature itself is hardly influenced by any extraneous factors, apart from the passage of time and geographical location. Hence, we adjusted the models for the underlying secular trends in temperature and diabetes incidence by adjusting for time passage. Also, by first determining the association for each state separately before pooling for obtaining the overall effect estimate, we minimized the effect of potential geographically determined confounding factors, and in this way we also precluded all other differences between states that could influence or bias the association between temperature and diabetes incidence. Furthermore, the distribution of the magnitudes and directions of the beta coefficients over the USA (Figure 2) appeared to be random as we could not distinguish a pattern overlapping with geographic (e.g. sea influences, biomes or altitudes) or demographic characteristics, indicating that these factors do not explain the association. Our results are therefore unlikely to be merely due to interstate demographic, socioeconomic or other differences.

On the global scale, detailed longitudinal international data on diabetes incidence rates were unfortunately unavailable for a large number of countries. Therefore, we used country-wise estimates of the prevalence of raised fasting blood glucose instead. In accordance with the results of the USA, we found that on a global level the prevalence of glucose intolerance is higher in warmer countries. This association between temperature and raised fasting blood glucose cannot be merely due to international differences in age, sex, income or obesity prevalence, as our analyses were adjusted for these variables. Although we cannot exclude residual confounding by population stratification, the fact that this association closely parallels our findings in the USA supports the notion that the ambient temperature affects the occurrence of glucose intolerance worldwide.

The associative design of our study does not allow us to draw conclusions on causality.

However, it is tempting to speculate that the mechanism underlying our present findings is related to an interplay between BAT activity and glucose clearance from the circulation by metabolically active tissues. Recently, peripheral insulin sensitivity in patients with type 2 diabetes was shown to increase markedly after ten days of mild cold exposure.[12] These patients had an increased glucose uptake by skeletal muscle, probably explained by an increased overall flux of fatty acids towards BAT, reducing the fatty acid flux to other tissues.[13] Interestingly, in this short-term cold acclimation study body weight was unaffected[12], indicating that the effects of temperature on glucose metabolism can occur independently of body weight. This is further supported by another study showing that higher BAT activity after cold exposure was associated with lower blood glucose and HbA1c levels independent of body fat.[33] In line with these findings we found that adjustment for obesity prevalence only slightly attenuated the association of outdoor temperature with both diabetes incidence in the USA and prevalence of glucose intolerance worldwide. Therefore, our data are consistent with the hypothesis that a decrease in BAT activity with increasing environmental temperature may deteriorate glucose metabolism and increase the incidence of diabetes. The role of BAT activity as an underlying pathway in the association between outdoor temperature and diabetes incidence, may even be genetic in origin. According to the cold climate genes hypotheses, DNA areas involved in thermogenesis may have encountered selection pressure during evolution in areas with cold climates.[34] The biological effects of the gene products may also decrease the susceptibility to diabetes. Interestingly, DNA areas that regulate uncoupling proteins, including uncoupling protein 1 that drives heat production in BAT, have been proposed as candidates for these cold climate genes.[34]

Besides BAT activity, physical activity could be a potential mediator in the association between temperature and diabetes incidence, as physical activity varies with environmental temperature.[35] Although a decrease in physical activity with increasing mean annual temperature may theoretically explain our findings, physical activity has been shown to be highest between daily temperatures of 15°C and 20°C and decreases with higher but also lower temperatures.[36,37] Therefore, we do not expect that physical activity completely explains the positive association between outdoor temperature and diabetes incidence. Unfortunately, data on physical activity for the USA were not available for the period of 1996-2009 and therefore we were not able to further elucidate the mediating effects. This topic evidently requires further investigation.

There are several potential limitations to our study. First, we used state-level and country-level aggregated data on incidence and prevalence rates. Although individual-level data would have yielded more accurate results, acquiring individual-level data on such a large

scale was not feasible. Moreover, we used statistical techniques, that is meta-regression and meta-analysis, which can appropriately account for the uncertainty in the aggregated effect estimates. Second, available data on BMI were categorically organized (i.e. obesity prevalence) and BMI could therefore not be applied as a continuous variable in the models. Although continuous data would have been more accurate, the prevalence of obesity is by definition closely related to the average BMI and its distribution in the general population, implying that obesity prevalence can be used as a valid surrogate measure for average BMI in a given population. It should be emphasized that we were not able to evaluate the linearity of the association between BMI and diabetes incidence or prevalence in our models, as continuous data on BMI were not available. Although adjustment for obesity prevalence only slightly attenuated the association between outdoor temperature and diabetes, mediation via BMI should be further evaluated in future studies to assess whether a non-linear association between BMI and diabetes might account for our results. Third, owing to the observational nature of our analyses we cannot exclude residual confounding. For the USA, however, we largely circumvented this issue by analysing longitudinal state-level data for each state separately before pooling the results. Furthermore, adjustment for important intercountry differences, that is age, sex, income and obesity, did not materially change the results on the global level. Importantly, adjusting for income also takes along other inter-country differences, as many surrogate measures which are often applied for adjusting for global inequalities in the degree of social development are based on the gross national income (GNI) per capita. In fact, the World Health Organization uses a four-group classification scheme, based on the Work Bank Atlas methodology to estimate GNI per capita,[38] according to which each country is categorized as either low-, lower-middle-, upper-middle- or high-income.[22]

In conclusion, diabetes incidence rate in the USA and prevalence of glucose intolerance worldwide increase with higher outdoor temperature. On the basis of our results, a 1°C rise in environmental temperature would account for over 100,000 new diabetes cases per year in the USA alone, given a population of nearly 322 million people in 2015.[16] These findings emphasize the importance of future research into the effects of environmental temperature on glucose metabolism and the onset of diabetes, especially in view of the global rise in temperatures with a new record set for the warmest winter in the USA last year.[37]

Acknowledgements

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Appendix

7.A. Supplementary figures

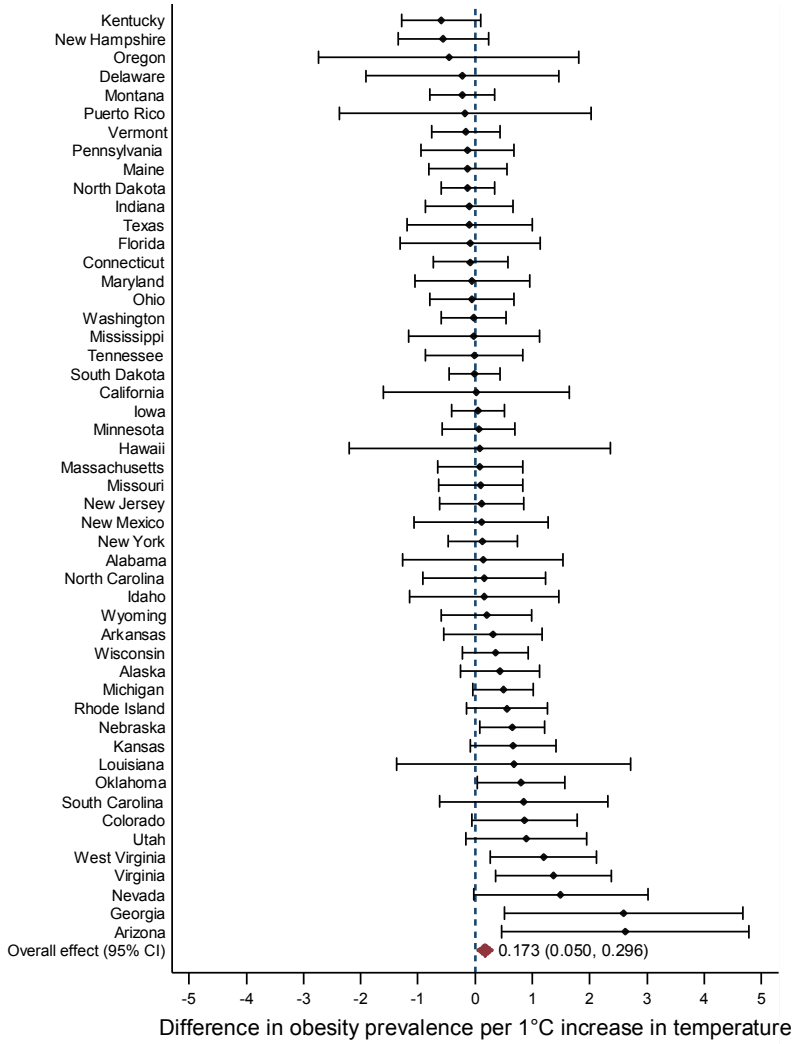


Figure 7.A.1: The association between mean annual temperature and obesity prevalence in the United States of America over the period 1996-2009. The forest plot represents the difference[a,b] in obesity prevalence[c] per 1°C increase in temperature.

[a] Beta coefficient from meta-regression analysis. Error bars represent 95%CI.
[b] Adjusted for the effect of time passage.
[c] Obesity prevalence is expressed as percentage.

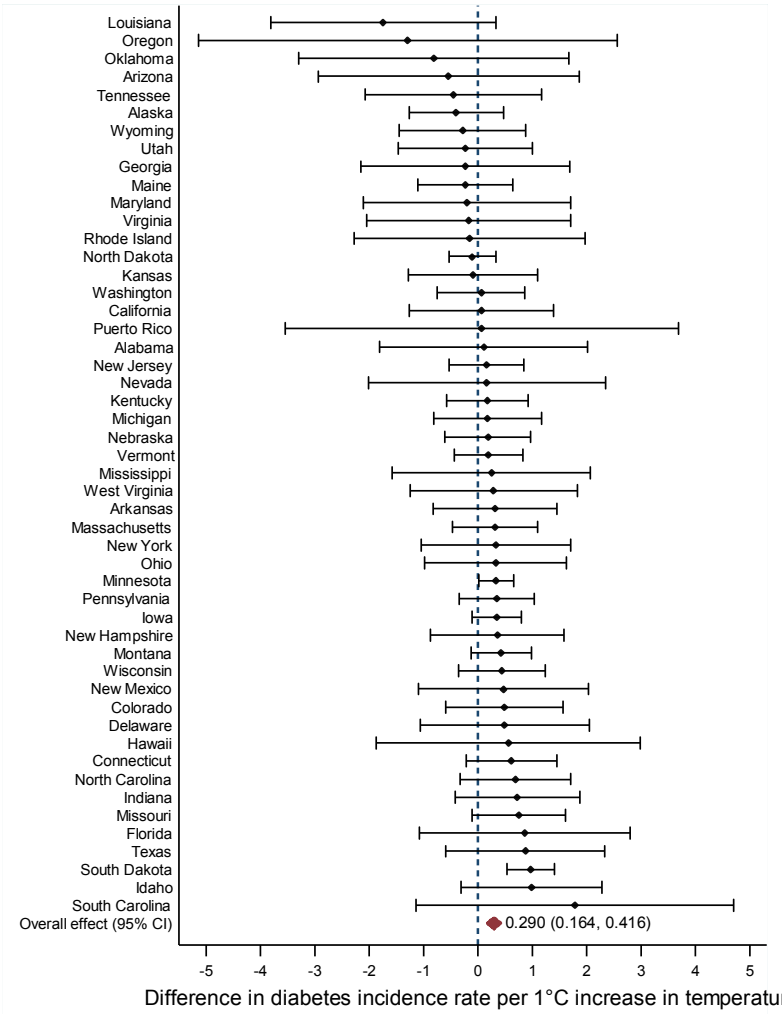


Figure 7.A.2: The association between mean annual temperature and diabetes incidence in the United States of America over the period 1996-2009, adjusted for obesity prevalence. The forest plot represents the difference[a,b] in diabetes incidence rate[c] per 1°C increase in temperature.

[a] Beta coefficient from meta-regression analysis. Error bars represent 95%CI.
[b] Adjusted for the effect of time passage.
[c] Diabetes incidence rate is the age-adjusted diabetes incidence rate per 1000.

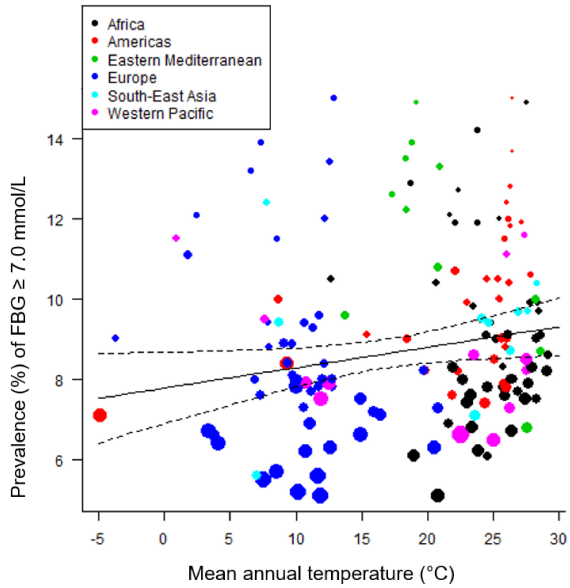


Figure 7.A.3: The worldwide association between mean annual temperature and age-, sex-, income- and obesity-adjusted prevalence of raised fasting blood glucose (FBG), for 161 countries in 2014, after exclusion of countries with a prevalence of raised fasting blood glucose >15%. Colours indicate the six World Health Organization regions. The size of each circle is inversely proportional to the standard error of the estimate of the prevalence of raised fasting blood glucose.

8

Smoking is associated with increased resting energy expenditure in the general population: The NEO study

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Metabolism 2015; 64:1548-55

Abstract

Introduction

Animal studies and human studies in small selected populations have shown a positive association between nicotine smoking and resting energy expenditure (REE), but data in large cohorts are lacking. We aimed to investigate the association between smoking behaviour and REE in a large, population-based study.

Methods

In this cross-sectional analysis of baseline measurements from the Netherlands Epidemiology of Obesity (NEO) study (n=6,673), we included participants with REE measurement by indirect calorimetry who were not using lipid or glucose lowering drugs (n=1,189). We used linear regression analysis to examine the association of smoking status (never, former, occasional, current smoker) and smoking quantity (pack years) with REE per kilogram (kg) fat free mass (FFM) and with REE adjusted for FFM. Models were adjusted for age, sex, ethnicity, educational level, physical activity, energy intake and body mass index (BMI).

Results

Mean (standard deviation, SD) age was 55.2 (5.9) years and BMI was 26.3 (4.4) kg/m^2. 60% of the participants were women. Mean (SD) REE/FFM (kcal/day/kg FFM) was for male never smokers 25.1 (2.0), male current smokers 26.4 (2.8), female never smokers 28.9 (2.5) and female current smokers 30.1 (3.7). After adjustment, only current smokers had a higher REE/FFM (mean difference 1.28, 95% CI 0.64, 1.92), and a higher REE adjusted for FFM (mean difference 60.3 kcal/day, 95% CI 29.1, 91.5), compared with never smokers. There was no association between pack years and REE/FFM (mean difference -0.01, 95% CI -0.06, 0.04) or REE adjusted for FFM (mean difference 0.2, 95% CI -2.4, 2.8) in current smokers.

Conclusion

Current smoking is associated with a higher resting energy expenditure compared with never smoking in a large population-based cohort.

Introduction

The effects of smoking on body composition have been studied extensively.[1–4] Cigarette smoking decreases body weight[1] and smoking cessation is associated with weight gain.[1,2] These effects of smoking on the body's energy balance can be explained by either a decrease in energy intake or an increase in energy expenditure and a possible inverse effect after cessation.

Nicotine is thought to be the most important mediator of the effects of smoking on body composition.[1,3] Nicotine acts by various mechanisms on the body's energy balance and affects both the central nervous system and peripheral tissues by regulating the release of a wide range of neurotransmitters and hormones.[1] Studies in mice suggest that the effects of nicotine on energy metabolism mostly involve the central nervous system. More specifically, a recent mouse study showed that nicotine-induced weight loss was associated with inactivation of hypothalamic AMP-activated protein kinase (AMPK). Inactivation of AMPK results in decreased orexigenic signalling and thus decreased food intake, while energy expenditure is increased as a result of increased locomotor activity and increased thermogenesis in brown adipose tissue.[4]

In humans, smoking has been shown to suppress appetite.[3] Smoking also seems to have a direct effect on energy expenditure. A study with eight healthy cigarette smokers showed an increase in total 24-hour energy expenditure after the participants smoked 24 cigarettes in 24 hours, compared with a 24-hour period without cigarette smoking.[5] In another study with 18 male smokers there was an increase in resting energy expenditure (REE) demonstrated directly after a nicotine dose via nasal spray, compared with placebo.[6] Further studies have shown that cigarette smoking increases REE in small (i.e. $n \geq 147$) selected patient populations.[7–10]

Overall, the studies mentioned above found a positive association between smoking and REE, which may contribute to the lower body weight of smokers and weight gain after smoking cessation.[1,2,5,6] However, such a relationship has not been confirmed in a large cohort, neither have long-term effects of smoking been investigated at the population level. The aim of the present study, therefore, was to examine the association between smoking and REE in a large population-based cohort.

Materials and Methods

Study design and study population

The Netherlands Epidemiology of Obesity (NEO) study is a population-based cohort study. Participants were recruited from September 2008 until September 2012, resulting in a co-

hort of 6,673 individuals with an oversampling of individuals with overweight or obesity. Men and women aged between 45 and 65 years with a self-reported body mass index (BMI) of 27 kg/m^2 or higher living in the greater area of Leiden (in the West of The Netherlands) were eligible to participate in the NEO study. In addition, all inhabitants aged between 45 and 65 years from one municipality (Leiderdorp) were invited, irrespective of their BMI, allowing for a reference distribution of BMI. Baseline data were collected at the NEO study center of the Leiden University Medical Center (LUMC). Prior to the NEO study visit, participants completed a questionnaire about demographic and clinical information and fasted for at least 10 hours. Participants came to the research site in the morning to undergo several baseline measurements including anthropometric measurements and blood sampling. Records were made of all medication used in the month preceding the visit to the study center. More extensive measurements were taken in random subsets of participants, including magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS), indirect calorimetry, dual energy X-ray absorptiometry (DXA) and accelerometry.

More detailed information on the study design and data collection has been described elsewhere.[11] This study was approved by the medical ethics committee of the Leiden University Medical Center (LUMC) and all participants gave written informed consent.

For the present analyses we included the random subsample of participants of whom REE was measured by indirect calorimetry (n=1,434) and of those we excluded the participants who were using lipid or glucose lowering drugs (n=245), as there are indications in literature that these classes of drugs affect energy expenditure.[12–15] This brings the total study population to 1,189 NEO study participants, of which six participants had missing data on fat free mass (FFM) (one non-smoking participant and five former smokers).

Data collection

Questionnaires on health and lifestyle factors were sent to all participants. Smoking status was classified as never-smoker, former smoker, occasional smoker or current smoker on the basis of a questionnaire, in which the participants could answer the question “Do you smoke?” as either: “No, I never smoked”; “No, but I did smoke in the past”; “Yes, occasionally” or “Yes, currently”. Long-term tobacco exposure was expressed in pack years of smoking, calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the person smoked. A pack year is defined as twenty cigarettes smoked every day for one year. Questionnaires were used to assess energy intake (semi-quantitative food frequency questionnaire, FFQ[16]) and physical activity (Short Questionnaire to Assess Health-enhancing physical activity, SQUASH[17]). Energy intake was calculated from the FFQ using the 2011 version of the Dutch food composition table (NEVO-2011). Physical

activity was expressed as metabolic equivalents of task (MET)*hours/week, with MET indicating the intensity of an activity. Weight was measured without shoes and one kilogram (kg) was subtracted to correct for the weight of clothing. BMI was calculated by dividing the weight in kilograms by the height in meters squared. FFM was calculated from body weight and body fat percentage estimated with the Tanita bio impedance balance (TBF-310, Tanita International Division, UK).

Resting energy expenditure

The basal metabolic rate (BMR) is responsible for approximately 60% of total energy expenditure in persons with sedentary occupations.[18] REE, which is more feasible to assess, can be used as a representation of BMR. Generally, REE is within 10% of the corresponding BMR.[18] REE was measured for 30 minutes by indirect calorimetry (ventilated hood system, Oxycon Pro), which was performed after an overnight fast and a resting period of 15 minutes. During the measurement, participants were awake while lying on a bed in a quiet room. The volume of oxygen inspired and carbon dioxide exhaled were measured every minute. REE and substrate oxidation rates were calculated using standard formulas.[11]

Statistical analysis

Because individuals with a BMI of 27 kg/m^2 or higher were oversampled in the NEO study adjustments were made to correctly represent associations for the general population.[19] This was done by weighting individuals according to the BMI distribution of participants from the Leiderdorp municipality[20], whose BMI distribution was similar to the BMI distribution of the general Dutch population[21]. All presented results are based on weighted analyses. Consequently, the results apply to a population-based study without oversampling of participants with a BMI of 27 kg/m^2 or higher.

Baseline characteristics were expressed as mean (standard deviation, SD) or percentage. Results concerning or related to body composition and REE are presented for men and women separately, as these variables are influenced by sex. Differences in baseline characteristics between the four categories of smoking status were tested by a chi-squared test for categorical variables and by analysis of variances (ANOVA) for continuous variables with post hoc Bonferroni correction if statistical differences in baseline characteristics between the four smoking categories were found.

Since the amount of metabolically active tissue is the major determinant of REE, REE is often adjusted for FFM by using the ratio of REE and FFM (REE/FFM).[22–26] Therefore, weighted linear regression analysis was performed to evaluate the association between smoking status and REE/FFM, and the association between long-term tobacco exposure,

expressed as pack years, and REE/FFM. Alternatively, linear regression analyses have frequently been performed with REE as dependent variable and FFM included as independent variable in the models.[23,27,28] This method was therefore additionally used to assess the association of REE adjusted for FFM with both smoking status and tobacco exposure. For all linear regression analyses mean differences and corresponding 95% confidence intervals (CI) were reported. Crude models (model 1: not adjusted in models using REE/FFM; adjusted for FFM in models using REE) were adjusted for sex (model 2), age, ethnicity and educational level (model 3). In a fourth model (model 4) physical activity, energy intake and BMI were added as potential confounding variables. Analyses to assess statistical interaction between smoking behaviour and sex, between smoking behaviour and age and between smoking behaviour and physical activity were performed. This was done by including product terms into the models. All analyses were performed using STATA Statistical Software (Statacorp, College Station, Texas, USA), version 12.0.

Results

Table 8.1 shows the demographic and clinical characteristics of the study population (n= 1,189. Overall, women had a considerably lower REE than men (mean difference -368 kcal/day; 95% CI -396, -339). In men mean BMI and total body fat were significantly different between the four categories of smoking status. Post-hoc analyses showed that only the difference in BMI between former and never smokers was statistically significant ($P<0.001$), and that the difference in total body fat was significantly different between former smokers and never smokers ($P<0.001$) and between current smokers and never smokers ($P=0.033$). The difference in total body fat between current and never smokers disappeared after adjustment for age, ethnicity and educational level ($P=0.105$). Unadjusted REE was not clearly different between never, occasional, former and current smokers for both men and women. REE/FFM was higher in current smokers than never smokers for both men and women (Figure 8.1).

In Table 8.2 the mean and difference in REE/FFM of the association between smoking status and REE/FFM are presented. After adjustment for sex (model 2) the REE/FFM of current smokers was 1.25 kcal/day/kg FFM higher compared with never smokers. Further adjustment for age, ethnicity and educational level (model 3) showed a similar higher REE/FFM for current smokers. Likewise, model 4 (further adjusted for physical activity, energy intake and BMI) showed a higher REE/FFM of 1.28 kcal/day/kg FFM for current smokers. This corresponds to a 4.7% higher REE/FFM relative to the adjusted mean of REE/FFM of never smokers. There was no statistical interaction with sex, age or physical

activity for the association between smoking status and REE/FFM.

No difference in REE/FFM was demonstrated between never and occasional smokers in the adjusted models (Table 8.2). There was also no difference in REE/FFM between never and former smokers, neither unadjusted nor in the adjusted models (Table 8.2). Pack years ranged from 0 to 139. There was no association between long-term tobacco exposure (expressed as pack years) and REE/FFM, neither in former smokers, occasional smokers or current smokers (Table 8.3).

Table 8.1: Demographic and clinical characteristics of the NEO study population stratified by smoking status.

Characteristic Characteristic	**Never smokers (40.2%)**	**Occasional smokers (4.5%)**	**Former smokers (44.6%)**	**Current smokers (10.7%)**	**P-value**
Sex (% women)	63	43	61	52	0.1337
Ethnicity (% whites)	96	97	96	92	0.5500
Age (year)	54.6 (5.9)	55.1 (5.6)	55.8 (5.9)	54.7 (6.0)	0.1544
Quantity of smoking (pack years)	-	7.0 (9.4)	13.0 (14.4)	24.6 (13.2)	<0.0001
Educational level (% academic degree)	15	9	13	5	0.2650
Net monthly income (% high[a])	13	13	11	16	0.5832
Alcohol use (% non-users)	14	15	8	9	0.1802
BMI (kg/m^2)					
Men	25.7 (3.1)	26.8 (4.9)	27.5 (4.0)	26.9 (3.6)	0.0007
Women	25.9 (4.6)	26.3 (5.7)	26.1 (4.9)	25.2 (4.7)	0.7709
Total body fat (%)					
Men	22.6 (4.8)	24.0 (7.9)	25.8 (5.9)	25.2 (5.0)	0.0002
Women	36.8 (6.0)	36.9 (6.7)	36.3 (7.7)	35.8 (7.8)	0.9211
FFM (kg)					
Men	66.0 (6.0)	65.3 (6.8)	66.1 (7.4)	64.7 (6.6)	0.6800
Women	45.2 (4.3)	46.1 (6.0)	45.1 (4.5)	44.3 (4.9)	0.6356
Physical activity (MET hours/week)					
Men	39.7 (37.7)	36.8 (27.0)	36.3 (34.8)	28.9 (25.8)	0.3042
Women	35.0 (27.4)	23.8 (13.9)	35.1 (26.9)	26.7 (25.3)	0.0035
REE (kcal/day)					
Men	1,658 (199)	1,655 (202)	1,689 (233)	1,703 (235)	0.5699
Women	1,304 (167)	1,283 (197)	1,312 (171)	1,334 (217)	0.7437
REE/FFM (kcal/day/kg FFM)					
Men	25.1 (2.0)	25.4 (2.3)	25.6 (2.6)	26.4 (2.8)	0.0310
Women	28.9 (2.5)	27.8 (2.3)	29.1 (2.5)	30.1 (3.7)	0.0058

Results are based on weighted analyses with n=1,189 (n=1,183 when characteristic comprises measurements of fat (free) mass). Values are presented as mean (SD) or percentage.

[a] High net monthly income: >€3,000/month.

BMI, body mass index; FFM, fat free mass; MET, metabolic equivalents of task; NEO, Netherlands Epidemiology of Obesity; REE, resting energy expenditure.

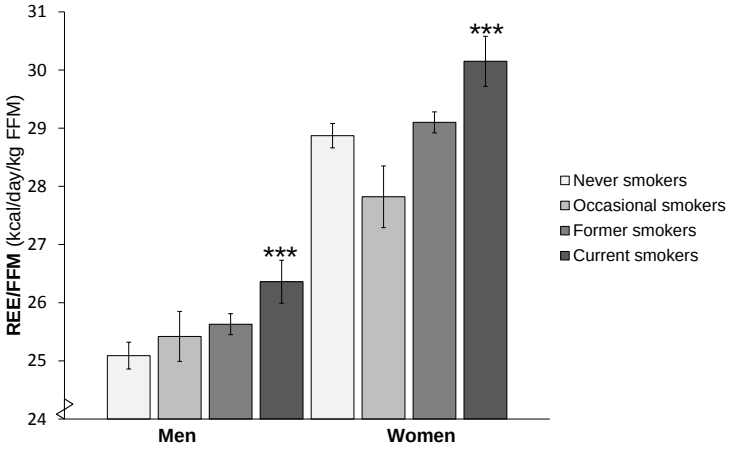


Figure 8.1: Association between mean REE/FFM and smoking status stratified by sex. Results are based on weighted analyses (n= 1,183). *** p ≤ 0.001 compared with non-smokers in linear regression analysis adjusted for sex (model 2). Error bars represent standard errors. FFM, fat free mass; REE, resting energy expenditure.

The associations between REE and smoking status and REE and long-term tobacco exposure, using linear regression to adjust REE for FFM, are presented in Table 8.4 and 8.5, respectively. Results were similar to those obtained with the REE/FFM ratio method (Table 8.2 and 8.3). Table 8.4 shows that, compared with never smokers, only current smokers had a higher REE of 60.3 kcal/day, even after adjustment for confounding variables. This corresponds to a 3.9% higher REE relative to the adjusted mean of never smokers. No association between pack years and REE adjusted for FFM was found (Table 8.5).

Table 8.2: Mean and difference in REE/FFM of never smokers compared with occasional smokers, former smokers and current smokers.

	Model 1	Model 2	Model 3	Model 4
REE/FFM, adjusted mean of never smokers (95% CI) kcal/day/kg FFM	27.49 (27.15, 27.83)	27.28 (27.02, 27.54)	27.28 (27.02, 27.54)	27.52 (27.27, 27.76)
Occasional smokers[a]				
Difference in REE/FFM[b](95% CI), kcal/day/kg FFM	-1.03 (-1.96,-0.11)	-0.30 (-1.05, 0.46)	-0.29 (-1.06, 049)	-0.28 (-1.02, 0.46)
P-value	0.029	0.441	0.471	0.455
Former smokers[a]				
Difference in REE/FFM[b](95% CI), kcal/day/kg FFM	0.28 (-0.24, 0.80)	0.34 (-0.06, 0.75)	0.35 (-0.06, 0.75)	0.32 (-0.09, 0.73)
P-value	0.293	0.094	0.093	0.124
Current smokers[a]				
Difference in REE/FFM[b](95% CI), kcal/day/kg FFM	0.85 (0.01, 1.69)	1.25 (0.61, 1.89)	1.24 (0.60, 1.89)	1.28 (0.64, 1.92)
P-value	0.046	<0.001	<0.001	<0.001

Results are based on weighted analyses (n=1,183).Model 1: unadjusted (n=1,183). Model 2: adjusted for sex (n=1,183). Model 3: adjusted for sex, age, ethnicity and educational level (n=1,173; ethnicity was missing in 1 participant, 9 missing data on educational level). Model 4: adjusted for sex, age, ethnicity, educational level, physical activity, energy intake and BMI (n=1,159; 14 missing data on physical activity).

[a] Compared with never smokers.

[b] Beta coefficient from weighted linear regression.

BMI, body mass index; FFM, fat free mass; REE, resting energy expenditure.

Table 8.3: Association between pack years of smoking and REE/FFM for occasional smokers, former smokers and current smokers separately.

	Model 1	Model 2	Model 3	Model 4
Occasional smokers				
Difference in REE/FFM per pack year[a] (95% CI), kcal/day/kg FFM	-0.02 (-0.07, 0.04)	0.02 (-0.03, 0.06)	0.01 (-0.05, 0.06)	-0.01 (-0.07, 0.06)
P-value	0.605	0.543	0.791	0.813
Former smokers				
Difference in REE/FFM per pack year[a] (95% CI), kcal/day/kg FFM	-0.02 (-0.04, 0.01)	0.01 (-0.01; 0.03)	0.01 (-0.00, 0.03)	0.01 (-0.01, 0.03)
P-value	0.210	0.174	0.141	0.200
Current smokers				
Difference in REE/FFM per pack year[a] (95% CI), kcal/day/kg FFM	-0.01 (-0.06,0.05)	-0.01 (-0.05, 0.03)	-0.01 (-0.06, 0.03)	-0.01 (-0.06, 0.04)
P-value	0.767	0.738	0.535	0.628

Results are based on weighted analyses (current smokers: n=126; occasional smokers: n=53; former smokers n= 555). Model 1: unadjusted. Model 2: adjusted for sex. Model 3: adjusted for sex, age, ethnicity and educational level (former smokers: ethnicity was missing in 1 participant, 4 missing data on educational level; current smokers: 2 missing data on educational level). Model 4: adjusted for sex, age, ethnicity, educational level, physical activity, energy intake and BMI (former smokers: 7 missing data on physical activity).

[a] Beta coefficient from weighted linear regression.

BMI, body mass index; FFM, fat free mass; REE, resting energy expenditure.

Table 8.4: Mean and difference in REE adjusted for FFM of never smokers compared with occasional smokers, former smokers and current smokers.

	Model 1	Model 2	Model 3	Model 4
REE, adjusted mean of never smokers (95% CI), kcal/day	1,504 (1,489, 1,519)	1,516 (1,502, 1,530)	1,513 (1,499, 1,527)	1,539 (1,526, 1,551)
Occasional smokers[a]				
Difference in REE[b] (95% CI), kcal/day	-19.1 (-61.1, 23.0)	-12.0 (-53.4, 29.4)	-11.0 (-53.3, 31.4)	-13.4 (-50.9, 24.1)
P-value	0.374	0.571	0.611	0.484
Former smokers[a]				
Difference in REE[b] (95% CI), kcal/day	17.7 (-3.7, 39.2)	18.5 (-2.0, 39.0)	18.9 (-1.24, 39.1)	13.7 (-6.3, 33.8)
P-value	0.105	0.078	0.066	0.180
Current smokers[a]				
Difference in REE[b] (95% CI), kcal/day	53.5 (18.6, 88.4)	63.2 (29.7, 96.7)	61.7 (28.2, 95.2)	60.3 (29.1, 91.5)
P-value	0.003	<0.001	<0.001	<0.001

Results are based on weighted analyses (n=1,183). Model 1: adjusted for FFM (n=1,183). Model 2: adjusted for FFM and sex (n=1,183). Model 3: adjusted for FFM, sex, age, ethnicity and educational level (n=1,173; ethnicity was missing in 1 participant, 9 missing data on educational level). Model 4: adjusted for FFM, sex, age, ethnicity, educational level, physical activity, energy intake and BMI (n=1,159; 14 missing data on physical activity).

[a] Compared with never smokers.

[b] Beta coefficient from weighted linear regression.

BMI, body mass index; FFM, fat free mass; REE, resting energy expenditure.

Table 8.5: Association between pack years of smoking and REE adjusted for FFM, for occasional smokers, former smokers and current smokers separately.

	Model 1	Model 2	Model 3	Model 4
Occasional smokers				
Difference in REE per pack year[a] (95% CI), kcal/day	1.9 (-1.3, 5.1)	1.7 (-1.5, 4.9)	1.2 (-2.2, 4.6)	-0.1 (-3.7, 3.5)
P-value	0.237	0.297	0.481	0.967
Former smokers				
Difference in REE per pack year[a] (95% CI), kcal/day	1.0 (-0.2, 2.1)	1.0 (-0.1, 2.1)	1.1 (-0.1, 2.2)	0.8 (-0.3, 1.9)
P-value	0.115	0.081	0.061	0.152
Current smokers				
Difference in REE per pack year[a] (95% CI), kcal/day	-0.3 (-2.0, 1.4)	-0.5 (-2.2, 1.3)	-0.8 (-2.9, 1.3)	0.2 (-2.4, 2.8)
P-value	0.690	0.614	0.475	0.899

Results are based on weighted analyses (current smokers: n=126; occasional smokers: n=53; former smokers n= 555). Model 1: adjusted for FFM. Model 2: adjusted for FFM and sex. Model 3: adjusted for FFM, sex, age, ethnicity and educational level (former smokers: ethnicity was missing in 1 participant, 4 missing data on educational level; current smokers: 2 missing data on educational level). Model 4: adjusted for FFM, sex, age, ethnicity, educational level, physical activity, energy intake and BMI (former smokers: 7 missing data on physical activity).

[a] Beta coefficient from weighted linear regression.

BMI, body mass index; FFM, fat free mass; REE, resting energy expenditure.

Discussion

In this large population-based cohort study we observed a higher REE/FFM (1.28 kcal/-day/kg FFM) and a higher REE adjusted for FFM (60.3 kcal/day) in current smokers compared with never smokers, after adjustment for age, sex, ethnicity, educational level, physical activity, energy intake and BMI. These results corroborate findings in previous studies investigating short-term effects of smoking on energy expenditure in selected study populations. These studies also observed a positive association between smoking or nicotine administration and REE.[5–10] However, as the habit of smoking often lasts many years, the question is whether results from short-term intervention studies are generalizable to prevalent smokers in the general population. The results of the current study show that in the general population smoking is associated with a higher REE.

The observed association between smoking and REE could contribute to a lower body weight in smokers and weight gain after smoking cessation. Compared with never smokers the REE adjusted for FFM was 3.9% higher in current smokers. In the literature, a range of 3-10% increase in REE has been reported directly after nicotine administration,[6] which is in line with our findings.

The mechanisms underlying the effects of nicotine on the body's energy balance have not yet been fully elucidated. It has been recognized for a long time that nicotine has sympathoadrenal stimulatory effects, thereby increasing energy substrate utilization, especially by enhancing the mobilization of free fatty acids through lipolysis in white fat tissue.[2,29] Besides this adrenergic pathway, nicotine also directly enhances lipolysis in white fat cells via nicotinic cholinergic receptors.[30] More recently it has been proposed that nicotine also increases energy combustion in brown adipose tissue (BAT).[4] BAT generates heat by burning triglycerides and glucose and thereby contributes to total energy expenditure.[31] We recently showed that in smoke-exposed mice, compared with air-exposed mice, plasma triglyceride-derived fatty acids were more rapidly taken up by BAT (MR Boon & PCN Rensen, unpublished). In addition, there are indications that the metabolic effects of nicotine change during physical activity in comparison with resting state. A study with thirty male participants, investigating the effects of smoking on energy expenditure during physical activity, concluded that smoking during light physical activity doubles the enhancing effect of smoking on energy expenditure.[32] Furthermore, there is still dissension about the contribution of a possible decreased REE after smoking cessation to body weight and composition, as some studies reported a decrease in REE after smoking cessation,[2,33] while others found no decline in REE.[2,33]

In this study there was no association between long-term tobacco exposure (pack years)

and REE/FFM or REE adjusted for FFM, neither in occasional smokers, former smokers or current smokers. These findings suggest that the effects of smoking on REE are primarily acute. The finding that never and former smokers, and especially never and occasional smokers did not differ in REE strongly supports this hypothesis that smoking has no long-term effects on REE. However, the current data did not allow us to confirm this hypothesis. There were no data available concerning the timespan between the last moment a participant smoked and the moment of blood sampling. Literature about this subject is scant. A small study examining the acute effects of smoking on energy expenditure, found that an acute cigarette-induced increase of energy expenditure ceased the morning after smoking.[5]

The main strength of our study is the extensive phenotyping of a large population-based cohort. It should be considered though that the NEO cohort is predominantly white, and different results might be obtained in other ethnic groups. Literature about the effect of race on the association between smoking and REE is scarce and ambiguous. It has been suggested that smoking increases REE to a greater extent in African American women compared to white women,[34] but others found no effects of racial differences on the association between smoking and REE.[8]

NEO study participants underwent a wide range of examinations and measurements and completed a variety of questionnaires, giving detailed information about smoking habits, REE and body composition. This made it possible to include many confounding factors into the models. However, we could not completely rule out the occurrence of any residual confounding. The reasons for this are that the study was set up as an observational cross-sectional study and that some confounding variables were self-reported (educational level, physical activity and dietary intake) which could have resulted in some extent of misclassification by under or overreporting. It should also be taken into consideration that pack years were self-reported, possibly leading to underreporting. Underreporting of pack years would lead to an underestimation of the association with REE/FFM or REE adjusted for FFM. However, there was no association between pack years and REE/FFM or REE adjusted for FFM in occasional, former and current smokers and we do not expect that some extent of underreporting would alter these highly non-significant results.

We conclude that current smoking is associated with an increased resting energy expenditure per kg fat free mass and an increased resting energy expenditure adjusted for fat free mass, compared with never smoking. The magnitude of the difference in resting energy expenditure between never and current smokers shows the considerable effect of smoking on the body's energy balance. There was no difference in resting energy expenditure per kg fat free mass or resting energy expenditure adjusted for fat free mass between never

and former smokers or between never and occasional smokers and no association with pack years, which implies that smoking has primarily acute effects on resting energy expenditure. To support this hypothesis future research into the duration and persistence of a smoking-induced increase in resting energy expenditure is necessary.

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9

Genetic variation in the obesity gene FTO is not associated with decreased fat oxidation: The NEO study

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Abstract

Introduction

The FTO gene harbours the strongest common genetic variant associated with obesity. Recently, rs1421085-T to -C substitution mapped in FTO was shown to induce a developmental shift of human adipocytes from an energy-combusting beige to an energy-storing white phenotype *in vitro*. As browning of adipocytes selectively enhances fat oxidation (FatOx), we hypothesized that rs1421085-C in FTO is associated with deceased FatOx compared with carbohydrate oxidation (CarbOx) and an increased respiratory quotient (RQ).

Methods

In the Netherlands Epidemiology of Obesity (NEO) study, a population-based cohort study of middle-aged individuals (45-65 years), anthropometry and genotyping was performed (n=5,744), in addition to indirect calorimetry (n=1,246). With linear regression analyses we examined associations of rs1421085 genotype with FatOx, CarbOx and RQ.

Results

In the total study population, 36.7% carried the rs1421085-TT genotype, 47.6% rs1421085-CT and 15.7% rs1421085-CC. Mean (SD) age was 56 (6) years, mean (SD) BMI was 26.3 (4.4) kg/m^2, and 56% of the total population were women. Measures of adiposity (difference, 95% CI) were higher in CC carriers than in rs1421085-TT carriers: BMI +0.56 (0.15, 0.98) kg/m^2; waist circumference +1.25 (0.02, 2.49) cm; total body fat mass +1.21 (0.28, 2.14) kg. However, no differences in mean FatOx (+2.5 (-2.4, 7.4) mg/min), CarbOx (-6.1 (-17.4, 5.2) mg/min) or RQ (-0.01 (-0.02, 0.01)) were observed between the two genotypes.

Conclusion

We observed no evidence for associations of rs1421085 in FTO with FatOx and RQ. This indicates that the rs1421085 C-allele in FTO induces obesity likely via other pathways than via reduced FatOx.

Introduction

With the global rise in the prevalence of obesity and accompanying increased risks of metabolic disorders, cardiovascular disease and cancer, the search for preventive strategies and therapies for obesity is of crucial importance.1 Environmental factors play a major role in the onset of obesity, and lifestyle interventions are therefore the keystone of treatment and prevention.[1] However, because 40-70% of the inter-individual differences in obesity risk in the general population is explained by genetics,[2,3] it is of great interest for future intervention strategies to unravel genetic factors and related biological pathways involved in obesity.

In the search for genetic factors contributing to inter-individual differences in obesity risk, genetic variation in the fat mass and obesity-associated gene (FTO) was identified through genome-wide association studies (GWAS) as being the top common genetic determinant of body mass index (BMI) and obesity risk.[4–6] Single nucleotide polymorphisms (SNPs) that cluster in the first intron of this gene are robustly associated with obesity. In addition, these SNPs associate with cardiovascular disease, metabolic syndrome and diabetes, mostly mediated by BMI.[7] Of the different common SNPs identified in relation to adiposity traits, rs1558902 showed the largest increase in BMI (0.39 kg/m^2) and obesity risk (1.2-fold) per additional copy of the risk allele.[6]

Although FTO was already identified in 2007 as an obesity susceptibility gene,[4,5] the biological explanation for the association of genetic variation in FTO with BMI and the risk of developing obesity remained largely unclear. Importantly, a potential mechanism was recently illuminated by Claussnitzer and colleagues.[8] Rs1421085-T to -C allele substitution, which is in perfect linkage disequilibrium with rs1558902, was shown to disrupt the binding of the transcriptional repressor ARID5B to IRX3 and IRX5, which increased their expression specifically in pre-adipocytes. This resulted in a developmental shift from energy-combusting beige adipocytes towards energy-storing white adipocytes *in vitro*, characterized by decreased mitochondrial thermogenesis and increased lipid storage. This study thereby provides evidence for a genetic basis of disturbed adipocyte beiging in humans. Previous studies have shown that decreased rates of fat oxidation are associated with obesity risk.[9–11] On the other hand, prolonged acclimation to cold, which selectively increases fat oxidation,[12] decreases body weight explained by a decrease in body fat mass.[13] Thus, a possible lower fat oxidation as a consequence of decreased browning in rs1421085 risk allele carriers may explain their increased obesity risk.

Previous studies that examined the association between FTO gene variants and resting energy expenditure (REE) obtained contradictory results.[14–19] Only one study observed

a lower REE in risk allele carriers independent of body size [14], while others found a higher REE in risk allele carriers which abolished after adjustment for fat free mass (FFM), [15–18] or observed no association. [19] Associations with specific components of substrate utilization have not been investigated to date. In light of the findings by Claussnitzer and colleagues, [8] it is of particular interest to study the association of rs1421085 with fat oxidation (FatOx), carbohydrate oxidation (CarbOx) and respiratory quotient (RQ) for several reasons: 1) brown adipose tissue activation selectively enhances the oxidation of fat over glucose, both in mice [20] and humans; [12] 2) long term exposure to cold that activates brown adipose tissue progressively increases lipid oxidation while reducing glucose oxidation; [21] 3) conversion of white adipocytes to beige adipocytes changes the substrate preference from glucose to lipid. [22] Because the rs1421085 variant in FTO was shown to be involved in adipocyte beiging in vitro, [8] we hypothesized that specifically the FTO rs1421085-C risk allele is associated with decreased whole-body FatOx compared with CarbOx, and a higher RQ in resting state in humans.

In the present study, we aimed to examine these associations in a large, population-based cohort of middle-aged participants with indirect calorimetry measurements performed in 1,246 participants. To the best of our knowledge, this is the largest study so far to determine the association of the FTO rs1421085 variant with REE, and the first to study the association of this SNP with substrate oxidation rates.

Materials and Methods

Study design and population

The study population was part of the Netherlands Epidemiology of Obesity (NEO) study, which is a population-based prospective cohort study including 6,671 participants recruited from September 2008 until September 2012. The NEO study was designed to study overweight and obesity-associated disease, and therefore persons with a BMI of ≥27 kg/m^2 were oversampled. Both men and women in the age of 45 to 65 years, living in the greater area of Leiden (The Netherlands), and with a self-reported BMI of ≥27 kg/m^2 were able to participate. To acquire a reference distribution for BMI, inhabitants from the nearby municipality Leiderdorp (The Netherlands) were asked to participate irrespective of their BMI. In the NEO study center extensive baseline measurement were performed after an overnight fasting period of at least ten hours. A random subgroup of participants underwent indirect calorimetry. Questionnaires on demographic and clinical characteristics were completed at home, preceding the study visit. The present study is a cross-sectional analysis of the baseline measurements of the NEO study. For this study we excluded 927 participants because of missing data on genotype, being a first-degree relative or being from non-

European ancestry. Therefore, the present study population comprised 5,744 NEO study participants, of which 1,246 participants underwent indirect calorimetry to measure REE and substrate oxidation rates (Supplementary figure 9.A.1).

The medical ethics committee of the Leiden University Medical Center approved the NEO study and written informed consent was given by all participants. The complete study design and detailed information on data collection can be found elsewhere.[23]

FTO genotype

DNA was extracted from venous blood samples obtained from the antecubital vein. Genotyping was performed using the Illumina HumanCoreExome-24 BeadChip (Illumina Inc., San Diego, California, United States of America). From these data, we extracted the rs1421085 (C/T) polymorphism from the whole-genome data. The rs1421085 polymorphism was in Hardy-Weinberg equilibrium (p-value=0.97).

Resting energy expenditure and substrate oxidation

In a random subsample of NEO study participants REE was measured by indirect calorimetry (ventilated hood system, Oxycon Pro) for 30 minutes under standardized conditions. The measurement was performed between 8 and 9 a.m., after an overnight fast of at least ten hours and a resting period of 15 minutes. During the measurement, participants were awake while lying on a bed in a quiet room. The volumes of oxygen inspired (VO_2) and carbon dioxide exhaled (VCO_2) were measured every minute. REE was calculated using standard formulas.[24] RQ is defined as VCO_2/VO_2.[24] FatOx was calculated as $1.69*VO_2 – 1.69*VCO_2 – 2.03$*urinary nitrogen excretion (N) and CarbOx as $4.57*VCO_2 – 3.23*VO_2 – 2.6$*N, considering urinary nitrogen excretion negligible (zero).[25]

Anthropometry

We assessed anthropometry to be able to associate rs1421085 genotype with measures of adiposity (i.e. BMI, total body fat percentage, waist circumference, hip circumference, fat mass and FFM), in order to show that the NEO study population is a valid and representative cohort to study genetic variation in the obesity-associated gene FTO. Body weight and total body fat were measured with the Tanita bio-impedance balance (TBF-310, Tanita International Division, UK), without shoes and with a one kilogram (kg) subtraction to correct for weight of clothing. BMI was calculated as the weight in kilograms divided by the height in meters squared. Fat mass and FFM were calculated from body weight and body fat percentage as estimated with the Tanita bio-impedance balance (TBF-310, Tanita International Division, UK). Waist circumference was measured mid-way between the lower

costal margin and the iliac crest. Hip circumference was measured at the maximum circumference of the buttocks.

Covariates

Smoking status was reported on the questionnaire and grouped into never smoker, former smoker and current smoker. Long-term tobacco exposure was expressed in pack years of smoking, calculated as the number of packs of cigarettes smoked per day multiplied by the number of years the person smoked. A pack year is defined as smoking twenty cigarettes every day during one year. Pre-existing cardiovascular disease was defined as reporting a medical history of myocardial infarction, congestive heart failure, angina, peripheral vascular disease, or stroke. Diabetes was defined as self-reporting the disease via the questionnaire, using anti-diabetic medication, or having a fasting glucose measurement ≥7.0 mmol/L at baseline. With the short questionnaire to assess health-enhancing physical activity (SQUASH)[26] physical activity during leisure time was quantified and expressed as metabolic equivalents of task (MET)*hours/week, which indicates the intensity of an activity. Intake of alcohol was determined with a semi-quantitative food frequency questionnaire,[27] and calculated with the 2011 version of the Dutch food composition table (NEVO-2011).[28] At the study center, research nurses recorded current medication use.

Statistical analyses

represent baseline associations in the general population.[29] All participants were weighted towards the BMI distribution of the reference population from Leiderdorp (The Netherlands)[30], of which the BMI distribution corresponded to the BMI distribution of the Dutch general population aged 45 to 65 years.[31] As all analyses were weighted, the results apply to a population-based study without oversampling of persons with overweight or obesity. Descriptive characteristics of the participants were reported as mean (standard deviation, SD) or proportion and stratified by rs1421085 genotype, i.e. TT, CT and CC with C being the rs1421085 allele associated with a higher BMI.[5]

As an initial step, we attempted to replicate in the NEO study population the previously described associations between rs1421085-C and measures of increased adiposity. After verification, we determined the association of rs1421085 genotype with REE, RQ, FatOx and CarbOx. All associations were assessed using linear regression analysis assuming an additive model. Data were presented as weighted mean differences per additional rs1421085-C allele, with TT as the reference. Models were adjusted for age, sex, and four principal components to correct for population substructures. Importantly, the amount of metabolically active tissue is a major determinant of REE.[32–36] To study the association with REE irrespective of FFM we adjusted for FFM in two ways: 1) using the ratio of

REE and FFM (REE/FFM) as dependent variable and 2) adding FFM as a covariate in the linear regression models. Considering a power of 0.80 and an alpha of 0.05, we were able to detect a mean difference between rs1421085-CC and -TT carriers of 64 kcal/day REE, 0.012 unit RQ, 6 mg/min FatOx, and 13 mg/min CarbOx in our study population.

REE is usually higher in persons with obesity, probably because the absolute amount of FFM increases with body size.[37] As the FTO risk genotype is associated with obesity,[4–6] we expect it also to be associated with an increase in REE. On the other hand, disturbed adipocyte browning may play a role in the increased obesity risk of rs1421085 risk allele carriers via a decrease in energy combustion.[8] As these opposing associations may conceal the true association, we also studied effect modification by BMI by examining the associations of rs1421085 with measures of REE, RQ, FatOx and CarbOx stratified by BMI tertile.

All analyses were performed using STATA Statistical Software (Statacorp, College Station, Texas, USA), version 12.0.

Results

Population characteristics

The minor allele frequency of rs1421085 was 0.39, 47.6% of the population was heterozygous and 15.7% was homozygous for the rs1421085-C allele. Descriptive characteristics of the study population are shown in Table 9.1. Characteristics were similar for the three genotypes. Demographic and clinical characteristics of the random subsample in which indirect calorimetry was performed (n=1,246) were comparable to characteristics of the total study population (results not shown). In the random subsample in which indirect calorimetry was performed, mean (SD) REE was 1,476 (270) kcal/day. Mean FatOx was 59.8 (24.5) mg/min and mean CarbOx was 123.4 (55.4) mg/min. Mean RQ was 0.84 (0.05). RQ ranged from 0.69 to 1.11, with 8 participants having a RQ above 1.

Associations of rs1421085 genotype with measures of body fat

In the total study population (Figure 9.1 and Supplementary table 9.A.1), BMI was higher (+0.56 kg/m^2; 95% CI 0.15, 0.98) in participants that carried the rs1421085-CC genotype than in rs1421085-TT carriers. In addition, waist circumference (+1.25 cm; 95% CI 0.02, 2.49), hip circumference (+1.01 cm; 95% CI 0.10, 1.92), fat mass (+1.21 kg; 95% CI 0.28, 2.14), and FFM (+0.59 kg; 95% CI 0.05, 1.13) were higher in rs1421085-CC genotype carriers than in rs1421085-TT carriers. Associations were similar when we restricted to the subpopulation of participants in whom indirect calorimetry was performed (Supplementary table 9.A.2).

Table 9.1: Demographic and clinical characteristics of the genotyped Netherlands Epidemiology of Obesity study population (aged 45-65 years), stratified by rs1421085 genotype.

Characteristic	rs1421085 genotype			Total
	TT	CT	CC	
Proportion of participants (%)	36.7	47.6	15.7	100
Demographic				
Sex (% women)	55	57	56	56
Age (year)	56 (6)	56 (6)	56 (6)	56 (6)
Body mass index (kg/m^2)	26.1 (4.1)	26.3 (4.4)	26.7 (5.0)	26.3 (4.4)
Total body fat (%)	31.4 (8.5)	31.7 (8.6)	32.2 (9.1)	31.6 (8.7)
Educational level (% high)[a]	46	49	45	47
Tobacco smoking (% current and former smokers)	60	62	66	62
Quantity of smoking (pack years)	9.7 (16.5)	9.4 (14.2)	9.9 (14.5)	9.6 (15.2)
Physical activity during leisure time (MET hours/week)	37 (30)	38 (33)	36 (33)	38 (32)
Alcohol intake (g/day)	15 (16)	15 (16)	14 (15)	15 (16)
Comorbidity and medication				
Diabetes (%)	5	5	7	5
Cardiovascular disease (%)	5	6	8	6
Lipid lowering drug use (%)	11	10	12	11

Results are based on analyses weighted towards the normal BMI distribution and presented as percentage or mean (SD), with n=5,744[b].

[a] High educational level: higher secondary education (according to Dutch educational system), higher vocational education, university, PhD.

[b] Missing data: n=56 for educational level, n=5 for smoking status, n=104 for physical activity, n=3 for alcohol intake, n=30 for total body fat, n=32 for diabetes, n=22 for cardiovascular disease.

MET, metabolic equivalents of task.

Associations of rs1421085 genotype with measures of REE, RQ, fat oxidation and carbohydrate oxidation

In figure 9.2 the differences in measures of REE, RQ, FatOx and CarbOx are shown per additional rs1421085-C allele. In our study population, rs1421085 genotype was not associated with REE, REE/FFM, REE adjusted for FFM, RQ, FatOx and CarbOx (Supplementary table 9.A.3). The difference between CC and TT (reference) carriers was for REE +0.9 kcal/day (95% CI -38.6, 40.4), for REE adjusted for FFM -4.6 kcal/day (95% CI -33.4, 24.3), for REE/FFM -0.05 kcal/day/kg FFM (95% CI -0.58, 0.48), for RQ -0.007 (95% CI -0.018, 0.005), for FatOx +2.49 mg/min (95% CI -2.41, 7.38), and for CarbOx -6.12 mg/min (95% CI -17.43, 5.19). Also, there was no effect modification by BMI, as associations were not different between BMI tertiles (Supplementary table 9.A.4). The BMI range per tertile was 18.21-28.14 kg/m^2 for tertile 1 (n=416); 28.15-31.16 kg/m^2 for tertile 2 (n=415); and 31.17-55.93 kg/m^2 for tertile 3 (n=415). Within each tertile the rs1421085 genotype was not associated with REE, REE adjusted for FFM, REE/FFM, RQ, FatOx and CarbOx.

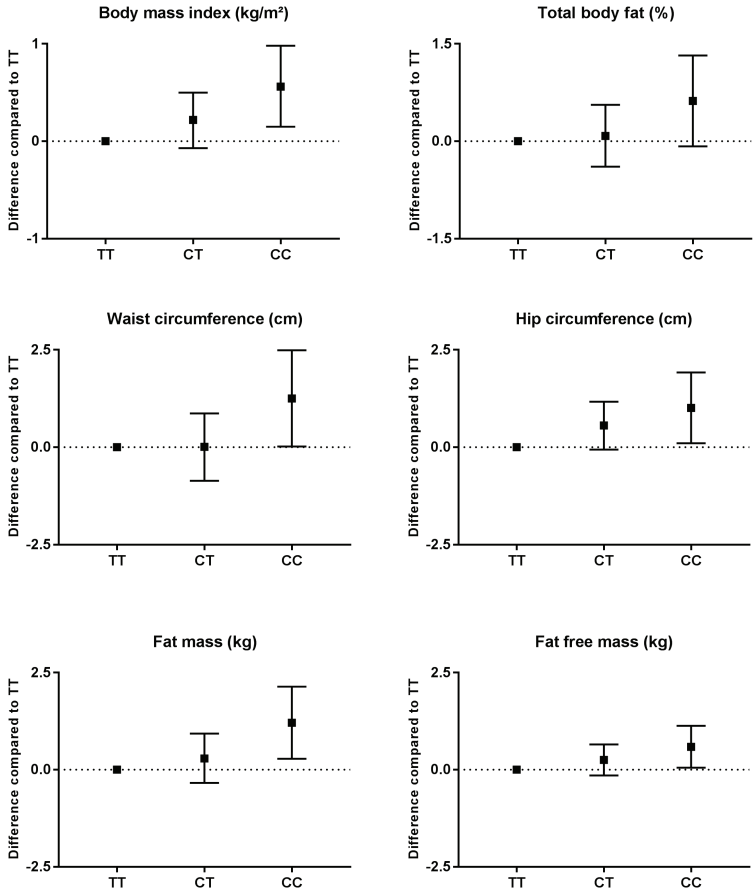


Figure 9.1: Difference in measures of body fat per additional rs1421085-C allele, with the TT genotype as reference. Results are based on analyses weighted towards the normal BMI distribution, with n=5,744[a]. Bars represent beta coefficients from linear regression calculated with respect to the adjusted mean of the reference group (TT genotype carriers; reference line y=0). Models were adjusted for age, sex and population structure. Error bars represent 95% confidence intervals.

[a] Missing data: n=30 for total body fat, n=5 for waist circumference, n=5 for hip circumference, n=30 for fat mass, n=30 for fat free mass.

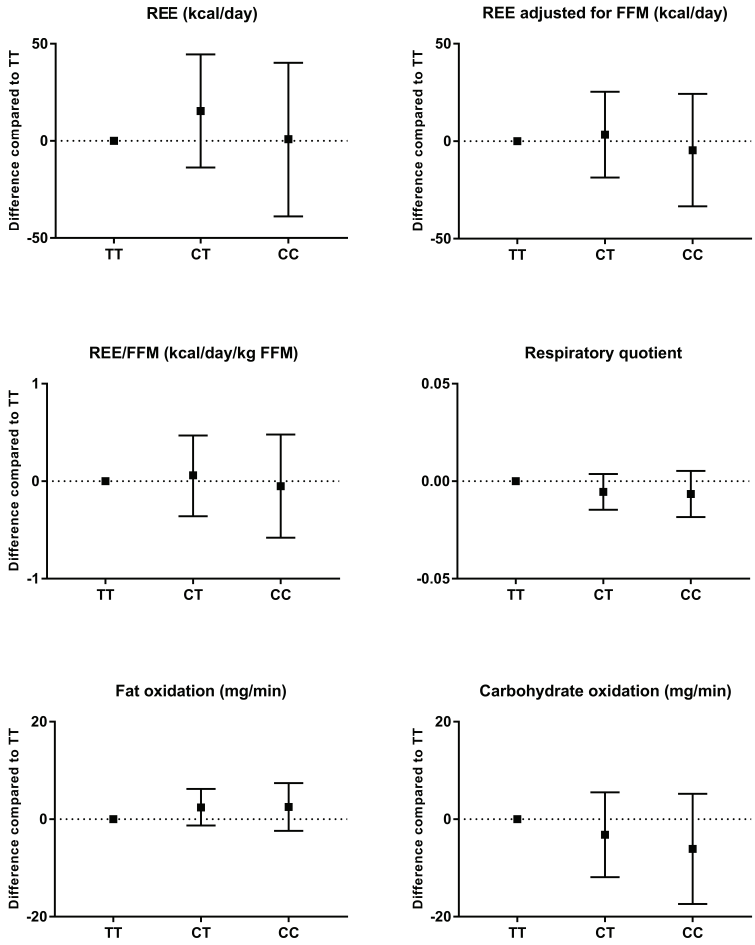


Figure 9.2: Difference in measures of REE, respiratory quotient, fat oxidation and carbohydrate oxidation per additional rs1421085-C allele, with the TT genotype as reference. Results are based on analyses weighted towards the normal BMI distribution, with n=1,246[a]. Bars represent beta coefficients from linear regression calculated with respect to the adjusted mean of the reference group (TT genotype carriers; reference line y=0). Models were adjusted for age, sex and population structure. Error bars represent 95% confidence intervals.

[a] Missing data: n=8 fat free mass.
FFM, fat free mass; REE, resting energy expenditure.

Discussion

Increased expression of IRX3 and IRX5 following rs1421085-T to -C substitution in the FTO gene was recently shown to skew human adipocyte browning *in vitro*, leading to reduced mitochondrial combustion of lipids for thermogenesis and increased intracellular lipid storage.[8] As decreased oxidation of lipids in rs1421085-C allele carriers may be an underlying mechanism explaining the association between FTO and obesity, we aimed to study the association between rs1421085 genotype and whole-body lipid oxidation. In the present study, we did not find evidence for an association between the rs1421085 polymorphism and whole-body FatOx, in a large population-based cohort of 1,246 middle-aged individuals.

In the NEO study population, we replicated the association between rs1421085 genotype and BMI that was previously shown in GWAS.[5] The effect sizes (+0.22 kg/m^2 for CT and +0.56 kg/m^2 for rs1421085-CC, relative to the rs1421085-TT genotype) were comparable with those determined in a large GWAS on BMI, in which BMI increased with 0.39 kg/m^2 per additional copy of the rs1558902 risk allele that is in perfect linkage disequilibrium with rs1421085.[6] For BMI and other markers of adiposity (i.e. total body fat, waist and hip circumference, fat mass and FFM) we observed an additive (i.e. per allele) effect of the rs1421085-C allele. These results imply that associations of genetic variation in the FTO gene with measures of REE and substrate oxidation rates can be validly studied in the NEO study population.

In the present study, there was no evidence for an association of rs1421085 with whole-body crude REE and FFM-adjusted REE. This is in line with several previous studies.[15–17,19,38] It has been shown before that obesity risk is associated with decreased rates of fat oxidation.[9–11] In contrast to our hypothesis, there was also no evidence for an association between rs1421085-C and FatOx. Claussnitzer and colleagues[8] showed a 5-fold reduction in mitochondrial thermogenesis in perirenal adipose tissue of rs1421085 risk allele carriers *in vitro*. Also, they showed a 4-fold increase in cellular metabolic rate after altering the causal nucleotide from rs1421085-C to -T with targeted genome editing technology (CRISPR-Cas9) in pre-adipocytes of risk allele carriers. Despite these considerable effects on a cellular level, the contribution of browning of white adipocytes to total energy metabolism is apparently too small to affect resting whole-body energy expenditure and fat oxidation, as implicated by the present study. Although our results indicate that genetic variation in FTO does not contribute to whole-body fat oxidation, there is evidence that other SNPs outside the FTO gene do affect fat oxidation, indicating that there is a genetic basis for whole-body fat oxidation in humans.[39]

Although we performed indirect calorimetry measurements in a very large population of 1,246 participants, based on power calculations it is possible that the effect size of rs1421085-C is too small to be detected in our study. In theory, small effect sizes could be of clinical relevance. An additive effect of small changes in FatOx over time could eventually contribute to a significant increase in adiposity, although no studies have yet explored this hypothesis. However, several factors argue against the existence and relevance of such a long-term additive effect. In the first place, the direction of the effect size of FatOx in CT and CC carriers was positive, which is opposite to what we hypothesized. The same holds for the effect sizes of the RQ, which was negative. Secondly, in the present study there was no evidence for an allele-dosage effect between rs1421085-C and measures of REE and FatOx, supporting the finding that there is no association of rs1421085 genotype with these measures. Thirdly, as a rule of thumb, a cumulative energy deficit of 3500 kcal is required to lose a pound (0.45 kg) of body weight.[40,41] By way of illustration, with an effect size of -4.6 kcal/day for the REE adjusted FFM in rs1421085-CC carriers, it would take over 760 days (2 years) to have an increase in body weight of 0.45 kg compared to rs1421085-TT carriers. The biological and clinical relevance of these effects should be further explored and GWAS meta-analyses on REE and substrate oxidation rates are eagerly awaited to further elucidate the genetic regulation of resting energy metabolism.

Our results do not provide further insight in the mechanistic basis for the association between FTO and obesity. However, they do indicate that FTO-associated obesity is induced via other mechanisms than reduced resting energy expenditure and whole-body fat oxidation in risk allele carriers. There is growing evidence that higher food intake via increased hunger and decreased satiety underlies the association between FTO and obesity.[38,42] The highest expression of FTO is in the brain4, and it was recently shown that the central nervous system plays a key role in genetic obesity susceptibility.[43] However, it is far from understood by which mechanisms the FTO gene regulates appetite and these pathways should be further explored.

The main strength of the present study is the large size of the genotyped population in which REE and substrate oxidation rates were measured with indirect calorimetry (n=1,246). Indirect calorimetry is a highly extensive and time-consuming measurement and was therefore previously performed in much smaller study populations. The RQ range resided in the physiological range of 0.67 to 1.2, which indicates a reliable measurement without technical errors.[44] However, it should be realized that the study population is from European-ancestry. The minor allele frequency of rs1421085 is high in populations from European ancestry compared with other ethnical groups.[45] Our results may therefore not be generalizable to populations of other ancestries.

In conclusion, in this large population-based study there was no evidence for an association between the FTO rs1421085 variant and whole-body FatOx measured with indirect calorimetry. Our findings imply that rs1421085-induced obesity is not attributable to decreased FatOx in rs1421085 risk allele carriers. Thus, the strong association between genetic variation in this FTO variant and obesity risk is probably induced via other pathways than via reduced whole-body FatOx. Further research is needed to elucidate the underlying mechanism of the strong association between the FTO region and obesity, in order to advance the search for new targets of anti-obesity intervention strategies.

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Appendix

9.A. Supplementary tables

Table 9.A.1: Association of rs1421085 genotype with measures of body fat.

Characteristic	rs1421085 genotype		
	TT	CT	CC
	Reference		
	Adjusted mean (95% CI)	Difference in measure of body fat compared with reference[a] (95% CI)	
Body mass index (kg/m^2)	26.2 (26.0, 26.4)	0.22 (-0.07, 0.50)	0.56 (0.15, 0.98)
Total body fat (%)	31.3 (31.0, 31.7)	0.08 (-0.39, 0.56)	0.62 (-0.08, 1.32)
Waist circumference (cm)	92.6 (92.0, 93.1)	0.01 (-0.86, 0.87)	1.25 (0.02, 2.49)
Hip circumference (cm)	103.6 (103.2, 104.0)	0.56 (-0.06, 1.17)	1.01 (0.10, 1.92)
Waist-to-hip ratio	0.89 (0.89, 0.90)	-0.005 (-0.011, 0.000)	0.003 (-0.005, 0.010)
Fat mass (kg)	25.2 (24.8, 25.6)	0.29 (-0.34, 0.93)	1.21 (0.28, 2.14)
Fat free mass (kg)	54.6 (54.3, 54.9)	0.25 (-0.15, 0.65)	0.59 (0.05, 1.13)

Results are based on analyses weighted towards the normal BMI distribution, with n=5,744[b]. Models were adjusted for age, sex and population structure.

[a] Beta coefficients from linear regression calculated with respect to the adjusted mean of the reference group (TT genotype carriers).

[b] Missing data: n=30 for total body fat, n=5 for waist circumference, n=5 for hip circumference, n=5 for waist-to-hip ratio, n=30 for fat mass, n=30 for fat free mass.

Table 9.A.2: Association of rs1421085 genotype with measures of body fat, in the random subsample of participants in which indirect calorimetry was performed.

Characteristic	rs1421085 genotype		
	TT	CT	CC
	Reference		
	Adjusted mean (95% CI)	Difference in measure of body fat compared with reference[a] (95% CI)	
Body mass index (kg/m^2)	26.5 (26.1, 27.0)	0.01 (-0.64, 0.66)	0.58 (-0.24, 1.40)
Total body fat (%)	31.3 (30.6, 32.0)	0.16 (-0.92, 1.25)	1.21 (-0.11, 2.54)
Waist circumference (cm)	93.0 (91.6, 94,3)	-0.51 (-2.44, 1.43)	0.97 (-1.64, 3.59)
Hip circumference (cm)	104.2 (103.3, 105.1)	-0.06 (-1.40, 1.27)	0.37 (-1.46, 2.21)
Waist-to-hip ratio	0.89 (0.88, 0.90)	-0.005 (-0.017, 0.007)	0.006 (-0.009, 0.021)
Fat mass (kg)	25.5 (24.5, 26.5)	0.22 (-1.24, 1.67)	1.44 (-0.49, 3.36)
Fat free mass (kg)	54.9 (54.4, 55.5)	0.49 (-0.28, 1.26)	0.29 (-0.90. 1.48)

Results are based on analyses weighted towards the normal BMI distribution, with n=1,246[b]. Models were adjusted for age, sex and population structure.

[a] Beta coefficients from linear regression calculated with respect to the adjusted mean of the reference group (TT genotype carriers).

[b] Missing data: n=8 for total body fat, n=1 for waist circumference, n=1 for hip circumference, n=1 for waist-to-hip ratio, n=8 for fat mass, n=8 for fat free mass.

Table 9.A.3: Association of rs1421085 genotype with measures of body fat, in the random subsample of participants in which indirect calorimetry was performed.

Characteristic	rs1421085 genotype		
	TT	CT	CC
	Reference		
	Adjusted mean (95% CI)	Difference in measure of body fat compared with reference[a](95% CI)	
REE (kcal/day)	1,494 (1,473, 1,516)	15.5 (-13.5, 44.6)	0.9 (-38.6, 40.4)
REE adjusted for FFM (kcal/day)	1,557 (1,541, 1,574)	3.4 (-18.6, 25.4)	-4.6 (-33.4, 24.3)
REE/FFM (kcal/day/kg FFM)	27.5 (27.2, 27.8)	0.06 (-0.36, 0.47)	-0.05 (-0.58, 0.48)
Respiratory quotient	0.84 (0.83, 0.84)	-0.005 (-0.015, 0.004)	-0.007 (-0.018, 0.005)
Fat oxidation (mg/min)	60.0 (57.4, 62.7)	2.41 (-1.33, 6.15)	2.49 (-2.41, 7.38)
Carbohydrate oxidation (mg/min)	126.2 (120.0, 132.5)	-3.21 (-11.90, 5.48)	-6.12 (-17.43, 5.19)

Results are based on analyses weighted towards the normal BMI distribution, with n=1,246[b]. Models were adjusted for age, sex and population structure.

Models were adjusted for age, sex and population structure.

[a] Beta coefficients from linear regression calculated with respect to the adjusted mean of the reference group (TT genotype carriers).

[b] Missing data: n=8 fat free mass.

FFM, fat free mass; REE, resting energy expenditure.

Table 9.A.4: Association of rs1421085 genotype with measures of REE, RQ, fat oxidation and carbohydrate oxidation, stratified by tertiles of BMI.

Characteristic	BMI tertile[a]	rs1421085 genotype		
		TT	CT	CC
		Reference		
		Adjusted mean (95% CI)	Difference in measure of REE compared to reference[b](95% CI)	
REE (kcal/day)	1	1,433 (1,409, 1,457)	12.7 (-19.8, 45.2)	-4.8 (-48.2, 38.6)
	2	1,569 (1,536, 1,601)	23.7 (-17.9, 65.3)	-3.9 (-62.4, 54.6)
	3	1,771 (1,730, 1,812)	-1.8 (-57.7, 54.0)	-15.3 (-85.9, 55.2)
REE adjusted for FFM (kcal/day)	1	1,517 (1,491, 1,543)	2.5 (-25.2, 30.3)	-6.9 (-42.0, 28.2)
	2	1,574 (1,544, 1,604)	5.5 (-33.0, 44.0)	-3.2 (-57.2, 50.8)
	3	1,667 (1,631, 1,703)	7.4 (-39.4, 54.2)	-18.8 (-79.3, 41.7)
REE/FFM (kcal/day/kg FFM)	1	27.3 (26.9, 27.6)	-0.01 (-0.55, 0.54)	-0.07 (-0.75, 0.60)
	2	27.8 (27.2, 28.5)	-0.07 (-0.85, 0.70)	-0.06 (-1.09, 0.98)
	3	28.7 (28.1, 29.2)	0.17 (-0.61, 0.94)	-0.30 (-1.29, 0.70)
Respiratory quotient	1	0.84 (0.83, 0.85)	-0.006 (-0.019, 0.006)	-0.009 (-0.024, 0.007)
	2	0.84 (0.83, 0.85)	-0.003 (-0.016, 0.011)	-0.006 (-0.024, 0.013)
	3	0.83 (0.82, 0.84)	-0.006 (-0.020, 0.008)	0.002 (-0.015, 0.019)
Fat oxidation (mg/min)	1	57.4 (53.8, 60.9)	2.75 (-2.12, 7.63)	2.93 (-3.46, 9.32)
	2	61.5 (57.4, 65.7)	0.89 (-4.89, 6.67)	2.07 (-6.32, 10.45)
	3	73.4 (68.2, 78.7)	2.31 (-4.89, 9.52)	-0.87 (-9.62, 7.87)
Carbohydrate oxidation (mg/min)	1	121.6 (113.4, 129.8)	-4.60 (15.91, 6.70)	-8.31 (-23.05, 6.43)
	2	136.2 (127.6, 144.9)	2.16 (-10.70, 15.03)	-5.96 (-23.67, 11.76)
	3	143.9 (132.6, 155.2)	-6.19 (-21.72, 9.33)	-0.64 (-19.23, 17.94)

Results are based on analyses weighted towards the normal BMI distribution, with n=1,246[c].Models were adjusted for age, sex and population structure.

[a] BMI range: tertile 1 (n=416) 18.21-28.14 kg/m^2; tertile 2 (n=415) 28.15-31.16 kg/m^2, tertile 3 (n=415) 31.17-55.93 kg/m^2.

[b] Beta coefficients from linear regression calculated with respect to the adjusted mean of the reference group (TT genotype carriers).

[c] Missing data: n=8 fat free mass.

BMI, body mass index; FFM, fat free mass; REE, resting energy expenditure.

10

General discussion and future perspectives

The aim of this thesis was to further disentangle the interwoven physiological, environmental and genetic factors that determine cholesterol and energy metabolism to increase our understanding of their contribution to cardiometabolic disease, thereby facilitating the search for preventive and curative strategies. In the first part of this thesis, we focussed on the biological function of cholesteryl ester transfer protein (CETP) and on the role of this protein in cholesterol metabolism and cardiovascular disease (CVD). In the second part, we studied environmental and genetic factors that may influence cardiometabolic health outcomes via their effects on energy metabolism. Here, we discuss and interpret the results from the previous chapters to further understand the human biology of cholesterol and energy metabolism, and we describe the implications of our findings for future preventive and curative strategies to reduce the burden of cardiometabolic disease.

To what extent can our findings be causally interpreted

Before we discuss the study results described in this thesis and their implications for cardiometabolic health, it is important to realize to what extend our findings can be interpreted as causal. In general, one of the important limitations of observational studies is the fact that conclusions on causality can never be drawn, because we cannot exclude the presence of reverse causality or residual confounding by unknown, unmeasured or not accurately measured confounding factors. Residual confounding can be minimized by extensive characterizing of the study population, which makes it possible to adjust for most known confounding factors (**Chapter 2 and 8**). In the case of a less well-phenotyped population (**Chapter 3**), more caution should be taken when interpreting the results. An ecological study (**Chapter 7**), which is an observational study using population-based data instead of individual data, is even more prone to confounding. Nevertheless, observational studies are crucial for the insight in the etiological aspects of risk factors and disease outcomes, and may provide indications for underlying disease mechanisms that can then be further elucidated in experimental and intervention studies. In contrast to observational study designs, Mendelian randomization (**Chapter 4, 6 and 9**) is able to assess causality based on the second law of Mendel, which states that the segregation of alleles during gamete formation is random.[1,2] With Mendelian randomization, genetic variation is used to define an exposed and a control group of a certain phenotypic trait within a population. Based on the random distribution of alleles in a population, which is not influenced by any environmental factor, it can be assumed that there is no confounding and reverse causation in the association of single nucleotide polymorphisms (SNPs) and disease or trait outcomes. Therefore, conclusions on causality can directly be drawn when comparing the exposed and unexposed groups on the disease or trait outcome.

Cholesteryl ester transfer protein

The potential of CETP inhibition for cardiovascular disease risk reduction

CETP was identified in human plasma back in 1978, as a protein that catalyses the exchange of cholesteryl esters between lipoproteins.[3,4] As a logical consequence, the focus was thereafter primarily on its function in lipoprotein metabolism. With the discovery of a point-mutation in the CETP gene, leading to CETP deficiency, which markedly increased the number and size of high-density lipoprotein (HDL) particles,[5] the relevance of CETP in circulating lipid metabolism was further substantiated. At the same time, observational studies convincingly showed an inverse association between HDL-cholesterol (C) concentration and CVD risk.[6] Although we are nowadays aware that the concentration of HDL-C is not causally involved in CVD risk based on Mendelian randomization studies,[7–10] in the beginning of the 21th century it was reasoned that pharmacological inhibition of CETP could be a novel therapeutic strategy to prevent the development of CVD by raising HDL-C concentration. The HDL-C increasing properties of CETP inhibition were regarded to reduce the residual CVD risk that remained after low-density lipoprotein (LDL)-C lowering by statin treatment.[11] However, three initial trials with the CETP inhibitors torcetrapib[12], dalcetrapib[13] and evacetrapib[14] were all terminated, as they did not show positive effects on CVD risk reduction, while torcetrapib even increased mortality due to off-target side effects.[13,15] The failure of these clinical trials provided space for the reconsideration of the position of CETP in CVD risk management.

To determine the causal effects of CETP on CVD and the circulating lipoprotein profile, we first performed a genome-wide association study (GWAS) to identify the genetic determinants of CETP concentration (**Chapter 4**). We identified three independent single nucleotide polymorphisms (SNPs) mapped to the CETP gene that account for 16.4% of the total variation in CETP concentration. With the identification of genetic variants that are associated with phenotypic traits, in this case circulating CETP, true causal associations with disease outcomes can be assessed, according to the concept of Mendelian randomization.[1,2] We used the three identified CETP SNPs as genetic instruments in a Mendelian randomization study to study the causal effects of an increase in CETP concentration on lipoprotein composition and coronary artery disease risk, as described in **Chapter 6** and **Chapter 4**. We showed that an increase in circulating CETP causally decreases the concentration of mainly large HDL particles, and increases the classes of small very-low-density lipoprotein (VLDL) particles without increasing the classes and composition of LDL particles. The absence of a causal association between CETP concentration and LDL concentration or composition is highly remarkable since the current dogma is that CETP increases LDL-C. However, it should be realized that LDL-C concentration is generally not

measured directly but calculated from the Friedewald formula.[16] Estimation of LDL-C by this Friedewald formula may well misclassify cholesterol contained within small VLDL subclasses, which we now found to be causally increased by CETP (**Chapter 6**), as LDL-C. Interestingly, it was previously reported that small and very small VLDL particle concentrations (reflecting triglyceride-rich lipoprotein remnants) and cholesterol in VLDL (reflecting remnant cholesterol) are among the lipoprotein components that most strongly associate with an increased CVD risk.[17–19] Thus, the effects of CETP on small VLDL particles may explain the increased CVD risk associated with higher CETP concentrations, as they likely reflect atherogenic VLDL remnants.

Besides a causal association between increased CETP concentration and lower circulating large HDL and higher circulating small VLDL, a 1 µg/mL increased CETP concentration also associated with coronary artery disease in the CARDIoGRAMplusC4D consortium with an odds of 1.08 (95% CI 0.94, 1.23) (**Chapter 4**). However, the three initial CETP inhibitors did not reduce the risk of cardiovascular events.[12–14] It is important to note that all these CETP inhibitors were tested in patients who already received statin treatment to lower LDL-C concentration. A recent Mendelian randomization study showed that genetically-modelled CETP inhibition on top of genetically-modelled statin treatment, was associated with a discordant reduction in LDL-C and apolipoprotein B (ApoB) concentrations. In other words, the reduction in ApoB concentration, which reflects the number of VLDL and LDL particles, was less compared to the reduction in LDL-C concentration.[20] On the other hand, in the case that genetically-modelled statin treatment was not taken into account, genetically-modelled CETP inhibition associated with a concordant reduction in ApoB and LDL-C concentration, as well as a significant reduction in cardiovascular events.[20] Indeed, the CETP inhibitor evacetrapib, which did not reduce CVD risk, caused a reduction in LDL-C concentration without a concordant decrease in ApoB,[21,22] which indicates that evacetrapib causes LDL particle remodelling rather than removal of LDL from the circulation. Therefore, the success of CETP inhibitors may be dependent on their capability to reduce the absolute number of VLDL, intermediate-density lipoprotein (IDL) and LDL (i.e. non-HDL) particles, which would be reflected by a reduction of ApoB concentration in the blood, rather than by a reduction of the LDL-cholesterol concentration.[20] Interestingly, the most recent CETP inhibitor trial (REVEAL) with anacetrapib on top of statin treatment showed a concordant reduction in non-HDL and ApoB concentration of -18%, which was accompanied by a reduced rate ratio for major coronary events of 0.91 (95% CI 0.85, 0.97).[23] Our results described in **Chapter 6** imply that specifically small VLDL particles underlie the association between CETP and CVD risk, which suggests that anacetrapib reduces CVD risk by causing a decrease in these alleged atherogenic remnants.

Future perspectives of CETP inhibition

Despite the positive outcome of the REVEAL trial, the pharmaceutical company that developed anacetrapib (Merck Sharp and Dohme (MSD), Kenilworth, United States of America) announced a few weeks after the publication of the trial results that they will stop the anacetrapib development program and not submit applications for regulatory approval.[24] Indeed, the 10% relative reduction in major coronary events in patients with pre-existing CVD, as observed with anacetrapib treatment, seems to be of minor clinical relevance. In comparison, with statin treatment that is relatively cheap, a risk reduction of 20 is achieved per 1 mmol/L decrease in LDL-C, and more intensive statin regimens can even further reduce the incidence of cardiovascular events.[25] Besides, more promising drugs are in development. In the beginning of 2017, the FOURIER trial with the proprotein convertase subtilisin–kexin type 9 (PCSK9) inhibitor evolocumab was completed, showing an additional 59% reduction in LDL-C on a background of statin therapy, and a large additional reduced risk of cardiovascular events (hazard ratio 0.85; 95% CI 0.79, 0.92).[26] However, the long journey of the development of CETP inhibitors has not ended as yet. There are indications that specific patient populations may benefit from CETP inhibition therapy. Recent studies showed that the CETP inhibitor dalcetrapib reduces CVD risk dependent on the rs1967309 polymorphism in the ADCY9 gene.[27,28] Patients carrying the AA genotype had a 39% relative reduction in cardiovascular events when treated with dalcetrapib compared with placebo, whereas an increased risk of 27% was observed in patients with the GG genotype. Currently, the dal-GenE randomized phase III clinical trial (NCT number: NCT02525939) is being performed to confirm whether the CETP inhibitor dalcetrapib is able to reduce CVD risk in patients with the AA genotype, who are hospitalized for acute coronary syndrome. In addition, CETP monotherapy may be a promising strategy to reduce CVD risk in the subgroup of patients that do not tolerate or respond to statins, however, this hypothesis has not been substantiated yet. Taken together, although the future of CETP inhibition may not lie within generalized large scale treatment of patients at risk for CVD, selected patient populations may possibly benefit from CETP inhibitory treatment.[29]

The biological function of CETP: a new hypothesis

To fully understand the physiology of CETP, it is important to consider the ultimate purpose of this protein. Previously, it has been proposed that CETP is involved in indirect reverse cholesterol transport by enriching ApoB-containing triglyceride-rich lipoproteins, mainly VLDL particles, with cholesteryl esters that are then taken up by the liver via the LDL receptor. This lipid clearance pathway is in potential antiatherogenic.[30,31] On the other hand, CETP activity leads to triglyceride-enrichment of triglyceride-poor ApoB-containing lipoproteins, which may lead to the formation of proatherogenic small lipoprotein rem-

nants.[30–33] As the balance between the anti- and proatherogenic effects of CETP on ApoB-containing lipoproteins is precarious, we regard it unlikely that the ultimate purpose of CETP is related to modulation of these particles. Interestingly, we show in **Chapter 6** by performing a Mendelian randomization analysis that CETP most of all determines the concentration and composition of HDL particles, and to a far lesser extent affects ApoB-containing lipoproteins. This is in line with results from clinical trials with CETP inhibitors, which showed considerably large effects on HDL-C concentration, and no or relatively small effects on LDL-C concentration.[12–14,34] Therefore, we propose that the HDL-decreasing capacity of CETP should be considered its primary function, which brings us back to the basics of CETP biology: the source of CETP production.

The cellular origin of CETP was unclear for a long period of time. With adipose tissue and the liver being the most abundant sources of CETP mRNA expression, both were postulated to contribute substantially to the circulating CETP concentration.[35–37] In **Chapter 2**, we describe that measures of body fat were not relevantly associated with serum CETP concentration, which strongly indicates that adipose tissue does not substantially contribute to the circulating CETP pool. Indeed, our research group recently showed that CETP is predominantly derived from the liver.[38] An interesting finding was that, contrary to the current dogma at that time and information that is still provided by the Human Protein Atlas (https://www.proteinatlas.org/), CETP appeared not to be produced by hepatocytes but specifically by resident hepatic macrophages, referred to as Kupffer cells.[38] Kupffer cells comprise the largest set of resident tissue macrophages in the body, and play a pivotal role in host defence and inflammation.[39,40] In light of the identification of innate inflammatory cells as main determinants of CETP, findings from early (pre)clinical studies can now be placed in better perspective. Results from early studies in CETP expressing animals showed that inflammatory cytokines and the bacterial endotoxin lipopolysaccharide (LPS) reduce hepatic CETP expression and circulating CETP concentrations.[41–43] In addition, we show in **Chapter 3** in obese individuals from a bariatric surgery cohort that histologically-determined liver inflammation tended to be associated with a smaller number of CETP positive cells in the liver as well as lower circulating CETP concentrations, suggesting that CETP is also down-regulated by inflammatory stimuli in humans. Taken together, these data shed a different light on the biological function of CETP.

Combining the findings that 1) LPS reduces hepatic CETP expression and circulating CETP concentrations,[41–44] liver inflammation seems to be associated with less hepatic and circulating CETP (**Chapter 3**), 3) less circulating CETP is causally associated with considerable higher concentrations of HDL constituents (**Chapter 6**), and 4) HDL has recently been reported to activate cytokine responses in macrophages, thereby enhancing their ca-

pacity to clear infections,[45] we postulate that inflammatory stimuli activate resting Kupffer cells to decrease CETP production and as a consequence raise HDL to combat invading microorganisms. In this context, it is highly interesting that in humans hospitalized for sepsis the CETP concentration was decreased in proportion to the severity of sepsis, and that lower HDL-C concentrations were associated with an increased risk of developing severe sepsis.[46,47] Biologically, decreasing hepatic CETP expression in sepsis may thus be a protective response of the host by increasing HDL. In fact, severe sepsis dramatically decreased CETP concentration (-25%, i.e. from 1.52 to 1.14 µg/mL),[47] while we observed only a trend towards a negative association between metabolically-induced liver inflammation and CETP-related outcomes, as described in **Chapter 3**. This suggests that metabolically-induced and infection-related inflammation may have different effects on the expression and production of CETP by Kupffer cells. It is tempting to speculate that acute and/or whole-body inflammatory responses to invading pathogens are required to induce a robust reduction in CETP production by Kupffer cells, and a consequent increase in HDL.

The role of HDL in host defence has been elaborately studied.[48] HDL has the capacity to bind LPS[49–52] and to promote the release of LPS from monocyte cell surfaces,[53] thereby preventing endotoxemia. However, we hypothesize that a constant high concentration of HDL may be energetically unfavourable and, presumably, CETP is therefore abundantly present when overall infection pressure is low in order to keep the amount of HDL particles at a low level. Interestingly, CETP expression in human macrophages is not only decreased by LPS from Gram-negative bacteria, but is also suppressed by interferon γ (IFN-γ),[42] which has important antiviral functions.[54] Also, in vitro exposure of murine macrophages to tumor necrosis factor α (TNF-α) reduced CETP expression,[42] and in hamsters TNF-α and interleukin-1β (IL-1β) decreased CETP expression and CETP concentration.[41] Thus, the purpose of the HDL-increasing capacity of CETP is possibly broader than the immune defence against Gram-negative bacterial infections, as elicited by LPS. In this perspective, it is interesting to note that the role of HDL in host defence extends far beyond LPS binding. This is illustrated by analyses of the HDL proteome, which may contain over ninety different proteins, the majority of which have functions unrelated to lipid metabolism, including host defence and recovery regulation.[55**?**] The HDL proteome is highly dynamic, and LPS administration changes the HDL proteome within hours.[56]

In light of our hypothesis that the main purpose of CETP is to regulate the concentration and possibly functionality of HDL particles in response to infection pressure on the body, it is biologically plausible that CETP is primarily produced by liver macrophages. The liver has a strategic location to function in the first line of host defence, as blood from the stomach and intestines directly drains into the liver via the portal vein.[57,58] Kupffer cells are the

first macrophage population in the body to encounter pathogens and endotoxins from the intestine, as they are located in the liver sinusoids and are therefore directly exposed to blood from the portal vein.[39,59] As such, Kupffer cells play a central role in host defence, being able to rapidly clear bacteria from the circulation and recruit additional immune cells to the liver to function in host defence.[39,59] The primary production of CETP by innate immune cells that function in the first line of defence against pathogens strengthens the hypothesis that CETP production is regulated by infection pressure. Further evidence for this hypothesis may be provided by the difference in functionality of the CETP gene between different mammalian species. The CETP gene seems to be conserved between several mammalian species,[60**?**] and functional CETP has been identified in plasma of monkeys, hamsters, and rabbits.[60–62] However, functional CETP is absent in mice, rats, pigs and dogs,[60,62,63] which are species that also have a higher HDL-to-total cholesterol ratio than mammals with functional CETP present in their circulation.[62] It may be well-possible that specifically mice, rats, pigs and dogs lack functional CETP, as they live in environments where infection pressure is in general high, for which they need high levels of HDL to combat invading microorganisms.

To summarize, we postulate that the concentration of circulating CETP is dependent on the infection pressure on the body. As infection pressure can be sensed by the liver as a crucial organ in the first line of host defence,[57] it is biologically plausible that CETP is primarily produced by resident liver macrophages. A high infection pressure may lead to downregulation of CETP, with the primary purpose to increase the number of HDL particles and potentially to improve their functionality. These HDL particles can then function in host defence and activate macrophages to combat invading microorganisms. An overview of our hypothesis on the role of CETP in host defence is depicted in Figure 10.1.

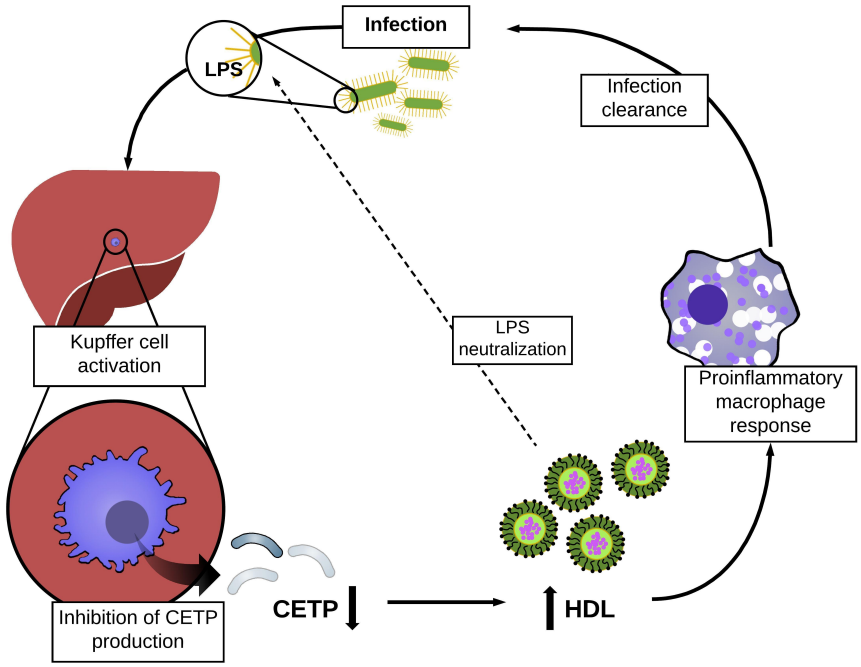


Figure 10.1: We hypothesize that the concentration of circulating CETP is dependent on the infection pressure on the body: a gram-negative bacterial infection leads to activation of Kupffer cells in the liver and a consequent decrease in CETP production by these cells, with the primary purpose to increase the number of HDL particles. These HDL particles then activate macrophages and bind LPS to ameliorate infection clearance. CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; LPS, polysaccharides.

Energy metabolism

Brown adipose tissue as a therapeutic target for cardiometabolic disease

In response to cold, brown adipose tissue (BAT) combusts energy stored in glucose and triglycerides into heat instead of adenosine triphosphate (ATP), by virtue of the unique presence of uncoupling protein 1 (UCP1) that uncouples the respiratory chain from ATP synthesis.[64,65] For neonates, activation of BAT is an important mechanism to keep their body temperature stable when exposed to cold, and therefore they have a large interscapular BAT depot. In 2009, the common notion that BAT disappears when age increases was invalidated by the discovery of functional BAT depots in adults.[66–68] In adults, BAT is located around the aorta and subclavian arteries, such that generated heat can be easily supplied to the circulation. Apart from the classical BAT depots, so called beige (or 'brite') adipocytes are scattered within white adipose tissue.[69] These beige cells have a low basal expression of UCP1, but upon prolonged cold stimulation UCP1 expression is markedly increased and their thermogenic capacity is induced.[69]

Simultaneously with the discovery of BAT in humans, it was also shown that a higher BAT activity, as determined by the uptake of the glucose tracer [18F]fluorodeoxyglucose ([18F]FDG), associates with a lower body mass index (BMI) and body fat mass.[66,67] From these data it was suggested that BAT contributes to energy metabolism and may prevent against excess adiposity. Indeed, it has been estimated that BAT contributes up to 5% of the basal metabolic rate,[70] and prolonged exposure to cold increases BAT activity accompanied by a reduction in fat mass.[71] Since then, BAT activation is regarded as a promising tool to combat cardiometabolic disorders, and extensive research into the beneficial effects of BAT activation on cardiometabolic health was initiated in preclinical settings. In mice, it was shown that BAT activation lowers plasma triglyceride as well as cholesterol concentrations.[72–74] Mechanistically, this was explained by an increased uptake of triglyceride-derived fatty acids by BAT from triglyceride-rich lipoproteins, including chylomicrons and VLDL, leading to the formation of cholesterol-enriched remnants that are taken up by LDL receptors of the liver.[65] As a result of lowering plasma cholesterol, treatment of hyperlipidemic APOE*3-Leiden.CETP mice, a well-established model of human-like lipoprotein metabolism, with a β3 adrenergic receptor agonist that potently activates BAT, reduces the development of atherosclerosis.[73]

Although BAT activation has thus become a potential therapeutic target in the prevention and treatment of obesity and CVD, it may be questioned if BAT can be activated to such an extent that its contribution to total energy expenditure is sufficient to have therapeutic potential. The most straightforward therapeutic option seems to be cold exposure, since this is the physiological activator of BAT. However, whereas cooler indoor temperatures may have some beneficial cardiometabolic effect by activating BAT, maximal BAT activation can only be reached within the range of uncomfortable environmental temperatures. It has been shown that people who can freely regulate indoor temperature prefer a range between 18°C and 20°C within winter time.[75] Although the exact temperature threshold for optimal BAT activation in humans is unknown, it is certainly reached far below comfortable room temperatures. In research settings, personal cooling protocols are used to maximally activate BAT just above the shivering temperature to study the uptake of [18F]FDG by BAT at a condition at which skeletal muscles do not take up the radiolabel.[76] In Caucasians shivering temperature is around 9°C, although this may vary somewhat between individuals.[77] There is no reason to assume that BAT activation does not further increase when cooling is continued, and presumably maximal BAT activation is reached at even lower temperatures. Besides being uncomfortable, it should also be realized that cold exposure increases sympathetic outflow not only to BAT, but also to other organs including white adipose tissue and the liver, of which the collective result is an increase in the hepatic

VLDL-triglyceride production.[78] As a consequence, short-term cold activation increases plasma triglycerides,[79] which may thus be a disadvantageous side effect of cold exposure. Thus, for several reasons physiological stimulation of BAT is no desired treatment option, and the development of pharmacological agents that potently activate BAT is warranted. Promisingly, the β3 adrenergic receptor agonist mirabegron has been shown to activate BAT and increase energy expenditure to a similar extent as cold exposure,[80] and β3 adrenergic agonists are now tested in clinical trials (NCT number: NCT03012113; NCT02354807; NCT02811289) for their effects on body weight and lipid metabolism.

Apart from fatty acids, BAT takes up glucose, which is the reason why BAT has been discovered as a metabolically active organ in human adults.[66–68] However, while BAT activation generates heat by direct β-oxidation of fatty acids, internalized glucose is not directly used for thermogenesis but rather for de novo lipogenesis and ATP synthesis.[65] Therapeutic implications for glucose utilization by BAT were provided by mouse studies, which showed improved glucose tolerance and insulin sensitivity upon cold exposure or BAT transplantation.[72,81] More recently, the translational value of these results was demonstrated in humans by exposing BAT positive (i.e. positive for cold-induced [18F]FDG uptake in the supraclavicular region) and BAT negative individuals to cold.[82] Whole-body glucose disposal and insulin sensitivity only increased in BAT positive individuals, which marked BAT as a potential anti-diabetes target. Lately, acclimatization of patients with type 2 diabetes to moderate cold for only ten days was shown to improve their insulin sensitivity.[83] In this study, a large enrichment of the glucose transporter type 4 (GLUT4) was observed in skeletal muscle cell membranes, whereas glucose uptake by BAT was only marginally increased.[83] This does not necessarily rule out BAT activation as the primary target of cold acclimatisation. It should be noted that glucose uptake by BAT is reduced in the context of insulin resistance due to long-term fasting or type 2 diabetes, while under these conditions the uptake of fatty acids by BAT is not impaired.[84,85] Therefore, it is conceivable that in type 2 diabetes patients, in whom [18F]FDG uptake by BAT is impaired, cold exposure led to an increased flux of fatty acids towards BAT, which results in a compensatory increased flux of glucose to other metabolically active tissues, such as muscle, thereby improving systemic insulin sensitivity.[86] Inspired by these findings, we reasoned that outdoor temperature, which has been associated with BAT activity,[87–89] may influence systemic insulin sensitivity, and we hypothesized that the global increase in temperature contributes to the current type 2 diabetes epidemic. In **Chapter 7** we indeed describe that the diabetes incidence rate in the United States of America (USA) and the prevalence of glucose intolerance worldwide increase with higher outdoor temperature. On the basis of our results, a 1°C rise in environmental temperature would account for over 100,000 new diabetes cases per year

in the USA alone, given a population of nearly 322 million people in 2015.[15] Although the ecological design of this study does not allow us to draw conclusions on causality, it is tempting to speculate that the mechanism underlying these findings is related to BAT: an increase in outdoor temperature may redirect the overall clearance of fatty acids from BAT towards other metabolically active tissues, which then rely less on glucose leading to systemic glucose intolerance. Regardless of the exact mechanism, our findings emphasize the importance of future research into the effects of environmental temperature on BAT functionality, glucose metabolism and the onset of diabetes, especially in view of the global rise in temperatures.

Future perspectives on the quantification of BAT activity

Until today, the golden standard to determine BAT activity is still positron emission tomography-computed tomography (PET-CT) using the glucose tracer [18F]FDG. Given that this technique is both costly and time consuming, large-scale human studies assessing BAT activity are not feasible. As discussed previously (section 2.1), another downside of this method is that [18F]FDG uptake insufficiently reflects BAT activity in case of insulin resistance that may arise from e.g. obesity, diabetes or old age.[86]. Interestingly, insulin resistance does not seem to reduce fatty acid uptake by BAT, since it has been shown that BAT fatty acid uptake upon cold exposure, as determined by the fatty acid tracer [18F]fluoro-thiaheptadecanoic acid ([18F]FTHA), does not differ between patients with type 2 diabetes and age-matched controls.[84] Therefore, alternative methods using [18F]FTHA for PET-CT are under development. Also, other PET-CT tracers have been evaluated for BAT quantification, including [11C]acetate to reflect oxidative capacity and [15O] to quantify blood flow and oxygen consumption.[86] However, with the use of different tracers the problem of the high costs for performing PET-CT scans is not solved, let alone the exposure to radioactive tracers. Combined magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS), which can quantify the fat content of BAT depots in the cervical region,[90] may be an alternative to overcome the problem of exposure to ionizing radiation, although costs remain high.[86] In this case, a reduced fat fraction in cervical BAT regions after cold exposure indicates cold-induced BAT activation, since combustion of fatty acids leads to the depletion of intracellular fat droplets.[91] The disadvantages of PET-CT scanning may also be overcome by the measurement of skin temperature, as it has been shown that cold-induced BAT activity measured with [18F]FDG PET-CT correlates with supraclavicular skin temperature.[92**?**] Determination of skin temperature may be a potential technique to measure BAT functionality in large populations at lower costs in the future. Another promising way to estimate BAT activity in large cohorts is by finding specific biomarkers. Our research group recently identified LysoPC-acyl C16:0 as the first

biomarker,[93] although relatively low sensitivity still precludes application on large scale.

In **Chapter 8** and **Chapter 9** we used whole-body fat oxidation, which was measured with indirect calorimetry in around 1,200 individuals, as rough surrogate marker for BAT activity. Specifically fat oxidation can be argued to reflect BAT activity for several reasons: 1) short term BAT activation by β3-adrenergic receptor stimulation or cold exposure selectively enhances the oxidation of fat over glucose, both in mice and humans;[77?] 2) long term BAT activation by cold exposure progressively increases lipid oxidation while reducing glucose oxidation in humans;[94] 3) conversion of human white adipocytes to beige adipocytes changes the substrate preference from glucose to lipid.[95] Although indirect calorimetric measurements can provide directions in the search for the underlying pathways and determinants of diseases related to energy metabolism, concluding statements about BAT functionality can never be made based on these measures. For example, we describe in **Chapter 8** that smoking is associated with a higher resting energy expenditure, and it is tempting to speculate that this association is explained by a nicotine-induced increase in BAT activity, as has been reported in rats.[96] However, nicotine has also been shown to enhance the mobilization of free fatty acids through lipolysis in white fat tissue,[97?] which may as well underlie the higher energy expenditure of smokers by enhancing substrate availability.

Genetic determinants of BAT functionality

In contrast to observational study designs, Mendelian randomization is able to assess causality,[1,2] as discussed previously. To identify the SNPs that can be used as robust genetic instruments in Mendelian randomization, preferably, GWAS is performed on the phenotypic trait of interest. Unfortunately, until today, a GWAS on BAT functionality has not been performed due the lack of BAT activity measurements in large populations. For that reason, the genetic basis of BAT activity is largely unknown. Based on a priori knowledge of BAT physiology, candidate gene studies mainly focussed on genetic variation in the UCP1 and β3AR gene, and showed that genetic variants in these genes were associated with an increased risk of obesity.[98,99] After identification of UCP1 and β3AR obesity-associated variants, it was shown that these SNPs also associate with resting energy expenditure, thermoregulatory sympathetic nervous system activity, and cold-induced thermogenesis.[100–102] Thereafter, these genes were linked to BAT activity in a study that showed that the UCP1 -3826A/G and the β3AR 64 Trp/Arg polymorphisms accelerated the age-related decrease in BAT activity.[103] This was the first study in humans to report that genetic variation affects BAT functionality. The search for a genetic basis of BAT went on, and more recently, the first evidence for a genetic effect on adipocyte browning in humans was provided. It was shown that T to C allele substitution in the rs1421085 variant of the fat

mass and obesity-associated gene (FTO), which is the top common genetic determinant of BMI,[104–106] disrupted the binding of the transcriptional repressor ARID5B to IRX3 and IRX5, which increased their expression specifically in pre-adipocytes.[107] This resulted in a developmental shift from energy-combusting beige adipocytes towards energy-storing white adipocytes in vitro, characterized by decreased mitochondrial thermogenesis and increased lipid storage. Although it was convincingly shown that this FTO variant reduced thermogenesis capacity on the cellular level, this finding was not translated to effects on whole-body energy metabolism. To make this translational step, we studied the effects of variation in rs1421085 on resting whole-body energy expenditure and fat oxidation, as described in **Chapter 9**. We found no evidence for an association of rs1421085 with measures of energy metabolism. This implies that the contribution of browning of white adipocytes to total energy metabolism is apparently too small to affect resting whole-body energy expenditure and fat oxidation, despite the 5-fold reduction in mitochondrial thermogenesis observed in white adipose tissue of rs1421085 risk allele carriers in vitro.[107] As an alternative explanation, it may be that effects of rs1421085 risk allele carriage on energy expenditure will only become apparent when the energy combusting capacity of beige adipocytes is stimulated by cold, which would be an interesting avenue for future research.

Thus, so far, the evidence for a genetic basis of BAT is fairly limited, and the search for genetic determinants for BAT has only just begun. Interestingly, according to the cold climate genes hypotheses, DNA areas involved in thermogenesis may have encountered selection pressure during evolution in areas with cold climates.[108] DNA areas that regulate uncoupling proteins, including UCP1, have been proposed as candidates for these cold climate genes. Furthermore, several genes have been identified as determinants of whole-body fat oxidation,[109] and future research needs to clarify whether the underlying mechanism for these genetic effects are related to BAT functionality. Identification of genetic determinants of BAT activity, hopefully assisted by reliable and cost-effective biomarkers, can aid in further understanding the individual risk differences for developing cardiometabolic disease. Eventually, future preventive and curative strategies may be adapted to an individual's genetic profile, as a part of personalized medicine, to more effectively prevent and treat disease.

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11

Addendum

Summary

While most infectious diseases have been combatted in the past centuries, mankind is now facing epidemics of non-communicable diseases. This is strikingly reflected in the global rise of cardiometabolic disorders, which mainly comprise obesity, cardiovascular disease (CVD) and type 2 diabetes. To increase human life span, spent in good health, the development and improvement of preventive and curative strategies for cardiometabolic disease is eagerly warranted. With the studies describes in this thesis, we aimed to disentangle the interwoven physiological, environmental and genetic factors that determine cholesterol and energy metabolism to increase our understanding of their contribution to cardiometabolic disease risk, thereby facilitating the search for novel preventive and curative strategies. **Chapter 1** serves as a general introduction to cholesterol and energy metabolism, and their role in cardiometabolic health.

The first part of this thesis focussed on the cholesteryl ester transfer protein (CETP). CETP is able to transfer cholesteryl esters from high-density lipoprotein (HDL) towards apolipoprotein B containing triglyceride-rich lipoproteins, mainly very low-density lipoproteins (VLDL), and triglycerides from VLDL and chylomicrons towards triglyceride-poor particles, such as HDL. Since these lipid transfer properties of CETP induce a proatherogenic lipoprotein profile, CETP inhibitory molecules have been developed and tested in clinical trials for their capability to improve the lipoprotein profile and reduce CVD risk. To fully understand the role of CETP in CVD, its physiology and biological function should be fully unravelled. Strikingly, although CETP inhibitors have already been tested in clinical trials for over a decade, there was no consensus on the cellular source of circulating CETP, as both the liver and adipose tissue were regarded to be responsible for the production of CETP.

In **Chapter 2** we examined the contribution of body fat to circulating CETP concentration. To this end, we studied in 6,606 participants of the Netherlands Epidemiology of Obesity (NEO) study the associations of total body fat, body mass index, waist circumference, waist-to-hip ratio, abdominal subcutaneous and visceral adipose tissue assessed with magnetic resonance imaging (N=2,547), and total and trunk fat mass assessed with dual-energy X-ray absorptiometry (N=909) with serum CETP concentration. We concluded that, in this population-based study, there was no evidence for clinically relevant associations between these measures of body fat and serum CETP concentration, which implies that adipose tissue does not contribute to the CETP pool in serum. These data support

previous findings from our research group that circulating CETP concentration does not associate with waist circumference, and that circulating CETP concentration is mainly determined by the number of resident macrophages in the liver, which are referred to as Kupffer cells.

Based on the primary expression of *CETP* by these cells of the innate immune system and our previous finding that activation of Kupffer cells by the bacterial endotoxin lipopolysaccharide strongly decreases CETP expression, we subsequently studied if metabolic liver inflammation is also associated with a decrease in hepatic expression and plasma concentrations of CETP, the results of which are described in **Chapter 3**. To this end, we collected plasma and liver biopsy samples at various stages of non-alcoholic fatty liver disease from 93 obese individuals who underwent bariatric surgery. Liver lobular inflammation was histologically determined, and liver *CETP* expression, CETP positive cells, circulating CETP concentrations were quantified. We found no strong evidence for a negative association between metabolic liver inflammation and CETP-related outcomes, although we observed consistent trends towards lower measures of CETP in the presence of metabolic liver inflammation. From this, we concluded that metabolic and infection-induced liver inflammation have different effects on the expression and production of CETP by Kupffer cells. Acute and/or whole-body inflammatory responses to invading pathogens are thus probably required to induce a robust reduction in CETP production by Kupffer cells.

Next, to elucidate the genetic basis of serum CETP concentration, we performed the first genome-wide association study (GWAS) and exome-wide analysis on serum CETP concentration to identify common and rare genetic variants that determine circulating CETP. In **Chapter 4**, we describe the identification of three common independent variants mapped to the CETP gene (i.e. rs247616, rs12720922 and rs1968905) that together explain 16.4% of the total variation in CETP concentration, in a discovery cohort of the NEO study (N=4,248). These genetic variants were replicated in a separate subpopulation of the NEO study (N=1,458). Furthermore, in an exome-wide gene-based aggregation analysis (N=6,094), we identified rare variants (i.e. minor allele frequency <0.03) in the ABCA6 gene as a novel determinants of CETP concentration, as described in **Chapter 5**, from which we concluded that abundance or functionality of the ABCA6 protein regulates the circulating CETP concentration. Since ABCA6 is involved in cellular lipid and sterol transport and CETP is regulated by sterol derivatives via a liver X receptor α responsive element in its promoter region, we speculate that ABCA6 determines CETP production via intracellular regulation of lipid and sterol trafficking.

We next used the GWAS-identified common CETP variants as genetic instruments in Mendelian randomization analyses to investigate the causal effects of an increase in CETP

concentration on the lipoprotein metabolite profile (NEO study) and coronary artery disease risk (CARDIoGRAMplusC4D consortium), which is relevant to better understand the outcomes of the CETP inhibitor trials that aim to reduce CVD. We showed in **Chapter 6** that an increase in circulating CETP causally decreases the concentration of mainly large HDL particles, and increases the classes of small VLDL particles without increasing the classes and composition of low-density lipoprotein (LDL) particles. Additionally, increased CETP causally associated with a higher risk of coronary artery disease with an odds of 1.08 (95% CI 0.94, 1.23) (**Chapter 4**). Interestingly, it was previously reported that small and very small VLDL particle concentrations (reflecting triglyceride-rich lipoprotein remnants) and cholesterol in VLDL (reflecting remnant cholesterol) are among the lipoprotein components that most strongly associate with an increased CVD risk. Thus, contrary to the current dogma that CETP increases CVD risk via increasing LDL-cholesterol, our results imply that the increased CVD risk associated with higher CETP concentrations is explained by an increase of small VLDL (remnant) particles.

The focus of the second part of this thesis was on the role of energy metabolism in cardiometabolic health. Specifically, we aimed to study the association of environmental and genetic factors, which were previously described to influence brown adipose tissue (BAT) activity, with energy expenditure and disease outcomes. Activation of BAT increases energy expenditure, as this tissue is capable to produce heat in response to cold by combusting energy that is stored in glucose and triglycerides. Based on a recent landmark paper, which showed that a 10 day cold acclimatisation period activates BAT and improves insulin sensitivity in patients with type 2 diabetes, we assessed in **Chapter 7** the association between mean annual temperature and diabetes incidence during 1996-2009 in the United States of America. On a global scale, we also assessed the association between mean annual temperature and the prevalence of glucose intolerance using data from 2014. We demonstrated that on average per 1°C increase in temperature age-adjusted diabetes incidence increased with 0.314 (95% CI 0.194, 0.434) per 1000. Similarly, the worldwide prevalence of glucose intolerance increased by 0.170% (95% CI 0.107, 0.234) per 1°C rise in temperature. These findings emphasize the importance of future research into the effects of environmental temperature on glucose metabolism and the onset of diabetes, especially in view of the global rise in temperatures.

Since it was shown in rats that nicotine increases energy combustion in BAT, we studied in **Chapter 8** the association between smoking and resting energy expenditure in 1,189 study participants from the NEO study. We showed that resting energy expenditure expressed per fat free mass was higher in current smokers compared with never smokers. There was no difference in resting energy expenditure between never and former smokers or

between never and occasional smokers and no association with pack years, from which we concluded that smoking has primarily acute effects on resting energy expenditure.

Finally, we studied in **Chapter 9** the association between rs1421085-risk allele carriage and whole-body fat oxidation in 1,246 participants of the NEO study. We initiated this study based on a landmark paper from 2015 that described a potential genetic basis for BAT activity, by showing that T-to-C substitution in the rs1421085 variant of the fat mass and obesity-associated gene (*FTO*) gene reduced browning of pre-adipocytes *in vitro*. We hypothesized that humans who carry the C-allele of rs1421085 may have reduced browning of their white fat depots, which possibly leads to a lower fat oxidation, since brown fat cells selectively enhance the oxidation of fat over glucose. However, we observed no evidence for associations of rs1421085 in *FTO* with resting whole-body fat oxidation, despite higher measure of obesity in risk allele carriers. This implies that the contribution of browning of white adipocytes to total energy metabolism is apparently too small to affect whole-body fat oxidation. Thus, likely, the rs1421085 C-allele in *FTO* induces obesity via other pathways than via reduced fat oxidation.

To conclude this thesis, the study findings, their implications for future research, and the perspectives for preventive and therapeutic strategies of cardiometabolic disease are discussed in **Chapter 10**. All in all, the results of the studies described in this thesis have increased our understanding of the physiological, environmental and genetic factors that determine cholesterol and energy metabolism, and provided insight in their contribution to cardiometabolic health.

Nederlandse samenvatting

Waar infectieziekten voorheen de voornaamste doodsoorzaak vormden, leven we nu in een tijdperk waarin de meeste infectieziekten onder controle zijn en de mensheid wordt getroffen door niet-besmettelijke, chronische welvaartsziekten. Dit wordt overduidelijk uit de wereldwijde toename van het aantal mensen dat leidt aan cardiometabole aandoeningen, waarvan obesitas, type 2 diabetes en hart- en vaatziekten de grootste boosdoeners zijn. Het is daarom van groot belang dat we onderzoek doen naar de verbetering van de preventie en genezing van cardiometabole aandoeningen om de levensverwachting in goede gezondheid te verlengen.

De studies beschreven in dit proefschrift waren erop gericht om meer inzicht te verwerven in de genetische, fysiologische en omgevingsfactoren die de cholesterolstofwisseling en energiestofwisseling beïnvloeden. Tevens werd getracht om de bijdrage van deze factoren aan het risico op het ontwikkelen van cardiometabole ziekten te begrijpen en zo handvatten te bieden voor de ontwikkeling van preventieve en curatieve behandelingen. In **Hoofdstuk 1** beschrijven we de algemene principes en huidige kennis van de rol van de cholesterol- en energiestofwisseling in cardiometabole gezondheid.

Het eerste deel van dit proefschrift richtte zich op de cholesterolstofwisseling. Cholesterol is een onoplosbare vetachtige stof die talloze belangrijke functies vervult in het lichaam. Zo is cholesterol onder meer een essentieel onderdeel van celmembranen en is het nodig voor de vorming van steroïdenhormonen, vitamine D en galzuren. Cholesterol is niet oplosbaar is in water en wordt daarom in het bloed getransporteerd in zogenaamde lipoproteïnen. Dit zijn bolvormige deeltjes die, zoals de naamgeving impliceert, zijn opgebouwd uit vetten (lipiden) en eiwitten (proteïnen). Lipoproteïnen hebben als doel om de in water onoplosbare vetten cholesteryl esters en triglyceriden te transporteren vanuit bijvoorbeeld de darmen en de lever naar overige weefsels via de bloedbaan. Triglyceriden en cholesteryl esters worden in de kern van de lipoproteïnen afgeschermd van het bloed, doordat ze zijn omgeven door een laag van zeepachtige moleculen die ‘fosfolipiden’ worden genoemd. Op basis van hun dichtheid worden lipoproteïnen ingedeeld naar toenemende dichtheid in chylomicronen, *very-low density lipoproteins* (VLDL), *low density lipoproteins* (LDL) en *high density lipoproteins* (HDL).

In dit proefschrift hebben we ons specifiek verdiept in de rol van het eiwit *cholesteryl ester transfer protein* (CETP) in de cholesterolstofwisseling. CETP is een eiwit dat circuleert in de bloedbaan en heeft als functie om vetten uit te wisselen tussen diverse lipoproteïnen.

CETP is in staat om cholesteryl esters uit HDL te transporteren naar VLDL en chylomicronen en andersom om triglyceriden vanuit VLDL en chylomicronen te transporteren naar HDL. Deze lipidenuitwisseling leidt tot een lipoproteïnenprofiel in het bloed (namelijk een hoog LDL en laag HDL) dat samengaat met een verhoogd risico op de ontwikkeling van slagaderverkalking. Om deze reden zijn in het verleden verscheidene geneesmiddelen ontwikkeld om de activiteit van CETP te remmen en daarmee het lipoproteïnenprofiel te verbeteren en het risico op hart- en vaatziekten te verlagen. Opvallend genoeg was het nog onbekend welke cellen in het lichaam verantwoordelijk zijn voor de aanmaak van CETP. Toen gestart werd met het testen van deze CETP remmers in klinische onderzoeken, werden zowel de lever als het vetweefsel verantwoordelijk gehouden voor de productie van CETP. Om de rol van CETP in het ontstaan van hart- en vaatziekten beter te begrijpen, was meer inzicht in de fysiologische en biologische functie van CETP vereist.

In **hoofdstuk 2** beschrijven we een studie waarin onderzocht werd of vetweefsel bijdraagt aan de concentratie van CETP in het bloed. Daartoe hebben we in een groep van 6606 deelnemers aan de Nederlandse Epidemiologie van Obesitas (NEO) studie bepaald wat het verband is tussen de hoeveelheid vetweefsel en de circulerende CETP concentratie. Als maten voor vetweefsel gebruikten we het totale lichaamsvetpercentage, de *body mass index* (BMI), de middelomtrek, de verhouding tussen middelomtrek en heupomtrek, het onderhuids en visceraal buikvet zoals bepaald met *magnetic resonance imaging* (MRI) en de totale vetmassa en de vetmassa in de romp zoals bepaald met *dual-energy X-ray absorptiometry* (DXA). Uit dit onderzoek konden we concluderen dat er geen bewijs is voor een klinisch relevant verband tussen de hoeveelheid vetweefsel en de circulerende CETP concentratie, wat impliceert dat vetweefsel niet bijdraagt aan de hoeveelheid CETP in het bloed. Deze resultaten ondersteunen een eerdere studie van onze onderzoeksgroep, waarin we aantoonden dat de CETP concentratie in het bloed voor het overgrote deel wordt bepaald door het aantal macrofagen in de lever, die ook wel Kupffer cellen worden genoemd.

Vanwege de primaire productie van CETP door deze Kupffer cellen, die een onderdeel vormen van het aangeboren immuunsysteem en de recente bevinding dat het bacteriële bestanddeel lipopolysaccharide de productie van CETP sterk vermindert, besloten we vervolgens te onderzoeken of ontsteking van de lever (zogenaamde ‘metabole leverinflammatie’) ook leidt tot een afname van de productie van CETP in de lever en de concentratie van CETP in het bloed, zoals is beschreven in **Hoofdstuk 3**. Daartoe verzamelden we bloed en leverbiopten van 93 personen met obesitas, die bariatrische chirurgie (bijvoorbeeld plaatsing van een maagband) ondergingen. Leverontsteking werd histologisch bepaald en de expressie van *CETP* in de lever, het aantal CETP-positieve levercellen en de circulerende

CETP concentratie werden gekwantificeerd. We zagen een trend richting lagere expressie van *CETP* in de lever, minder CETP-positieve cellen en een lagere circulerende CETP concentratie wanneer de biopten kenmerken van metabole leverontsteking vertoonden, maar het bewijs was niet overtuigend genoeg om te spreken van een robuuste omgekeerd verband tussen metabole leverontsteking en CETP-gerelateerde uitkomsten. Op basis van deze resultaten concludeerden we dat leverontsteking ten gevolge van een infectie een groter remmend effect heeft op de productie van CETP door Kupffer cellen dan metabole ontsteking. Wellicht is dus alleen een acute en uitgebreide ontstekingsreactie geïnduceerd door ziekteverwekkers in staat om een robuuste afname van de CETP productie teweeg te brengen.

Ondanks dat we steeds meer te weten komen over de biologie van CETP, is het nog onvoldoende duidelijk in welke mate genetische aanleg de hoeveelheid CETP in het bloed bepaalt. Daarom voerden we in **Hoofdstuk 4** een analyse uit van de genen van alle deelnemers aan de NEO studie om te bepalen welke genen bijdragen aan de circulerende CETP concentratie. Een dergelijke analyse wordt een genoomwijde associatiestudie (GWAS) genoemd. We zochten specifiek naar kleine veranderingen in de volgorde van basenparen in het DNA, zogenoemde genetische varianten ofwel *single nucleotide polymorphisms* (SNPs), die in bepaalde personen voorkomen en die samengaan met een hogere concentratie CETP in het bloed. Deze personen noemen we dan dragers van de 'risicovariant'. Met deze DNA-analyse identificeerden we drie veelvoorkomende genetische varianten in het *CETP* gen (op de posities rs247616, rs12720922 en rs1968905). Deze drie varianten bepaalden samen 16,4% van de totale variatie in de circulerende CETP concentratie in de NEO studie populatie. Daarnaast identificeerden we zeldzame genetische varianten in het *ABCA6* gen die de circulerende CETP concentratie beïnvloeden, zoals vermeld staat in Hoofdstuk 5. Uit deze bevindingen concludeerden we dat de aanwezigheid of de functionaliteit van het ABCA6 eiwit de concentratie van CETP in de bloedbaan reguleert.

Na de identificatie van de drie veelvoorkomende genetische varianten die de concentratie van CETP in het bloed beïnvloeden, gebruikten we deze varianten vervolgens om een zogenaamde Mendeliaanse randomisatie studie uit te voeren. Met deze methode is het mogelijk om de causale effecten te onderzoeken van een toename van de circulerende CETP concentratie op het lipoproteïnenprofiel (NEO studie) en op het risico op coronaire hartziekten (CARDIoGRAMplusC4D consortium). Dit is van belang om de uitkomsten van de klinische onderzoeken met CETP remmers beter te begrijpen. In **Hoofdstuk 6** laten we zien dat een toename van de concentratie van CETP in het bloed voornamelijk leidt tot een causale verlaging van de grote HDL deeltjes en een causale toename van de kleine VLDL deeltjes, zonder daarbij een effect te hebben op de LDL deeltjes. Bovendien ging een toe-

name van de CETP concentratie oorzakelijk samen met een 8% hoger risico op coronaire hartziekten (**Hoofdstuk 4**). In dit opzicht is het uiterst interessant dat uit eerdere studies is gebleken dat van alle lipoproteïnen juist de concentratie van de deeltjes en het cholesterol van (zeer) klein VLDL het sterkst samengaan met een verhoogd risico op hart- en vaatziekten. Onze resultaten impliceren dan ook dat het verhoogde risico op hart- en vaatziekten, dat samengaat met een toename van de circulerende CETP concentratie, wordt veroorzaakt door een specifieke toename in de kleine VLDL deeltjes. Dit is in tegenstelling tot het huidige dogma dat bepleit dat CETP het risico op hart- en vaatziekten verhoogt via een verhoging van het cholesterol in LDL deeltjes.

Het tweede deel van dit proefschrift was gericht op de rol van de energiestofwisseling in de cardiometabole gezondheid. Het specifieke doel was om te onderzoeken hoe omgevings- en genetische factoren, waarvan bekend is dat ze de activiteit van bruin vet beïnvloeden, samengaan met energieverbruik en cardiometabole ziekten. Bruin vet is een belangrijke speler in het energieverbruik. In tegenstelling tot het welbekende witte vet dat vetten opslaat en kan leiden tot vetzucht, is bruin vet juist in staat om vetten te verbranden. Bruin vet wordt actief wanneer het lichaam wordt blootgesteld aan kou. Het is dan in staat om warmte te produceren door energie te verbranden die is opgeslagen in suiker (glucose) en vetten (triglyceriden). Naast de bruine vetcellen, die zich bevinden rondom de grote lichaamsslagader en langs de grote bloedvaten in de hals, bestaan er ook zogenaamde beige vetcellen. Dit zijn witte vetcellen die zich kunnen gedragen als bruine vetcellen wanneer het lichaam wordt blootgesteld aan kou, een fenomeen dat we ‘bruining’ van vetcellen noemen.

Geïnspireerd door recent onderzoek dat aantoonde dat tien dagen blootstelling aan kou het bruin vet activeert en de insulinegevoeligheid van mensen met type 2 diabetes verbetert, hebben wij het verband onderzocht tussen de gemiddelde jaarlijkse buitentemperatuur en het ontstaan van diabetes. Hiervoor gebruikten we gegevens uit de Verenigde Staten omdat daar grote openbare archieven bestaan, waarin gegevens over de temperatuur en de ziektestatus van de bevolking nauwkeurig worden bijgehouden. In **Hoofdstuk 7** beschrijven we dat een toename van 1 graad Celsius in de buitentemperatuur 100.000 nieuwe diabetes gevallen per jaar tot gevolg kan hebben in de Verenigde Staten. Deze bevindingen benadrukken het belang van toekomstig onderzoek naar de effecten van omgevingstemperatuur op de ontwikkeling van diabetes, zeker in het licht van de problematiek rondom de opwarming van de aarde.

Omdat er aanwijzingen zijn vanuit preklinische studies dat nicotine bruin vet activeert, bestudeerden we in **Hoofdstuk 8** het verband tussen roken en het energieverbruik in rust in 1189 deelnemers van de NEO studie. We toonden aan dat energieverbruik in rust hoger

was in personen die op dat moment rookten in vergelijking met personen die nooit hadden gerookt. We konden geen verschil aantonen in energieverbruik tussen personen die nooit hadden gerookt en voormalige rokers en er was ook geen verband met de duur en intensiteit van het roken. Op basis van deze resultaten concludeerden we dat roken voornamelijk acute effecten heeft op het energieverbruik.

Tenslotte beschrijven we in **Hoofdstuk 9** het verband tussen het genetisch dragerschap van een genetische risicovariant in het *fat mass and obesity-associated gene* (FTO gen) en vetverbranding, waarvoor we gegevens van 1246 deelnemers van de NEO studie analyseerden. We baseerden deze studie op de hypothese van een recent artikel waarin een potentiële genetische basis voor de activiteit van bruin vet werd aangedragen. In deze studie werd aangetoond dat een bepaalde verandering in de volgorde van basenparen in het *FTO* gen (in dit geval substitutie van de nucleotide thymine (T) naar cytosine (C) op positie rs1421085) leidde tot een verminderde bruining van voorlopers van vetcellen (zogenaamde preadipocyten). We vormden daarom de hypothese dat mensen die de risicovariant (C-variant) van deze genetische variant dragen minder capaciteit hebben om hun witte vetcellen die vet opslaan om te zetten in bruine vetcellen, die vet juist verbranden. Dit leidt mogelijk tot een verminderde vetverbranding, omdat geactiveerde bruine vetcellen selectief de vetverbranding verhogen ten opzichte van de glucoseverbranding. Desalniettemin vonden we geen verband tussen het dragen van de risicovariant en vetverbranding, ondanks dat dragers van de risicovariant meer lichaamsvet hadden. Onze resultaten impliceren dat de bijdrage van mogelijke bruining aan de totale energiestofwisseling te klein is om een effect te hebben op de vetoxidatie in deze mensen. We concludeerden daarom dat de genetische risicovariant in het *FTO* gen waarschijnlijk leidt tot obesitas via een ander mechanisme dan door vermindering van de vetverbranding.

Tot slot bediscussiëren we onze studiebevindingen, de implicaties voor vervolgonderzoek en de vooruitzichten voor preventieve en curatieve behandelingsmethoden van cardiometabole aandoeningen in **Hoofdstuk 10**. Concluderend kunnen we stellen dat de studies die beschreven zijn in dit proefschrift ons begrip hebben vergroot over de fysiologische, omgevings- en genetische factoren die de cholesterol- en energiestofwisseling beïnvloeden en ons inzicht hebben gegeven in hun bijdrage aan de cardiometabole gezondheid.

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Blauw LL, Noordam R, Berbée JFP, et al. Genetic variation in MC4R does not affect atherosclerotic plaque phenotype and cardiovascular disease outcomes irrespective of sex, BMI and blood pressure. *In preparation*.

Curriculum vitae

Lisanne Louise Blauw werd geboren op 7 juni 1993 te Leiderdorp. Ze groeide op in Voorschoten en haalde in 2011 haar Gymnasiumdiploma aan het Stedelijk Gymnasium te Leiden (*cum laude*). In datzelfde jaar begon ze aan de opleiding Geneeskunde aan de Universiteit Leiden, waar ze in 2014 haar Bachelor of Science behaalde (*cum laude*).Tijdens haar bacheloropleiding werd haar interesse voor wetenschap gewekt en besloot ze zich aan te melden voor het MD/PhD-traject van het Leids Universitair Medisch Centrum (LUMC). Na haar toelating tot dit traject startte zij in 2013 met een onderzoekproject bij de sectie Endocrinologie binnen de afdeling Interne Geneeskunde in samenwerking met de afdeling Klinische Epidemiologie onder supervisie van Prof. dr. Patrick Rensen en Prof. dr. Olaf Dekkers.

Na het afronden van haar Bacheloropleiding Geneeskunde ontving zij begin 2015 een tweejarige beurs van de Raad van Bestuur van het LUMC voor het uitvoeren van haar promotieonderzoek bij de sectie Endocrinologie binnen de afdeling Interne Geneeskunde onder supervisie van haar promotor Prof. dr. Patrick Rensen en copromotor Dr. ir. Renée de Mutsert. Tevens besloot ze in 2015 haar studie Geneeskunde te onderbreken om een schakelprogramma Werktuigbouwkunde te starten aan de Technische Universiteit Delft. Na afronding van dit schakelprogramma werd zij toegelaten tot de masteropleiding Biomedical Engineering met als specialisatie Biomaterials and Tissue Biomechanics, waaraan ze in 2017 begon.

Lisanne presenteerde de resultaten van haar onderzoek op verscheidene (bio)medische congressen. Ze kreeg tweemaal de Young Investigator Award toegekend voor presentatie van haar werk tijdens de jaarlijkse conferentie van de European Atherosclerosis Society (EAS) en was board member van het Young Talent Forum van het Cardiovasculair Onderzoek Nederland (CVON) consortium ENERGISE. Ze rondde haar promotieonderzoek, waarvan de resultaten staan beschreven in dit proefschrift, af in 2018. In ditzelfde jaar begon Lisanne aan haar masterstage voor de studie Biomedical Engineering op de afdeling Applied Stem Cell Technologies van de Universiteit Twente onder begeleiding van Prof. dr. Robert Passier en Dr. Marcelo Ribeiro.

List of abbreviations

ABC ATP binding cassette
ALT alanine transaminase
AMPK AMP-activated protein kinase
ABC ATP binding cassette
ALT alanine transaminase
AMPK AMP-activated protein kinase
Apo apolipoprotein
ApoB apolipoprotein B
aSAT abdominal subcutaneous adipose tissue
AST aspartate transaminase
ATP adenosine triphosphate
BAT brown adipose tissue
BIA bioelectrical impedance analysis
BMI body mass index
BMR basal metabolic rate
C cholesterol
CAD coronary artery disease
CarbOx carbohydrate oxidation
CDC centers for disease control and prevention
CETP cholesteryl ester transfer protein
CI confidence interval
CMC combined multivariate and collapsing
CV coefficient of variation
CVD cardiovascular disease
DNA deoxyribonucleic acid
DXA dual-energy X-ray absorptiometry
ELISA enzyme-linked immunosorbent assay
En% energy percent
eQTL expression quantitative trait loci
FatOx fat oxidation
FBG fasting blood glucose
FDG fluorodeoxyglucose
FFM fat free mass
FFQ food frequency questionnaire
FTO fat mass and obesity-associated gene
GCTA genome-wide complex trait analysis
GLGC Global Lipids Genetics Consortium
GLUT4 glucose transporter type 4
GNI gross national income
GRS genetic risk score

GTEx genotype-tissue expression
GWAS genome-wide association studies
HbA1c glycated haemoglobin
HDL high-density lipoprotein
IFN-γ interferon γ
IL-1β interleukin-1β
kcal kilocalorie
LC-MS liquid chromatography-mass spectrometry
LDL low-density lipoprotein
LPS lipopolysaccharide
LUMC Leiden University Medical Center
LXRα liver X receptor α
MAF minor allele frequency
MET metabolic equivalents of task
MRI magnetic resonance imaging
MRS magnetic resonance spectroscopy
N urinary nitrogen excretion
NAFLD non-alcoholic fatty liver disease
NASH non-alcoholic steatohepatitis
NEO Netherlands Epidemiology of Obesity
NMR nuclear magnetic resonance
PC principal component
PCSK9 proprotein convertase subtilisin–kexin type 9
PET-CT positron emission tomography-computed tomography
REE resting energy expenditure
RQ respiratory quotient
SCORE systematic coronary risk estimation
SKAT sequence kernel association test
SNP single nucleotide polymorphism
SQUAH short questionnaire to assess health-enhancing physical activity
TNF-α tumor necrosis factor α
TG triglycerides
UCP1 uncoupling protein 1
USA United States of America
VAT visceral adipose tissue
VCO_2 volume of carbon dioxide exhaled
VLDL very low-density lipoproteins
VO_2 volume of oxygen inspired
WHR waist-to-hip ratio