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

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Aim and general introduction

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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

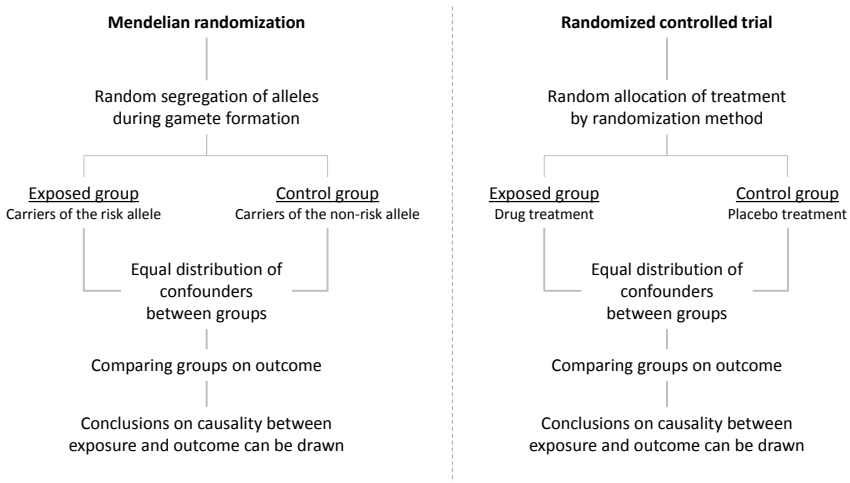

Figure 1.1: Schematic representation of the clear analogy between Mendelian randomization studies and randomized controlled trials. Adapted from Davey Smith G, Ebrahim S. BMJ 2005; 330: 1076-9.

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**).

References

[1] K. Christensen, G. Doblhammer, and R. Rau et al. Ageing populations: the challenges ahead. *Lancet*, 374(9696):1196–208, 2009.

[2] A. R. Omran. The epidemiologic transition. a theory of the epidemiology of population change. *Milbank Mem Fund Q*, 49(4):509–38, 1971.

[3] The GBD 2015 Obesity Collaborators. Health effects of overweight and obesity in 195 countries over 25 years. *N Engl J Med*, 377:13–27, 2017.

[4] M. F. Piepoli, A. W. Hoes, and S. Agewall et al. 2016 European Guidelines on cardiovascular disease prevention in clinical practiceThe Sixth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of 10 societies and by invited experts)Developed with the special contribution of the European Association for Cardiovascular Prevention and Rehabilitation (EACPR). *Eur Heart J*, 37(29):2315–2381, 2016.

[5] R. M. Conroy, K. Pyörälä, and A. P. Fitzgerald et al. Estimation of ten-year risk of fatal cardiovascular disease in Europe: the SCORE project. *Eur Heart J*, 24(11):987–1003, 2003.

[6] C. Reith and J. Armitage. Management of residual risk after statin therapy. *Atherosclerosis*, 245:161–170, 2016.

[7] B. A. Ference, H. N. Ginsberg, and I. Graham et al. Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. *Eur Heart J*, 38(32):2459–2472, 2017.

[8] M. S. Sabatine, R. P. Giugliano, and A.C. Keech et al. Evolocumab and clinical outcomes in patients with cardiovascular disease. *N Engl J Med*, 376(18):1713–1722, 2017.

[9] P. J. Barter, M. Caulfield, and M. Eriksson et al. Effects of torcetrapib in patients at high risk for coronary events. *N Engl J Med*, 357(21):2109–22, 2007.

[10] A. M. Lincoff, S. J. Nicholls, and J. S. Riesmeyer et al. Evacetrapib and cardiovascular outcomes in high-risk vascular disease. *N Engl J Med*, 376(20):1933–1942, 2017.

[11] G. G. Schwartz, A. G. Olsson, and M. Abt et al. Effects of dalcetrapib in patients with a recent acute coronary syndrome. *N Engl J Med*, 367(22):2089–99, 2012.

[12] T. Chajek and C. J. Fielding. Isolation and characterization of a human serum cholesteryl ester transfer protein. *Proc Natl Acad Sci U S A*, 75(7):3445–9, 1978.

[13] N. M. Pattnaik, A. Montes, and L. B. Hughes et al. Cholesteryl ester exchange protein in human plasma isolation and characterization. *Biochim Biophys Acta*, 530(3):428–38, 1978.

[14] D. Drayna, A.S. Jarnagin, and J. McLean et al. Cloning and sequencing of human cholesteryl ester transfer protein cDNA. *Nature*, 327(6123):632–634, 1987.

[15] M. L. Brown, A. Inazu, and C. B. Hesler et el. Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoproteins. *Nature*, 342(6248):448–51, 1989.

[16] M. A. Charles and J. P. Kane. New molecular insights into CETP structure and function: a review. *J Lipid Res*, 53(8):1451–8, 2012.

[17] X. Qiu, A. Mistry, and M. J. Ammirati et al. Crystal structure of cholesteryl ester transfer protein reveals a long tunnel and four bound lipid molecules. *Nat Struct Mol Biol*, 14(2):106–13, 2007.

[18] L. Zhang, F. Yan, and S. Zhang et al. Structural basis of transfer between lipoproteins by cholesteryl ester transfer protein. *Nat Chem Biol*, 8(4):342–9, 2012.

[19] D. Lei, M. Rames, and X. Zhang et al. Insights into the tunnel mechanism of cholesteryl ester transfer protein through all-atom molecular dynamics simulations. *J Biol Chem*, 291(27):14034–44, 2016.

[20] A. R. Tall. Plasma cholesteryl ester transfer protein. *J Lipid Res*, 34(8):1255–74, 1993.

[21] I. J. Goldberg. Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis. *J Lipid Res*, 37(4):693–707, 1996.

[22] K. Guendouzi, X. Collet, and B. Perret et al. Remnant high density lipoprotein2 particles produced by hepatic lipase display high-affinity binding and increased endocytosis into a human hepatoma cell line (HEPG2). *Biochemistry*, 37(42):14974–80, 1998.

[23] H. H. Newnham and P. J. Barter. Synergistic effects of lipid transfers and hepatic lipase in the formation of very small high-density lipoproteins during incubation of human plasma. *Biochim Biophys Acta*, 1044(1):57–64, 1990.

[24] K. Rye, M. A. Clay, and P. J. Barter. Remodelling of high density lipoproteins by plasma factors. *Atherosclerosis*, 145(2):227–238, 1999.

[25] C. Xiao, T. Watanabe, and Y. Zhang et al. Enhanced cellular uptake of remnant high-density lipoprotein particles: a mechanism for high-density lipoprotein lowering in insulin resistance and hypertriglyceridemia. *Circ Res*, 103(2):159–66, 2008.

[26] P. J. Barter, H. B. Brewer, and M. J. Chapman et al. Cholesteryl ester transfer protein: a novel target for raising HDL and inhibiting atherosclerosis. *Arterioscler Thromb Vasc Biol*, 23(2):160–7, 2003.

[27] M. J. Chapman, W. Le Goff, and M. Guerin et al. Cholesteryl ester transfer protein: at the heart of the action of lipid-modulating therapy with statins, fibrates, niacin, and cholesteryl ester transfer protein inhibitors. *Eur Heart J*, 31(2):149–64, 2010.

[28] J. Hulthe, L. Bokemark, and J. Wikstrand et al. The metabolic syndrome, LDL particle size, and atherosclerosis: the Atherosclerosis and Insulin Resistance (AIR) study. *Arterioscler Thromb Vasc Biol*, 20(9):2140–2147, 2000.

[29] K. J. Williams and I. Tabas. The response-to-retention hypothesis of atherogenesis reinforced. *Curr Opin Lipidol*, 9(5):471–4, 1998.

[30] D. J. Gordon, J. L. Probstfield, and R. J. Garrison et al. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation*, 79(1):8–15, 1989.

[31] L. A. Morehouse, E. D. Sugarman, and P. A. Bourassa et al. Inhibition of CETP activity by torcetrapib reduces susceptibility to diet-induced atherosclerosis in New Zealand White rabbits. *J Lipid Res*, 48(6):1263–72, 2007.

[32] H. Okamoto, F. Yonemori, and K. Wakitani et al. A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits. *Nature*, 406(6792):203–7, 2000.

[33] T. Radeau, P. Lau, and M. Robb et al. Cholesteryl ester transfer protein (CETP) mRNA abundance in human adipose tissue: relationship to cell size and membrane cholesterol content. *J Lipid Res*, 36(12):2552–61, 1995.

[34] T. Radeau, M. Robb, and P. Lau et al. Relationship of adipose tissue cholesteryl ester transfer protein (CETP) mRNA to plasma concentrations of CETP in man. *Atherosclerosis*, 139(2):369–76, 1998.

[35] Y. Wang, S. van der Tuin, and N. Tjeerdema et al. Plasma cholesteryl ester transfer protein is predominantly derived from Kupffer cells. *Hepatology*, 62(6):1710–1722, 2015.

[36] P. J. Kappelle, A. van Tol, and B. H. Wolffenbuttel et al. Cholesteryl ester transfer protein inhibition in cardiovascular risk management: ongoing trials will end the confusion. *Cardiovasc Ther*, 29(6):e89–99, 2011.

[37] M. Bilzer, F. Roggel, and A. L. Gerbes. Role of kupffer cells in host defense and liver disease. *Liver International*, 26(10):1175–1186, 2006.

[38] L. J. Dixon, M. Barnes, and H. Tang et al. Kupffer cells in the liver. *Compr Physiol*, 3(2):785–97, 2013.

[39] L. Masucci-Magoulas, P. Moulin, and X. C. Jiang et al. Decreased cholesteryl ester transfer protein (CETP) mRNA and protein and increased high density lipoprotein following lipopolysaccharide administration in human CETP transgenic mice. *J Clin Invest*, 95(4):1587–94, 1995.

[40] I. Hardardóttir, A. H. Moser, and J. Fuller et al. Endotoxin and cytokines decrease serum levels and extra hepatic protein and mRNA levels of cholesteryl ester transfer protein in syrian hamsters. *J Clin Invest*, 97(11):2585–2592, 1996.

[41] G. Arguello, E. Balboa, and M. Arrese et al. Recent insights on the role of cholesterol in non-alcoholic fatty liver disease. *Biochim Biophys Acta*, 1852(9):1765–78, 2015.

[42] G. N. Ioannou. The Role of Cholesterol in the Pathogenesis of NASH. *Trends Endocrinol Metab*, 27(2):84–95, 2016.

[43] N. Lanthier. Targeting kupffer cells in non-alcoholic fatty liver disease/non-alcoholic steatohepatitis: Why and

how? *World J Hepatol*, 7(19):2184–8, 2015.

[44] W. Liu, R. D. Baker, and T. Bhatia et al. Pathogenesis of nonalcoholic steatohepatitis. *Cell Mol Life Sci*, 73(10):1969–87, 2016.

[45] M. Nati, D. Haddad, and A. L. Birkenfeld et al. The role of immune cells in metabolism-related liver inflammation and development of non-alcoholic steatohepatitis (NASH). *Rev Endocr Metab Disord*, 17(1):29–39, 2016.

[46] W. Peverill, L. W. Powell, and R. Skoien. Evolving concepts in the pathogenesis of NASH: beyond steatosis and inflammation. *Int J Mol Sci*, 15(5):8591–638, 2014.

[47] M. Lazo and J. M. Clark. The epidemiology of nonalcoholic fatty liver disease: a global perspective. *Semin Liver Dis*, 28(4):339–50, 2008.

[48] R. V. Farese and T. C. Walther. Lipid droplets finally get a little R-E-S-P-E-C-T. *Cell*, 139(5):855–60, 2009.

[49] H. Min, A. Kapoor, and M. Fuchs et al. Increased hepatic synthesis and dysregulation of cholesterol metabolism is associated with the severity of nonalcoholic fatty liver disease. *Cell Metabolism*, 15(5):665–674, 2012.

[50] S. Honzumi, A. Shima, and A. Hiroshima et al. LXRalpha regulates human CETP expression in vitro and in transgenic mice. *Atherosclerosis*, 212(1):139–45, 2010.

[51] Y. Luo and A. R. Tall. Sterol upregulation of human CETP expression in vitro and in transgenic mice by an LXR element. *J Clin Invest*, 105(4):513–20, 2000.

[52] A. A. Bielska, P. Schlesinger, and D. F. Covey et al. Oxysterols as non-genomic regulators of cholesterol homeostasis. *Trends Endocrinol Metab*, 23(3):99–106, 2012.

[53] S. Gill, R. Chow, and A. J. Brown. Sterol regulators of cholesterol homeostasis and beyond: the oxysterol hypothesis revisited and revised. *Prog Lipid Res*, 47(6):391–404, 2008.

[54] N. J. Spann, L. X. Garmire, and J. G. McDonald et al. Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. *Cell*, 151(1):138–52, 2012.

[55] C. Zhao and K. Dahlman-Wright. Liver X receptor in cholesterol metabolism. *J Endocrinol*, 204(3):233–40, 2010.

[56] D. Lakomy, C. Rebe, and A. L. Sberna et al. Liver X receptor-mediated induction of cholesteryl ester transfer protein expression is selectively impaired in inflammatory macrophages. *Arterioscler Thromb Vasc Biol*, 29(11):1923–9, 2009.

[57] Institute of Medicine Committee on Assessing Interactions Among Social, Behavioral and Genetic Factors in Health. The National Academies Collection: Reports funded by National Institutes of Health. *Genes, Behavior, and the Social Environment: Moving Beyond the Nature/Nurture Debate*, National Academies Press, Washington (DC), 2006.

[58] James A. Levine and L. Norman et al. Eberhardt. Role of nonexercise activity thermogenesis in resistance to fat gain in humans. *Science*, 283(5399):212–214, 1999.

[59] W. D. van Marken Lichtenbelt and P. Schrauwen. Implications of nonshivering thermogenesis for energy balance regulation in humans. *Am J Physiol Regul Integr Comp Physiol*, 301(2):R285–96, 2011.

[60] M. R. Boon, L. E. Bakker, and A. E. Meinders et al. [brown adipose tissue: the body's own weapon against obesity?]. *Ned Tijdschr Geneeskd*, 157(20):A5502, 2013.

[61] G. Hoeke, S. Kooijman, and M.R. Boon et al. Role of brown fat in lipoprotein metabolism and atherosclerosis. *Circulation Research*, 118(1):173–182, 2016.

[62] J. Wu, P. Bostrom, and L. M. Sparks et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell*, 150(2):366–76, 2012.

[63] T. Yoneshiro, S. Aita, and M. Matsushita et al. Recruited brown adipose tissue as an antiobesity agent in humans. *J Clin Invest*, 123(8):3404–8, 2013.

[64] J. F. Berbee, M. R. Boon, and P. P. Khedoe et al. Brown fat activation reduces hypercholesterolaemia and protects from atherosclerosis development. *Nat Commun*, 6:6356, 2015.

[65] The Interagency Working Group on Climate Change and Health. *A Human Health Perspective On Climate Change*. Environmental Health Perspectives and the National Institute of Environmental Health ciences, 2010.

[66] A. G. Barnett. Temperature and cardiovascular deaths in the US elderly: changes over time. *Epidemiology*, 18(3):369–72, 2007.

[67] K. L. Bassil, D. C. Cole, and R. Moineddin et al. Temporal and spatial variation of heat-related illness using

911 medical dispatch data. *Environ Res*, 109(5):600–6, 2009.

[68] S. Hajat, A. Haines, and C. Sarran et al. The effect of ambient temperature on type-2-diabetes: case-crossover analysis of 4+ million GP consultations across England. *Environ Health*, 16(1):73, 2017.

[69] G. P. Kenny, R. J. Sigal, and R. McGinn. Body temperature regulation in diabetes. *Temperature (Austin)*, 3(1):119–45, 2016.

[70] M. J. Hanssen, J. Hoeks, and B. Brans et al. Short-term cold acclimation improves insulin sensitivity in patients with type 2 diabetes mellitus. *Nat Med*, 21(8):863–5, 2015.

[71] M. Schilperoort, G. Hoeke, and S. Kooijman et al. Relevance of lipid metabolism for brown fat visualization and quantification. *Curr Opin Lipidol*, 27(3):242–8, 2016.

[72] V. Ouellet, A. Routhier-Labadie, and W. Bellemare et al. Outdoor temperature, age, sex, body mass index, and diabetic status determine the prevalence, mass, and glucose-uptake activity of 18F-FDG-detected BAT in humans. *J Clin Endocrinol Metab*, 96(1):192–9, 2011.

[73] M. Saito, Y. Okamatsu-Ogura, and M. Matsushita et al. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes*, 58(7):1526–31, 2009.

[74] X. Shao, X. Shao, and X. Wang et al. Characterization of brown adipose tissue (1)(8)F-FDG uptake in PET/CT imaging and its influencing factors in the Chinese population. *Nucl Med Biol*, 43(1):7–11, 2016.

[75] P. Lee, R. Bova, and L. Schofield et al. Brown adipose tissue exhibits a glucose-responsive thermogenic biorhythm in humans. *Cell Metab*, 23(4):602–9, 2016.

[76] Centers for Disease, Control and Prevention and National Center for Chronic Disease, Prevention and Health, Promotion and Office on, Smoking and Health. *Publications and Reports of the Surgeon General*. Atlanta (GA), 2010.

[77] J.A. Fulkerson and S.A. French. Cigarette smoking for weight loss or control among adolescents: gender and racial/ethnic differences. *Journal of Adolescent Health*, 32(4):306–313, 2003.

[78] J. Audrain-McGovern and N. L. Benowitz. Cigarette smoking, nicotine, and body weight. *Clin Pharmacol Ther*, 90(1):164–8, 2011.

[79] C. Filozof, M. C. Fernandez Pinilla, and A. Fernandez-Cruz. Smoking cessation and weight gain. *Obes Rev*, 5(2):95–103, 2004.

[80] Y. H. Jo, D. A. Talmage, and L. W. Role. Nicotinic receptor-mediated effects on appetite and food intake. *J Neurobiol*, 53(4):618–32, 2002.

[81] P. B. Martinez de Morentin, A. J. Whittle, and J. Ferno et al. Nicotine induces negative energy balance through hypothalamic AMP-activated protein kinase. *Diabetes*, 61(4):807–17, 2012.

[82] A. Hofstetter, Y. Schutz, and E. Jequier et al. Increased 24-hour energy expenditure in cigarette smokers. *N Engl J Med*, 314(2):79–82, 1986.

[83] K. A. Perkins, L. H. Epstein, and R. L. Stiller et al. Acute effects of nicotine on resting metabolic rate in cigarette smokers. *Am J Clin Nutr*, 50(3):545–50, 1989.

[84] L. C. Collins, J. Walker, and B. A. Stamford. Smoking multiple high- versus low-nicotine cigarettes: impact on resting energy expenditure. *Metabolism*, 45(8):923–6, 1996.

[85] Z. H. Huang, L. P. Li, and H. L. Wu et al. The effect of smoking on resting energy expenditure in patients with early diabetic kidney disease. *Zhonghua Nei Ke Za Zhi*, 49(11):939–42, 2010.

[86] S. Y. Kimm, N. W. Glynn, and C. E. Aston et al. Effects of race, cigarette smoking, and use of contraceptive medications on resting energy expenditure in young women. *Am J Epidemiol*, 154(8):718–24, 2001.

[87] J. F. Walker and C. J. Kane. Effects of body mass on nicotine-induced thermogenesis and catecholamine release in male smokers. *Sheng Li Xue Bao*, 54(5):405–10, 2002.

[88] R. J. Loos and G. S. Yeo. The bigger picture of FTO: the first GWAS-identified obesity gene. *Nat Rev Endocrinol*, 10(1):51–61, 2014.

[89] T. L. Assimes and R. Roberts. Genetics: Implications for prevention and management of coronary artery disease. *J Am Coll Cardiol*, 68(25):2797–2818, 2016.

[90] C.K. Chow, S. Islam, and L. Bautista et al. Parental history and myocardial infarction risk across the world. *J Am Coll Cardiol*, 57(5):619–627, 2011.

[91] Y. Friedlander. *Familial Clustering of Coronary Heart Disease: A Review of its Significance and Role as a Risk*

Factor for the Disease, pages 37–53. Springer Netherlands, Dordrecht, 1994.

[92] M. Nielsen, C. Andersson, and T. A. Gerds et al. Familial clustering of myocardial infarction in first-degree relatives: a nationwide study. *Eur Heart J*, 34(16):1198–1203, 2013.

[93] Initial sequencing and analysis of the human genome. *Nature*, 409(6822):860–921, 2001.

[94] R. Sachidanandam, D. Weissman, and S. C. Schmidt et al. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature*, 409(6822):928–933, 2001.

[95] J.C. Venter, M.D. Adams, and E.W. Myers et al. The sequence of the human genome. *Science*, 291(5507):1304–1351, 2001.

[96] The International HapMap Consortium. The International HapMap Project. *Nature*, 426(6968):789–796, 2003.

[97] Consortium The International HapMap. A second generation human haplotype map of over 3.1 million SNPs. *Nature*, 449(7164):851–861, 2007.

[98] M. Cargill, D. Altshuler, and J. Ireland et al. Characterization of single-nucleotide polymorphisms in coding regions of human genes. *Nat Genet*, 22(3):231–8, 1999.

[99] L. Kruglyak and D.A. Nickerson. Variation is the spice of life. *Nat Genet*, 27(3):234–236, 2001.

[100] D.G. Wang, J. Fan, and C. Siao et al. Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. *Science*, 280(5366):1077–1082, 1998.

[101] J. K. Pritchard. Are rare variants responsible for susceptibility to complex diseases? *Am J Hum Genet*, 69(1):124–37, 2001.

[102] T. Flatt and P. S. Schmidt. Integrating evolutionary and molecular genetics of ageing. *Biochim Biophys Acta*, 1790(10):951–62, 2009.

[103] J. Hardy and A. Singleton. Genomewide association studies and human disease. *N Engl J Med*, 360(17):1759–1768, 2009.

[104] H. K. Tabor, N. J. Risch, and R. M. Myers. Candidate-gene approaches for studying complex genetic traits: practical considerations. *Nat Rev Genet*, 3(5):391–397, 2002.

[105] J. N. Hirschhorn. Genomewide association studies — illuminating biologic pathways. *N Engl J Med*, 360(17):1699–1701, 2009.

[106] A. Helgadottir, G. Thorleifsson, and A. Manolescu et al. A common variant on chromosome 9p21 affects the risk of myocardial infarction. *Science*, 316(5830):1491–3, 2007.

[107] R. McPherson, A. Pertsemlidis, and N. Kavaslar et al. A common allele on chromosome 9 associated with coronary heart disease. *Science*, 316(5830):1488–91, 2007.

[108] N. A. Almontashiri, D. Antoine, and X. Zhou et al. 9p21.3 Coronary Artery Disease Risk Variants Disrupt TEAD Transcription Factor-Dependent Transforming Growth Factor beta Regulation of p16 Expression in Human Aortic Smooth Muscle Cells. *Circulation*, 132(21):1969–78, 2015.

[109] O. Jarinova, A.F.R. Stewart, and R. Roberts et al. Functional analysis of the chromosome 9p21.3 coronary artery disease risk locus. *Arterioscler Thromb Vasc Biol*, 29(10):1671–1677, 2009.

[110] T. M. Frayling, N. J. Timpson, and M. N. Weedon et al. A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. *Science*, 316(5826):889–94, 2007.

[111] A. Scuteri, S. Sanna, and W. M. Chen et al. Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. *PLoS Genet*, 3(7):e115, 2007.

[112] E. K. Speliotes, C. J. Willer, and S. I. Berndt et al. Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. *Nat Genet*, 42(11):937–48, 2010.

[113] T. Fall, S. Hagg, and R. Magi et al. The role of adiposity in cardiometabolic traits: a Mendelian randomization analysis. *PLoS Med*, 10(6):e1001474, 2013.

[114] M. Claussnitzer, S. N. Dankel, and K. H. Kim et al. FTO Obesity Variant Circuitry and Adipocyte Browning in Humans. *N Engl J Med*, 373(10):895–907, 2015.

[115] C. Dachet, O. Poirier, and F. Cambien et al. New Functional Promoter Polymorphism, CETP/−629, in Cholesteryl Ester Transfer Protein (CETP) Gene Related to CETP Mass and High Density Lipoprotein Cholesterol Levels: Role of Sp1/Sp3 in Transcriptional Regulation. *Arterioscler Thromb Vasc Biol*, 20(2):507–515, 2000.

[116] D. Drayna and R. Lawn. Multiple RFLPs at the human cholesteryl ester transfer protein (CETP) locus. *Nucleic*

Acids Res, 15(11):4698, 1987.

[117] K. J. Rothman. A pictorial representation of confounding in epidemiologic studies. *J Chronic Dis*, 28(2):101–108, 1975.

[118] G. Davey Smith and S. Ebrahim. What can mendelian randomisation tell us about modifiable behavioural and environmental exposures? *BMJ*, 330(7499):1076–9, 2005.

[119] G. D. Smith and S. Ebrahim. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? *Int J Epidemiol*, 32(1):1–22, 2003.

[120] M. M. Glymour, E. J. Tchetgen Tchetgen, and J. M. Robins. Credible mendelian randomization studies: approaches for evaluating the instrumental variable assumptions. *Am J Epidemiol*, 175(4):332–9, 2012.

[121] N. Solovieff, C. Cotsapas, and P. H. Lee et al. Pleiotropy in complex traits: challenges and strategies. *Nat Rev Genet*, 14:483–495, 2013.

[122] B. F. Voight, G. M. Peloso, and M. Orho-Melander et al. Plasma hdl cholesterol and risk of myocardial infarction: a mendelian randomisation study. *Lancet*, 380(9841):572–80, 2012.

[123] A. Hingorani and S. Humphries. Nature's randomised trials. *Lancet*, 366(9501):1906–8, 2005.

[124] G. Thanassoulis and C. J. O'Donnell. Mendelian randomization: nature's randomized trial in the post-genome era. *JAMA*, 301(22):2386–8, 2009.

[125] B. A. Ference. How to use Mendelian randomization to anticipate the results of randomized trials. *Eur Heart J*, 39(5):360–362, 2018.

[126] R. de Mutsert, M. den Heijer, and T. J. Rabelink et al. The Netherlands Epidemiology of Obesity (NEO) study: study design and data collection. *Eur J Epidemiol*, 28(6):513–23, 2013.

[127] M. G. Wolfs, S. S. Rensen, and E. J. Bruin-Van Dijk et al. Co-expressed immune and metabolic genes in visceral and subcutaneous adipose tissue from severely obese individuals are associated with plasma HDL and glucose levels: a microarray study. *BMC Med Genomics*, 3:34, 2010.

[128] M. Nikpay, A. Goel, and H. H. Won et al. A comprehensive 1,000 genomes-based genome-wide association meta-analysis of coronary artery disease. *Nat Genet*, 47(10):1121–30, 2015.

[129] C. J. Willer, E. M. Schmidt, and S. Sengupta et al. Discovery and refinement of loci associated with lipid levels. *Nat Genet*, 45(11):1274–83, 2013.

[130] J. Kettunen, A. Demirkan, and P. Wurtz et al. Genome-wide study for circulating metabolites identifies 62 loci and reveals novel systemic effects of LPA. *Nat Commun*, 7:11122, 2016.