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

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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.

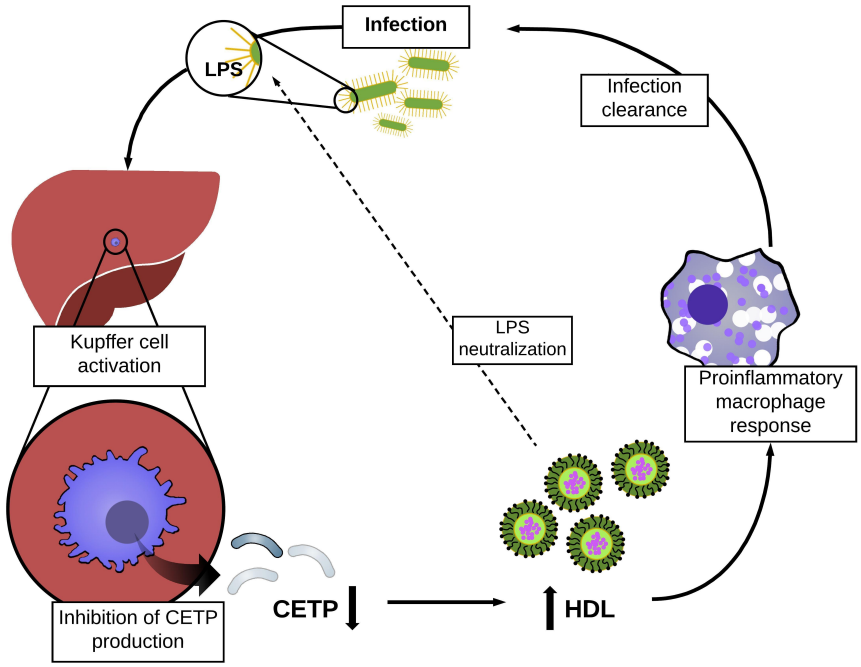

Figure 10.1: We hypothesize that the concentration of circulating CETP is dependent on the infection pressure on the body: a gram-negative bacterial infection leads to activation of Kupffer cells in the liver and a consequent decrease in CETP production by these cells, with the primary purpose to increase 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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