Targeting adipose tissue to improve cardiometabolic health

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General discussion and future perspectives

History is full of favorable annotations towards obesity, after all, it reflects one's ability to feed themselves well, it reflects prosperity. However, the ever increasing prevalence of obesity in modern society also revealed strong associations with diseases such as type 2 diabetes mellitus (T2DM), non-alcoholic fatty liver disease (NAFLD) and cardiovascular diseases (CVDs). CVDs are currently the leading cause of death worldwide, and pharmacological treatment options to lower atherosclerotic CVD (asCVD) risk are mostly limited to cholesterol-lowering medication, which only prevents one-third of all atherosclerotic cardiovascular events.

It is increasingly acknowledged that triglycerides (TGs) and TG-rich lipoprotein (TRL) remnants contribute to asCVD risk [1, 2]. Although life-style changes such as increasing physical activity and reducing caloric intake would be the preferred strategy to lower TGs, it is not always feasible and long-term adherence appears challenging. Novel therapeutic approaches are thus required to sustainably improve TG metabolism in order to attenuate atherosclerosis development. In this thesis, I therefore explored the potential of various therapeutic strategies to improve TG metabolism and cardiometabolic health, with special attention for an appropriate experimental design. The results of these studies are discussed in the current chapter.

Targeting adipose tissue to attenuate dyslipidemia

White adipose tissue (WAT) plays an important role in lipid metabolism. It can store nutritional energy in the form of TGs for use during fasting, but also acts as a buffer for postprandial TGs. Specifically, upon nutrient ingestion lipoprotein lipase (LPL) translocates from adipocytes to the luminal membrane of endothelial cells lining the adipose tissue, thereby increasing the uptake of TG-derived fatty acids (FAs) from TRLs by adipocytes for temporary storage. In the fasted state, intracellularly stored TGs are hydrolyzed and released as FAs into the blood for use by other tissues. This adaptive response of WAT to feeding status is essential for metabolic health and contributes to an anti-atherogenic metabolic profile [3].

In addition to WAT, the human body also contains brown adipose tissue (BAT). Compared to WAT, the TG storage-capacity of BAT is limited. Instead, BAT primarily uses TG-derived FAs as fuel for heat production (*i.e.* thermogenesis). Physiologically, cold exposure stimulates the release of norepinephrine by sympathetic nerve termini that innervate the BAT, leading to thermogenic activation of brown adipocytes via signaling through the β-adrenergic receptor (AR). The lipolytic activity in BAT not only lowers plasma TGs [4], but also leads to the formation of cholesterol-enriched TRL remnants that can be cleared by the liver, thereby indirectly lowering plasma cholesterol levels as well [5]. Adding to this, during lipolysis of TRLs, TRL-surface remnants intercalate into the high-density lipoprotein (HDL) pool, which increases the capacity of HDL for reverse cholesterol transport (*i.e.* transport of cholesterol from peripheral tissues towards the feces) [6]. Prolonged cold exposure or β-adrenergic agonism furthermore induces browning of WAT, which in turn also contributes to the accelerated lipolytic processing and hepatic clearance of TRLs. Taken together, thermogenic adipocytes are capable of reversing dyslipidemia and lowering atherosclerosis as shown in mice [5]. Strikingly, a negative association has recently been reported for the detectability of metabolically active BAT and asCVD risk in humans [7].

Novel therapeutic strategies that improve the lipid buffering capacity of WAT and/or stimulate thermogenic activity of BAT or WAT may thus be valuable to improve lipid metabolism and ultimately attenuate asCVD risk.

Inhibition of the endocannabinoid system

The cannabinoid type 1 receptor (CB1R) is present on adipocytes and inhibits the production of intracellular cAMP [8], which is required for the thermogenic activation of BAT and browning of WAT upon β-adrenergic signaling. The CB1R is furthermore expressed on termini of sympathetic nerves that innervate the adipose tissues, where its activation inhibits the release of the neurotransmitter norepinephrine [9].

Pharmacological blockage of the CB1R has correspondingly been shown to stimulate TG-derived FA uptake by BAT [10]. We have now shown that CB1R inhibition with rimonabant also induces browning of WAT, and potently lowers atherosclerosis development via improving dyslipidemia in APOE*3-Leiden.CETP mice (chapter 2). Others have reported that CB1R inhibition also lowers atherosclerosis development in apolipoprotein E (ApoE) deficient and low-density lipoprotein receptor (LDLr) deficient mice through a direct effect on immune cells resulting in lowered inflammation [11, 12]. Together, these findings implicate that inhibition of the endocannabinoid system (ECS) can lower asCVD risk both via improving dyslipidemia and via reducing inflammation. Interestingly, Krott *et al.* [13] reported that β3-AR agonism increases the levels of endocannabinoids in BAT of mice suggesting the existence of a negative feedback loop to prevent excessive thermogenic activation. This implicates that ECS inhibition might potentiate the effects of cold exposure or a sympathomimetic. In addition, we recently showed that high-fat diet feeding of mice acutely raises the circulating levels of endocannabinoids, coinciding with elevated expression of their synthesis enzymes in BAT and WAT [14]. Given the observed positive association between endocannabinoid tonus and obesity in humans [15], this suggests that targeting the ECS may also be useful to attenuate at least part of the consequences of obesity.

In humans, inverse agonism of the CB1R by rimonabant has shown great potential in lowering adiposity and attenuating dyslipidemia [16, 17], but unfortunately also led to psychiatric side effects within the central nervous system (CNS), resulting in its withdrawal from the market. For this reason, second generation CB1R inverse agonists and antagonists with less brain penetrance have been developed. Although at least some of the effects of rimonabant may be mediated by the CNS via lowering food intake, our group showed that the peripherally-restricted CB1R antagonist AM6545 can also potently activate BAT and attenuate dyslipidemia [10], therefore atheroprotective effects for peripherally restricted CB1R inhibitors are expected comparable to those we have observed for rimonabant. Some of these second generation compounds were also tested in humans in phase 1 clinical trials, which promisingly showed lower CB1R occupancy in the brain compared to rimonabant [18, 19]. However, these compounds initially did not move forward in clinical development, as may be related to difficulties in pharmacokinetics or bioavailability after all. An alternative reason hampering the clinical development of these compounds may be related to concerns amongst researchers regarding the potential of CNS-related side effects on the long term, as chronic use of these antagonist may still cause accumulation in the brain [20].

Nevertheless, the development of new therapies based on peripherally restricted CB1R inverse agonists is still actively ongoing [21]. Recent advancements include the development of third generation compounds with activity at two distinct receptors. For example, a hybrid CB1R inverse agonist and inducible nitric oxide synthase (iNOS) inhibitor has been developed for the treatment of liver fibrosis. In this combination, CB1R inhibition exerts growth-inhibitory and proapoptotic effects on hepatic myofibroblasts [22], while iNOS inhibition lowers the generation of proinflammatory reactive nitrogen species [23, 24], together resulting in stronger

anti-fibrotic effects than rimonabant [23, 24].

Compounds that inhibit the synthesis of endocannabinoids themselves may also be valuable to modulate ECS tonus. So far, inhibitors of diacylglycerol lipase (DAGL) [25] and N-arachidonoylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) [26] have been developed with proven efficacy in lowering endocannabinoid levels *in vivo*, and in our initial experiments their use seems to delay acute weight gain in mice as induced by a high-fat diet, by transiently lowering food intake (unpublished observations). Long-term studies will be needed to investigate tissue-specificity of these endocannabinoid synthesis inhibitors, as well as their safety and potential for attenuating adiposity and atherosclerosis development.

Targeting the incretin system

Long-acting glucagon-like peptide-1 (GLP1) receptor (GLP1R) agonists, used in the clinic as first-line treatment for T2DM [27], are currently also used as treatment for obesity as addition to life style intervention [28]. Studies have furthermore shown that analogues of the other main incretin hormone glucose-dependent insulinotropic polypeptide (GIP) can also lower body weight [29], at least in part via mechanisms different from GLP1R agonism [30, 31]. In order to further improve glycemic control and lower body weight, recent advances therefore include combining GLP1R agonism with the complementary actions of GIP receptor (GIPR) agonism. Clinical trials with the GIPR and GLP1R agonist NNC0090-2746 (RG7697) or tirzepatide (LY3298176; Mounjaro) in patients with T2DM showed superior glycemic control and body weight loss as compared to single GLP1R agonism [32-35]. In fact, Mounjaro was recently approved by the U.S. Food and Drug Administration (FDA) for improving glycemic control in adults with type 2 diabetes, as a complement to diet and exercise [36], and was recently admitted to an U.S. FDA Fast Track for treatment of obese or overweight adults with weight-related comorbidities [37].

Both GIPR and GLP1R agonism have also been implicated in lipid metabolism, that is, GLP1R agonism is capable of thermogenically activating BAT and inducing browning of WAT [38], while GIPR agonism enhances the lipid-buffering capacity of WAT by stimulating the uptake of lipids in the postprandial state [39-41] and increases intracellular lipolysis in the fasted state [42, 43]. Adding GIPR agonism to GLP1R agonism may thus further improve lipid metabolism as compared to GLP1R agonism alone by improving postprandial lipid handling, but possibly also by further stimulating thermogenic adipocytes by inducing a flux of FAs from WAT as fuel for combustion. Combined with the anti-inflammatory properties of both GIPR and GLP1R agonism [44-49], which could be additive as both receptors are expressed by most endothelial and immune cells, combined GIPR/GLP1R agonism is considered a suitable strategy to attenuate asCVD risk. Indeed, we observed that combined GIPR/GLP1R agonism lowers atherosclerosis severity in APOE*3-Leiden.CETP mice as related to a pronounced reduction in plasma TG levels and decreased markers of systemic low-grade inflammation (chapter 3). Notably, TG-lowering effects of combined GIPR/GLP1R agonism have also been reported in the beforementioned clinical trials. However, in both mice and humans the reduction in TG levels did not translate one-to-one to a decrease in cholesterol levels. This might be related to an increased hepatic secretion of cholesterol within very-low-density lipoprotein (VLDL) as we have demonstrated in mice (chapter 3). A phase 3 trial that investigates the effects of tirzepatide on major cardiovascular outcomes in obese individuals with T2DM is currently ongoing (trial registration no. NCT04255433), and will provide more insight into the effects of combined GIPR/GLP1R agonism on asCVD risk in humans. It may be worthwhile to investigate whether addition of a cholesterol-lowering agent (*e.g.* a statin) to combined GIPR/GLP1R agonism lowers

cholesterol levels compared to combined GIPR/GLP1R agonism by preventing the increased VLDL-cholesterol production and enhancing (V)LDL-cholesterol uptake by the liver.

As insulin resistance and obesity are strong risk factors for NAFLD development, combined GIPR/GLP1R agonism is also an interesting candidate to reduce NAFLD development. Indeed, we have demonstrated that combining GIPR and GLP1R agonism additively lowers hepatic steatosis as related to reduced food intake, increased uptake of circulating nutrients by BAT and increased fecal energy excretion, and also reduces hepatic inflammation in mice (chapter 4). Interestingly, treatment of patients with T2DM with the GIPR and GLP1R agonist tirzepatide also lowers the non-alcoholic steatohepatitis (NASH)-related biomarkers alanine aminotransferase (ALT), aspartate aminotransferase (AST), keratin-18 (K-18) and procollagen III (Pro-C3) [50]. Together, these findings hold promise for combined GIPR/GLP1R agonism as a future treatment modality for NAFLD and NASH in humans. Accordingly, an ongoing phase 2 trial (trial registration no. NCT04166773) currently investigates the effects of tirzepatide on NAFLD and hepatic fibrosis and will provide further insight into the relevance of combined GIPR/GLP1R agonism for treatment of NAFLD and NASH in humans.

Very interestingly, recent studies in rodents have shown that adding glucagon receptor (GCGR) agonism to combined GIPR/GLP1R agonism improves glycemic control and lowers body weight beyond the effect of combined GIPR/GLP1R agonism, even when comparing a triple agonist with a dual agonist at equimolar dose [51-53]. The catabolic and thermogenic properties of GCGR agonism increases energy expenditure as an additional mechanism to lower body weight [52, 54]. Indeed, a recent phase 1 trial in healthy individuals already showed a persistent body weight loss of approximately 3 kg upon administration of a single dose of a GIPR/GLP1R/GCGR agonist [53]. GCGR agonism by itself stimulates glucose release from glycogen stores in the liver to elevate blood glucose; nonetheless the glucose-lowering effects of both GIPR and GLP1R agonism seem to be sufficiently capable of preventing such an increase [52, 53]. Importantly, glucagon has been shown to directly bind to the GCGR on hepatocytes to increase hepatic beta oxidation and lower hepatic lipogenesis [55], which may help even further improving lipid metabolism when combined with concomitant GIPR/GLP1R agonism. Investigating the effects of concomitant GIPR/GLP1R/GCGR agonism on asCVD and NAFLD development in mice and humans is therefore an interesting topic for future research.

Translation of observations in BAT-targeted research in mice to humans

Choice of mouse model and experimental design

Experiments in APOE*3-Leiden.CETP mice consistently showed that improving the lipid buffering capacity of WAT and/or stimulating thermogenic activity of BAT or WAT coincides with an anti-atherogenic lipid profile and improvements in cardiometabolic health ([5]; chapters 2 and 3). A recent large retrospective study furthermore showed a negative association between the presence of metabolically active BAT and asCVD risk in humans [7]. The atheroprotective effects of promoting thermogenic activity in BAT and WAT are largely the result of the accelerated formation of delipidated, cholesterol-enriched TRL remnants that can be cleared by the liver, thereby leading to a reduction in circulating cholesterol in addition to lowered TGs as demonstrated in APOE*3-Leiden.CETP mice. As the clearance of TRL remnants by the liver is highly dependent on the acquisition of ApoE by the TRLs and the subsequent interaction of ApoE with the LDLR on hepatocytes, it is not entirely unexpected that activation

of BAT by cold [56] or by the β-adrenergic agonist mirabegron [57] in ApoE or LDLR knockout mice results in accumulation of these pro-atherogenic TRL remnants in the circulation and therefore aggravates atherosclerosis development in those mice. Such observations in ApoE and LDLR deficient mice are thus the result of an artifact of the models, rather than representing a biological effect. Indeed, we showed that a comparable treatment with mirabegron in female APOE*3-Leiden.CETP mice that have a delayed, but functional ApoE-LDLR pathway, resulted in a decrease in circulating cholesterol and attenuated atherosclerosis development upon BAT activation and WAT browning (chapter 6).

The APOE*3-Leiden.CETP mouse is thus an appropriate model for studying lipid-driven atherosclerosis as well as potential atheroprotective effects of interventions that improve lipid metabolism. In fact, together with single-transgenic APOE*3-Leiden mice it is the only mouse model that responds to lipid-lowering treatments such as statins and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors in a human-like manner. As atherosclerosis development in this mouse model is primarily lipid-driven, a concurring limitation is that it is challenging to investigate inflammation-driven atherosclerosis development in this mouse model. It should also be noted that only female APOE*3-Leiden.CETP mice develop atherosclerosis, likely because male APOE*3-Leiden.CETP mice fail to increase hepatic VLDL production in response to a cholesterol-containing Western-type diet and typically have a higher VLDL clearance rate when compared to their female counterparts [58]. These same properties cause male APOE*3-Leiden.CETP mice to be susceptible to developing NAFLD when fed a diet enriched in cholesterol and fat, making it a suitable model as also used in chapter 4 to investigate interventions that aim to attenuate diet-induced NAFLD.

Critical attention should also be paid to aspects of experimental design beyond the choice of animal model to ensure maximal validity of the results. This includes the use of randomization and blinded outcome assessment. While these methods are common practice in human trials, a worrisome two-third of preclinical studies do not report their use [59]. Implementation of blinding and randomization, however, has been found to reduce effect sizes [59], suggesting that results in the majority of animal studies may, at least to a certain degree, be subject to a bias. To overcome such bias, we have developed RandoMice as a user-friendly tool that can aid researchers in randomizing mice, and other experimental units, at the start of an experiment (chapter 7). Using this software, users can furthermore take randomization one step further by creating groups that are balanced for baseline variations in the outcome measures and potential confounders as we have done in chapters 3, 4 and 6, allowing the number of animals within an experiment to be kept at a minimum to detect an effect of a given size, which is a necessity from an ethical point of view. As long as the use of animals is inevitable to study the complex interactions between metabolic tissues, the use of tools such as RandoMice can add to minimizing the need of experimental replication and validation by increasing the validity of results (*i.e.* by reducing bias), leading to an overall reduction of the animals used.

The current version of RandoMice assumes that all data is continuous and normally distributed thereby limiting distribution of experimental units based on categorical or nominal variables or covariates (*e.g.* sex, tumor stage, *etc.*). In the future, approaches could additionally be incorporated to further improve group division when starting an experiment in multiple batches or cohorts. To further aid researchers, the software could also be extended to include a convenient method for blinded outcome assessment.

Studying the role of human BAT in cardiometabolic health

When investigating the role of BAT or the efficacy of a BAT-targeted intervention on lowering asCVD risk in humans, human BAT is ideally quantified using non-invasive methods. The current gold standard for BAT visualization in humans is by quantifying glucose uptake as traced with [^{18}F]fluorodeoxyglucose (FDG) PET-CT scans. However, studies in rodents suggest that quantifying TG-derived FA uptake better reflects metabolic BAT activity. A first underlying reason for this is that glucose uptake via glucose transporter 4 (GLUT4) is highly dependent on insulin signaling, therefore the labeled glucose analogue [^{18}F]FDG will be taken up less upon insulin-resistance, *e.g.* as a result of obesity or ageing. In addition, cold exposure has been shown to specifically enhance the oxidation of FAs rather than glucose [60]. As an alternative to [^{18}F]FDG PET-CT scans, attempts have therefore been made to visualize BAT by FA uptake as traced by [^{18}F]fluoro-6-thia-heptadecanoic acid (FTHA) PET-CT scans [61]. Although the uptake of these free FA tracers by metabolic tissues including BAT is not hampered by insulin resistance, upon intravenous injection free FAs are efficiently bound by albumin for transport to the liver thereby restricting its uptake by BAT and resulting in a strong hepatic background signal [62]. More importantly, we previously showed in mice that BAT primarily takes up FAs from TRL-derived TGs rather than in free form, taking advantage of the high expression and activity of the TG hydrolyzing enzyme LPL in BAT [63]. Ongoing research in our group therefore focuses on incorporating a PET-compatible [^{18}F]TG tracer into TRL-like particles [64] to ultimately specifically quantify TG-derived FA uptake by BAT in humans. As compared to GLUT4-mediated glucose uptake, LPL activity and therefore TG-derived FA uptake is only to some extent influenced by the actions of insulin. Furthermore, uptake by the brain which is typically high in [^{18}F]FDG PET-CT as the brain is strictly dependent on glucose oxidation, will be negligible, collectively allowing for lower radioactive dosing. The use of such PET-compatible TG tracers thus holds promise for future studies in humans to investigate TG-derived FA uptake by BAT in comparable intervention experiments as done in mice in the current thesis (chapters 2-6), using PET-CT as visualization method.

In both mice and humans, β-AR agonism has proven to be an effective strategy to activate thermogenesis. For example, treatment of APOE*3-Leiden.CETP mice with the highly selective β3-AR agonist CL316,243 increases the uptake of TG-derived FAs by BAT, stimulates browning of WAT, increases energy expenditure, and protects against atherosclerosis development [5]. Similarly in humans, use of the β3-AR agonist mirabegron, used in the clinic to treat overactive bladder, increases energy expenditure, stimulates [^{18}F]FDG uptake by BAT as measured by PET-CT and reduces supraclavicular BAT fat fraction as measured by MRI (*e.g.* 65-69). However, these effects of mirabegron were seen at the supra-pharmacological dose of 200 mg instead of the 50 mg that is used to treat hyperactive bladder. Recent studies have subsequently revealed that mirabegron at 50 mg does not activate BAT in humans, and that the β2-AR responsible for BAT activation in humans [70]. The BAT-activating effects of supra-pharmacological dosing of mirabegron are thus likely caused by off-target binding of mirabegron to the other β-ARs [70]. As the β2-AR is more broadly expressed than the β3-AR, including in the pulmonary and cardiovascular systems, β2-AR agonism results in unwanted side effects and is thus probably not a feasible strategy to activate BAT if the β2-AR agonists is not specifically directed to brown adipocytes. Other pharmacological strategies are thus required, such as those that activate BAT via indirect modulation (*e.g.* GIPR/GLP1R agonism) or via mechanisms independent of β-adrenergic signaling.

Identifying novel pharmacological targets to stimulate BAT thermogenesis

Given the limitations of current pharmacological targets in BAT, novel pharmacological strategies are desired that ideally would specifically target BAT with minimal risk of side effects. A recent study investigated which membrane associated G_s coupled G-protein coupled receptor was most upregulated upon cold-exposure, which led to the identification of G-protein coupled receptor 3 (GPR3) as a lipolysis-induced receptor with constitutive activity that can activate thermogenesis [71], which allows for finding (pharmacological) strategies that induce expression and membrane translocation of GPR3 in BAT to activate thermogenesis. An alternative method for revealing novel pharmacological strategies to target BAT is by identifying the naturally occurring switches within BAT that are responsible for the strong day-night rhythm in TG-derived FA uptake by the tissue. By studying gene expression patterns in BAT throughout a 24-hour period by RNA-sequencing, we found that the diurnal oscillation in TG-derived FA uptake by BAT is in synchrony with the diurnal expression of *Lpl*, in line with previous a report showing a crucial role of LPL in TG-derived FA uptake by BAT [4]. Interestingly, we also found that expression of the LPL inhibitor angiopoietin-like 4 (*Angptl4*) displays a strong oscillation in a phase opposite to that of *Lpl*, and that ANGPTL4 plays a critical role in the diurnal regulation of LPL on protein level (chapter 5). In fact, we demonstrated that ANGPTL4 is responsible for regulating LPL activity within the range of its physiological day-night rhythm (chapter 5). ANGPTL4 has indeed been considered as a target for lowering CVD risk, as loss-of-function gene variants in humans are associated with lower plasma TG levels and attenuated CVD risk. In fact, treatment of mice with anti-ANGPTL4 monoclonal antibodies or with antisense oligonucleotides causally improve dyslipidemia and atherosclerosis [72-79]. It should be noted, however, that whole body-deficiency of ANGPTL4 in mice results in high-fat diet-induced ascites by massive macrophage activation [80], therefore approaches that target ANGPTL4 expression should probably be tissue-specific. Given high expression of ANGPTL4 in hepatocytes, an ongoing phase 1 clinical trial investigates the effect of liver-specific downregulation of ANGPTL4 via antisense oligonucleotides that are targeted to the hepatocyte-specific asialoglycoprotein receptor [81]. In future studies that target ANGPTL4, the diurnal oscillations in LPL activity should be taken into account, as inhibition of ANGPTL4 at the onset of the resting phase (when expression of *Angptl4* in BAT is highest) may be clinically most relevant for stimulating TG-derived FA uptake by BAT.

We also revealed that expression of the oscillating genes in BAT that are in synchrony with TG-derived FA uptake by the tissue were predicted to be driven by peroxisome proliferator-activated receptor γ (PPARγ), and correspondingly, we could show oscillated binding of PPARγ to *Lpl*, preceding changes in *Lpl* expression (chapter 5). PPARs are known to play an important role in lipid metabolism, and in fact may function as FA sensors as (polyunsaturated) FAs are their natural ligands [82]. PPARγ has already been considered as a pharmacological target to improve metabolic health, and PPARγ agonists (*i.e.* thiazolidinediones) lower insulin resistance in humans [83]. Given that others have demonstrated that PPARα agonists (*i.e.* fibrates) are capable of improving dyslipidemia [84], which may in part be mediated via reductions in hepatic APOC3 production [85, 86], this led to the development of combined PPARα/γ agonists, which have been shown to effectively improve glucose and lipid homeostasis in clinical trials [87, 88]. Development of these combined PPARα/γ agonists, however, initially stalled owing to adverse cardiovascular events [87, 88]. Since then, novel PPARα/γ agonists have been developed which show less side effects [88]. In addition, novel developments include the linkage of a PPARα/γ

agonist to a GLP1R agonist for GLP1R-mediated cellular delivery of the PPARα/γ agonist [89]. As the GLP1R is not expressed by adipocytes, a comparable approach may be used to link a PPARγ agonist to a ligand of another receptor that is expressed by BAT, for example GPR3. Moreover, further insight into the role of PPARγ in the diurnal regulation of BAT may provide rationale for how to optimally target PPARγ in BAT. For example, the use of a short-lived PPARγ agonist at an optimized time of the day, as opposed to long-lived PPARγ agonist with constitutive activity, may be clinically valuable to maximize the desired response and minimize potential side effects.

An interesting notion is that during thermogenesis brown adipocytes excrete damaged parts of mitochondria as external vesicles to ensure efficient thermogenesis [90], and it was found that these extracellular vesicles induce autocrine signaling leading to disrupted PPARγ signaling [90]. Given that among the genes with the largest absolute diurnal amplitude in chapter 5 are several mitochondrial genes, this may indicate that large diurnal changes in mitochondrial dynamics are required throughout the day. If the content and/or excretion of those extracellular vesicles turns out to be rhythmic in future experiments, that could provide another clue regarding the diurnal regulation of metabolic BAT activity, and may provide novel therapeutic handles to stimulate the tissue.

Besides the potential targets within BAT, it may also be worthwhile to investigate what the main (external) driving force behind the diurnal activity of BAT is in order to identify novel targets. Our group has previously shown that the rhythm in glucocorticoids, that is in synchrony with TG-derived FA uptake by BAT, modulates oscillating BAT activity. However, this rhythm is likely indirect as expression of the glucocorticoid receptor in BAT itself was shown not to be involved [91]. Possibly, modulation of sympathetic outflow towards the tissue links glucocorticoids to oscillating BAT activity [91], and an initial stimulus from the sympathetic nervous system may be required to stimulate intracellular lipolysis in BAT, leading to increased thermogenesis and activation of PPARγ. It would therefore be interesting to investigate the rhythm in LPL activity in BAT-specific inducible PPARγ-knockout models.

Overall, the strong diurnal oscillations in BAT implicate that time of the day is essential when measuring its activity. Similarly, time of day should be taken into account when targeting the tissue for example by ANGPTL4 inhibition or PPARγ agonism. Given that for example shift work has been associated with an increased risk for asCVD [92], further studies are also required to elucidate the effects of rhythm disturbances on the diurnal oscillations in BAT and the subsequent consequences on metabolic health.

Concluding remarks and future prospectives

Over the course of the past decades, the number of obese and overweight people worldwide has reached pandemic proportions and with that, the associated risk for disease has become increasingly evident. This thesis has provided insights into various strategies that target the adipose tissue to improve lipid metabolism and cardiometabolic health.

We showed that targeting the ECS reduces atherosclerosis development in a humanized mouse model, and therefore may be a valuable strategy to lower asCVD risk in humans as well. Development of novel pharmacological strategies that target the ECS is ongoing, and focuses on peripherally restricted CB1R inhibitors and/or inhibitors of endocannabinoid synthesis enzymes, possibly combined with other pharmacological strategies. Using the humanized mouse model we also showed that combined GIPR/GLP1R agonism attenuates atherosclerosis severity and that GIPR and GLP1R agonism additively attenuate NAFLD development. Given that a dual

GIPR/GLP1R agonist has recently been approved for clinical use in T2DM by the U.S. Food and Drug Administration, and multiple phase 3 trials are underway, data on the atheroprotective and NAFLD-lowering effects of combined GIPR/GLP1R agonism in humans are expected within the next few years. In order to further improve the efficacy of incretin-based therapies, concomitant GIPR/GLP1R agonism can likely be combined with other pharmacological strategies. Current studies for example focus on the addition of GCGR agonism to combined GIPR/GLP1R agonism to beneficially modulate energy metabolism in the liver (via the GCGR) in addition to the brain (via the GLP1R) and adipose tissue (via the GIPR), which will likely prove to be valuable to further improve (cardio)metabolic health in the future.

We demonstrated that the effects of BAT-targeted interventions on atherosclerosis development and therefore also the translational value of experiments critically depends on a suitable choice of mouse model. To ensure maximal validity of the obtained results, attention should furthermore be paid to aspects of experimental design beyond the choice of mouse model. In this thesis, we have therefore developed RandoMice as a user-friendly tool to aid researchers with randomizing animals at the start of an experiment. Currently, efforts are actively made to facilitate a transition from animal models towards alternative models, which is a necessity from an ethical perspective. Until suitable *in vitro* models are available to study the complex interactions between metabolic tissues in relation to (cardio)metabolic health, *e.g.* those mediated via neuronal and hormonal signals, researchers should aim at minimizing the use of animals. Specifically, critical attention should be paid to the choice of animal model and other aspects of experimental design such as the use of randomization in order to maximize the translational value and validity of each experiment, which will result in an overall reduction of the required number of animals.

Finally we showed that ANGPTL4 plays a critical role in regulating LPL activity in BAT and thereby governs the rhythm in TG-derived FA uptake by the tissue, and revealed potential involvement of PPARγ in the regulation of diurnal gene expression. The importance of the day-night rhythm within metabolic tissues is increasingly acknowledged by researchers. Further insight into the regulatory mechanism behind these rhythms may yield valuable insights into how to optimally target these tissues. Specifically, it will provide rationale for optimal timing of existing (*e.g.* statins) and novel treatments (*e.g.* those targeting ANGPTL4), which will not only improve their efficacy, but also reduce the risk of side effects.

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