# Modulating energy metabolism: pathophysiological aspects and novel interventions

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# CHAPTER 10

## General discussion and future perspectives

# GENERAL DISCUSSION

Obesity is currently one of the most important public health problems worldwide and the number of people affected by obesity is still growing. In March 2022, the World Obesity Federation published the World Obesity Atlas in which they estimated that by 2030 globally 1 billion people will be living with obesity (defined as a body mass index (BMI) > 30 kg/m$^2$), which will correspond to 1 in 5 women and 1 in 7 men (1). Together with the steep increase in obesity prevalence, the incidences of obesity-associated cardiometabolic diseases, including type 2 diabetes (T2D) and cardiovascular diseases (CVD), will rise rapidly as well. Obesity is a complex disease, with a chronic and relapsing character, with multiple drivers and determinants. Yet, obesity is commonly seen as personal failure with little recognition from the society, making it difficult for individuals to fight. Identification of obesity-associated factors that drive cardiometabolic disturbances, especially in populations at risk for cardiometabolic diseases such as South Asians, is urgently needed to develop targeted therapy. Moreover, for the prevention and treatment of obesity and associated cardiometabolic diseases, better understanding of the regulation of energy metabolism and exploration of novel treatment strategies, including those improving energy metabolism, is highly warranted.

In the first part of this thesis, we described novel pathophysiological aspects of cardiometabolic conditions, including in the metabolically vulnerable South Asian population. To this end, in patients with genetic hypertriglyceridemia due to mutations in lipoprotein lipase (LPL) or its regulators, we comprehensively assessed the (apo) lipoprotein profile, to obtain insight in the consequences of dysfunctional LPL on lipoprotein metabolism. Moreover, we studied mRNA transcript levels of a large panel of immune genes in overweight-to-obese Dutch South Asian *versus* Dutch Europid patients with T2D, with the aim to elucidate the role of inflammation in the rapid progression of T2D in South Asians. Furthermore, we described new insights on the pathophysiology of adiposity in patients with narcolepsy type 1 and proposed a role for brown adipose tissue (BAT). In the second part of this thesis, we describe the possibilities to modulate energy metabolism by the use of cold exposure and the activation of BAT. Specifically, we studied the effect of cold exposure on the immune system and on whole-body lipid metabolism. Furthermore, we elaborated on the role and the consequences of the biological clock in BAT, followed by the assessment of the best timing of the day to apply cold exposure for the modulation of energy metabolism. Finally, we focused on the pharmacological activation of BAT, by the use of the β2-adrenergic receptor agonist salbutamol.

This thesis provides new insights in the regulation of energy metabolism, both in patients with cardiometabolic diseases as well as in healthy individuals. Moreover, promising novel therapeutic interventions to combat cardiometabolic diseases are further explored. In **this chapter**, new insights, their implications in current clinical practice, and future challenges will be discussed.

## Unraveling the high type 2 diabetes risk in South Asians

### *The role of inflammation*

People from South Asian descent are at particularly high risk to develop T2D and show a more rapid and more severe disease progression compared to Europids, including higher rates of macro- and microvascular complications that occur already at an earlier age (2). Although several factors have been proposed to be involved, including their disadvantageous fat distribution and higher insulin resistance state, the exact mechanism (s) that contribute (s) to their elevated T2D risk is still largely unknown (3). As described in **chapter 1**, inflammation is increasingly recognized as a central player in the development and progression of T2D. During the course of obesity, excessive accumulation of lipids within the white adipocytes, leading to hypertrophic adipocytes, can result in local hypoxia, dysfunctional adipocytes, the secretion of cytokines, and the infiltration of immune cells into the adipose tissue (4-6). Especially centrally located visceral adipose tissue appears prone for the development of inflammation and inflamed visceral adipose tissue is shown to be linked to obesity comorbidity (7, 8). Interestingly, healthy South Asians, compared to Europids, possess larger subcutaneous adipocytes and preferably store fatty acids in centrally located depots (9-11). Both factors thus may put the adipose tissue of South Asians at higher risk for adiposity-associated inflammation. In line with this, independent of adipose tissue mass, in nonobese South Asians compared to Europids, higher macrophage infiltration has been reported in abdominal subcutaneous adipose tissue (12). Apart from that study, up till now studies on the inflammatory phenotype of South Asians have been scarce. In **chapter 3**, we showed that many circulating mRNA transcripts of immune related genes are higher in Dutch South Asian than Dutch Europid patients with T2D. This particularly involved higher transcript levels of interferon (IFN) signaling genes, with the IFN signaling pathway being the most enhanced canonical pathway. The question remains, however, whether the increased pro-inflammatory state with an enhanced IFN signaling pathway actually contributes to the development of T2D or is merely a result of a longer disease duration in South Asians, as South Asians develop T2D approx. 5-10 years earlier than Europids (13, 14). Indeed, in **chapter 5**, we showed considerably less differences in circulating mRNA transcripts of immune genes in a cohort of lean Dutch South Asians and Dutch Europids, *i.e.*, before they have developed metabolic disturbances. Unfortunately, in both studies, we were unable to obtain information about immune cell composition and

number in blood nor within the metabolic tissues. Hence, it remains unclear 1) whether circulating mRNA transcripts represent higher gene expression per immune cell or higher immune cell numbers, 2) how circulating mRNA transcripts relate to immune cell phenotype and function and 3) whether local inflammation within metabolic tissues is present. To answer these questions and elucidate if an increased pro-inflammatory state is already present in healthy and lean South Asians, future research should focus on comparing immune cell composition and phenotype in blood as well as in insulin-target tissues. Furthermore, since monocyte-derived macrophages are one the of the hallmarks of dysfunctional adipose tissue as well as a central player in development of atherosclerotic CVD (15), it would be of high interest to assess monocyte phenotype in more detail, for instance by measuring *in vitro* inflammatory responses of monocytes derived from South Asians *versus* Europids upon an inflammatory challenge, *e.g.*, with the endotoxin lipopolysaccharide (LPS). Despite the fact that a lot still needs to be discovered, the immune system seems more triggered in South Asian patients with T2D, suggesting that South Asians are especially amenable to anti-inflammatory strategies.

Our data and those obtained from future studies may reveal opportunities for clinical treatments that may reduce obesity-associated cardiometabolic diseases in the South Asian population by impacting on the immune system. For instance, recent trials have shown that anti-inflammatory therapies, such as with the interleukin (IL)-1-receptor antagonist Anakinra (16) and the anti-inflammatory compound salsalate (17, 18), improve glycemic control in patients with T2D. In addition, salsalate reduces glycemia in nondiabetic individuals with obesity (19). In patients with previous myocardial infarction, the IL-1β inhibitor canakinumab (20) and anti-inflammatory compound colchicine (21) reduce recurrent major adverse cardiovascular events. Therefore, dedicated clinical trials are needed to reveal whether such anti-inflammatory therapies are especially effective for the treatment of cardiometabolic diseases in the South Asian population and how they can be safely used in the clinic.

*The role of endocrine factors*

Together with the preference to store fat in the centrally located adipose tissue depots, South Asians often possess a low lean mass, which together generally results in a 'normal' BMI. This characteristic phenotype is often referred to as the 'thin-fat phenotype' and is seen as one of the factors that contributes to the development of insulin resistance, both due to enhanced adiposity-associated inflammation as well as a reduced capacity for glucose uptake from the blood by skeletal muscle (9, 22). Intriguingly, this phenotype fits with the clinical presentation of patients with hypercortisolism, which is caused by elevated levels of the stress hormone and glucocorticoid cortisol. Chronic hypercortisolism leads to hyperglycemia and insulin

resistance and is associated with abdominal, predominantly visceral, adiposity as well as muscle wasting resulting in low muscle mass (23, 24). Remarkably, and in contrast what may be expected, previously unpublished results from our group showed that in South Asians circulating cortisol levels, as determined around their daily peak in the morning, are markedly lower compared to Europids (205±78 *versus* 307±76 nmol/L, P = 0.008), which is in line with data of others (25). Since glucocorticoid-binding globulin levels do not differ between ethnicities (25), this may suggest that South Asians have increased glucocorticoid sensitivity. This actually fits with a previous case report that suggested that South Asian patients are more likely to respond on dexamethasone treatment compared to Europids (26). Therefore, a have higher glucocorticoid sensitivity of South Asians compared to Europids may well underlie their unhealthy fat distribution and disadvantageous metabolic phenotype. Interestingly, selective glucocorticoid receptor modulators have been developed, which are compounds that induce a specific combination of agonistic and antagonistic glucocorticoid receptor properties by activating only a subset of downstream signaling pathways. Although the efficacy and safety for clinical use is still being tested, these compounds could potentially be used in the clinic to induce anti-inflammatory effects with minimal (metabolic) side effects (27). Hypothetically, such compounds could eventually also be developed to counteract unfavorable metabolic effects of endogenous cortisol, and be applied to 'correct' the adverse metabolic phenotype of South Asians.

Another stress-related factor that has obtained a lot of scientific interest in the field of metabolism is fibroblast growth factor 21 (FGF21). FGF21 is a hormone predominantly produced and released by the liver in response to metabolic stress (*i.e.*, extreme fasting, acute exercise), after which FGF21 can reach various target tissues via the circulation (28). Studies from our group (29) and other groups (30) (31) show that pharmacological administration of FGF21 has beneficial effects on lipid and glucose handling. Specifically, our group has shown that administration of long-acting FGF21 in mice is able to activate BAT and induce browning of white adipose tissue (WAT), which, together with a reduction in adipose tissue inflammation, improves dyslipidemia and glucose tolerance (29). Interestingly, by using a targeted proteomics panel, we found that FGF21 is much lower in plasma of overweight-to-obese South Asian compared to Europid patients with T2D (Fold change: 0.48, FDR =0.001; unpublished data from the cohort described in **chapter 3**). Since circulating FGF21 levels would actually be expected to be higher in South Asians, related to their generally higher state of metabolic stress, the finding that FGF21 is actually lower may implicate a causal relation to their disadvantageous metabolic phenotype. Moreover, as FGF21 is currently being developed as potential pharmacological approach to treat obesity-associated diseases such as T2D and CVD in humans, this approach may potentially be especially beneficial for South Asians.

It is important to note though that circulating levels of both glucocorticoids and FGF21 have a pronounced circadian rhythm, with a peak early in the morning at the start of the active period (32). In our studies, blood samples were taken in the morning after an overnight fast, with the purpose to take blood samples at the same 'circadian time' for each individual. Hypothetically, a dampened diurnal rhythm in glucocorticoids and/or FGF21 with lower amplitudes in South Asians *versus* Europids could explain lower circulating levels. In line with this hypothesis, it has been reported that migrant South Asians have a lower daily light exposure and higher night-time activity and sleep fragmentation than Europids (33). To test whether South Asians are subjected to perturbations in the stress response and/or disturbed circadian regulation of physiological metabolic processes, a dedicated clinical trial may be performed that includes the assessment of diurnal cortisol and FGF21, 24-hour cortisol excretion, long-term cortisol exposure (*i.e.*, by using a small hair sample (34)), and an overnight very-low dose (0.25 mg) dexamethasone suppression test. The latter tests how strongly the hypothalamic-pituitary-adrenal axis is suppressed after a very-low dose dexamethasone and could give an indication of glucocorticoid sensitivity (35). Since prevention and treatment of T2D in South Asians might require a different approach than in Europids, optimal understanding of its pathophysiology is highly warranted.

*Matching South Asians and Europids in clinical trials*

A major challenge for researchers who study and compare different ethnic populations in clinical trials is proper matching of the populations (**chapter 3**). In most clinical trials, cases and controls are matched according to age and BMI, to minimize variability in parameters other than the primary outcome of interest. However, for comparing South Asians with Europids with T2D, matching on BMI often means that South Asians have a higher fat mass and suffer from T2D for a longer period, including a longer exposure to T2D associated cardiometabolic risk factors and pharmacological treatment period. Instead, individuals may be matched on diabetes duration, *e.g.*, by including individuals directly after the onset of T2D. However, in that case, South Asians will be younger and have a lower BMI, both of which are important determinants in metabolic outcomes (36). As another alternative, one could use ethnicity-specific cut-offs for BMI. A recent study proposed that, in terms of age- and sex-adjusted incidences of T2D, for obesity a BMI cutoff of 30 kg/m$^2$ in white populations would be equal to a BMI cutoff of 23.9 kg/m$^2$ in South Asian populations (37). This BMI cutoff for obesity in South Asians is considerably lower than what the WHO proposes (*i.e.*, 27.5 kg/m$^2$) (38), illustrating the difficulty in defining such ethnicity-specific BMI cutoffs. Moreover, the BMI cutoffs are defined by T2D incidence which leads to unwanted correction when studying one of the factors that predisposes South Asians to the early development of T2D. We propose that BMI cutoffs are especially useful in the clinic, to identify individuals at risk for the

development of T2D and facilitate early prevention and treatment. For clinical trials, matching of South Asians and Europids should be carefully discussed depending on the study design and objective.

**Modulating energy metabolism through cold exposure**

Over the last decades a lot of scientific interest has emerged in the application of cold exposure as a non-pharmacological intervention to improve cardiometabolic health. Several physiological effects of short-term cold exposure were investigated in part 2 of this thesis (*i.e.*, **chapters 5**, **6**, and **8**) and will be further discussed in the sections below.

*Effects of cold exposure on the immune system*

Cold exposure stimulates the sympathetic nervous system, resulting in the release of epinephrine and norepinephrine that subsequently bind to adrenergic receptors (39). These receptors are present on many organs within the body, including heart, brown and white adipose tissue, skeletal muscle and blood vessels, which explains the large variety of effects induced by cold exposure. For instance, in **chapter 1** it is described that when noradrenaline binds to β-adrenergic receptors on thermogenic tissues, including BAT and skeletal muscles, this results in enhanced thermogenesis, which increases metabolic rate. Interestingly, also immune cells possess adrenergic receptors and can thus potentially be modified by cold exposure (40, 41). The interplay between the sympathetic nervous system and immune cells is further highlighted by the fact that some immune cells have the capacity to synthesize, store and release catecholamines themselves, to adapt the immune response (42, 43). Moreover, cold-induced alternations in hormone levels, such as cortisol, may additionally influence the inflammatory response of immune cells (44). In **chapter 5**, we found that short-term cold exposure affects mRNA transcripts of a large panel of immune genes in blood of healthy nonobese Europid and South Asian individuals. We showed that cold exposure increases circulating transcript levels of genes encoding cytotoxic proteins and pro-inflammatory chemokines. This may indicate that short-term cold exposure quickly provokes a pro-inflammatory state to prepare for an optimal immune response. Interestingly, it has been reported that when individuals are repeatedly exposed to short-term cold bouts (*i.e.*, cold water immersion at 14°C for 16 days) the initial immunomodulatory effects observed after the first cold water immersion are blunted (45). Moreover, when applying a combined intervention consisting of meditation, breathing techniques and long-term cold exposure (*i.e.*, 4 days extreme outdoor cold exposure followed by 5-9 days cold showers; 'Wim Hof Method'), healthy individuals show increased plasma IL-10 levels and an attenuated innate immune response upon intravenous administration of purified LPS (46). All taken together, this may indicate that the immunomodulatory effects of cold exposure are dependent on the intensity and

duration of the stimulus, with a higher pro-inflammatory responsiveness of the immune system after short-term cold exposure and the induction of an anti-inflammatory state after chronic cold exposure. Interestingly, this hypothesis nicely aligns with the effects of acute *versus* chronic exercise on the immune system. Acute aerobic exercise is thought to transiently redirect pro-inflammatory immune cells to the circulation, to allow an appropriate immune response (47). Chronic exercise modifies immune cells to a more anti-inflammatory state, among other characterized by increased levels of the anti-inflammatory cytokine IL-10, and is therewith proposed to offer protection against the chronic low-grade inflammatory state seen in obesity and its associated diseases (48). Whether chronic cold exposure, in a similar manner as chronic exercise, is able to sufficiently modify immune cells towards an anti-inflammatory phenotype remains to be elucidated, ideally in a clinical trial that includes isolation of immune cells from blood as well as metabolic tissues after long-term cold exposure combined with *in vitro* experiments to assess the inflammatory response of those immune cells after LPS challenge. Ultimately, the application of chronical cold exposure may be a promising intervention for the (preventive) treatment of cardiometabolic diseases that are characterized by low-grade inflammation.

*Effects of cold exposure on lipoprotein metabolism*

Cold exposure has been shown to beneficially modulate lipoprotein metabolism in mice (49-53), but the translation to humans remained controversial thus far. In mice, prolonged cold exposure enhances clearance of triglyceride (TG)-rich lipoproteins from the circulation by accelerating the uptake of TG-derived fatty acids by BAT and browned WAT, which is coupled to increased removal of TG-rich lipoprotein remnants by the liver, therewith reducing hyperlipidemia (49-53). In seeming contrast, previous studies in human adults with or without T2D reported that short-term cold exposure either did not affect (54, 55) or even increased (56) circulating TGs. In **chapter 8** we confirmed that short-term cold exposure increases circulating TG levels. By sequential lipidomic profiling of serum samples taken during cold exposure we now showed in **chapter 6** that cold exposure in fact transiently decreases total circulating TG levels at 30 min, which likely illustrates the uptake of TG-derived fatty acids by thermogenic tissues (*e.g.*, skeletal muscle and BAT) as fuel for oxidation. This finding nicely aligns with preliminary data from an unpublished study of our group, in which we show with dynamic magnetic resonance imaging (MRI) that, compared to baseline, the fat fraction of the supraclavicular BAT depot transiently increases after initiation of cold after 30 min, followed by a progressive decrease in fat fraction (Sardjoe-Mishre, in preparation). The initial increase in fat fraction may thus reflect an enhanced uptake of TG-derived FA by BAT from the circulation.

Notably, on a molecular level, saturated and mono-unsaturated TG species did show a progressive decrease during cold exposure while polyunsaturated fatty acid (PUFA)-containing TG species with ≥6 double bonds started to increase after 90-120 min of cooling. This combination of effects suggests that the circulating TGs are taken up from the circulation by thermogenic tissues, while the increase in PUFA-containing TG species likely illustrates enhanced hepatic VLDL-TG secretion into the circulation (57). This concurs with a previous study from our group showing an increased circulating concentration of large VLDL particles after short-term cold exposure (56). In addition to supplying the thermogenic tissues with fatty acids, we hypothesize that the rise in specifically PUFA-containing VLDL-TG species may promote thermogenesis in a similar way as how dietary (omega-3) PUFAs induce thermogenesis by BAT in mice (58), via sympathetic activation (59) or directly through the G-protein-coupled receptor (GPCR) GPR120 (60).

These observed dynamic changes in circulating TG species were accompanied by an increase in a broad spectrum of circulating free fatty acids (**chapter 6**, and also observed in **chapter 8**). As explained in **chapter 1**, sympathetic stimulation of WAT increases intracellular lipolysis, resulting in the liberation of free fatty acids that are, among other, taken up by the liver to drive the production of TG-rich VLDL particles (61, 62). By inhibiting intracellular lipolysis by blocking adipose TG lipase using Atglistatin during cold exposure of mice, we showed that the cold-induced increase in total TG concentration was largely attenuated by Atglistatin and thus concluded that the cold-induced hepatic VLDL-TG production is in fact driven by this liberation of free fatty acids (**chapter 6**).

Taken together, our findings coincide with a mechanism by which cold exposure activates intracellular lipolysis in WAT to drive hepatic VLDL-TG secretion to deliver fuel (*i.e.*, TG-derived fatty acids) to thermogenic tissues for combustion into heat. Although an increase in circulating TGs during short-term cold exposure may at first glance seem disadvantageous, we propose a model by which cold exposure on the long term decreases adipose stores and thus liberation of FFAs, while still accelerating lipoprotein turnover, and therewith benefits cardiovascular health (**Figure 1**). It would thus be of high interest to study the effects of long-term cold exposure (*i.e.*, multiple months) and to investigate whether the therapeutic potential of cold exposure to correct dyslipidemia and improve cardiometabolic health as reported in mice can be recapitulated in humans.

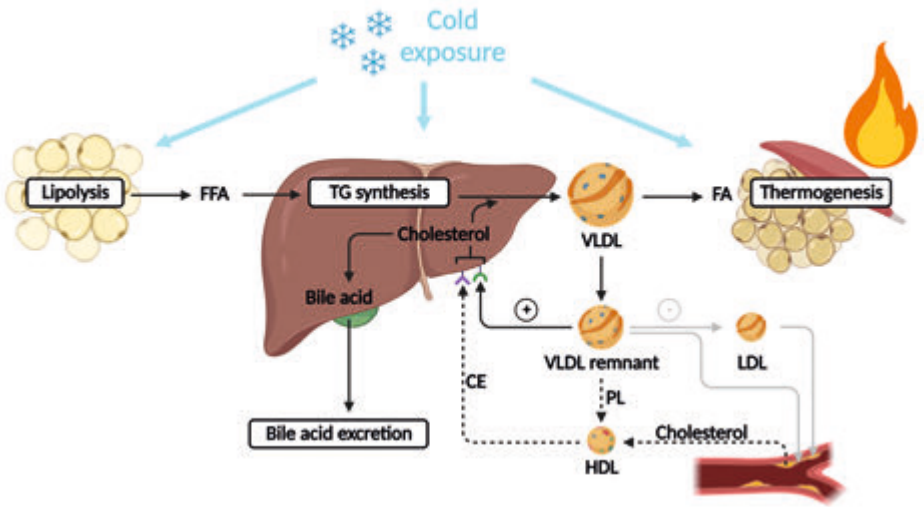


**Figure 1.** Proposed model of how cold exposure enhances whole-body lipoprotein metabolism in humans. Cold exposure induces intracellular lipolysis in white adipose tissue, increasing circulating free fatty acids (FFA) that subsequently drive hepatic biosynthesis and release of triglyceride (TG)-containing very-low-density lipoprotein (VLDL) particles. The cold-activated thermogenic tissues (i.e., brown adipose tissue and skeletal muscle) take up TG-derived fatty acids (FA) from VLDL particles as fuel for oxidation to generate heat, leading to the generation of delipidated and cholesterol-enriched VLDL remnants, which are rapidly taken up by the liver, preventing the generation of LDL. During lipolysis, the surface lipids (i.e., mostly phospholipids (PL)) from VLDL are transferred to circulating lipid-poor apolipoprotein A-I, initiating the formation of small high-density lipoprotein (HDL) particles. These HDL particles can in turn take up cholesterol from foam cells in atherosclerotic plaques in the vessel wall and deliver this in the form of cholesteryl esters (CE) to the liver. Within the liver, cholesterol derived from the VLDL remnants and HDL is subsequently partially used for VLDL synthesis and partially converted into bile acid and partly removed from the body.

Another question that needs to be unraveled is the ultimate way to apply long-term cold exposure so it is feasible for patients to implement in their daily life. Rather simple methods include taking daily cold showers, cold baths, or by wearing cooling vests that are broadly available for commercial use. A more rigorous method would be cold water dipping in for example pools, lakes, or the sea/ocean, also called 'winter swimming'. Winter swimming is within Europe for obvious reasons especially popular in Scandinavia and often alternated with taking a hot sauna (approx. 2-3 cold dips and 2 sauna visits per week). Interestingly, in thermoneutral state, winter swimmers compared to controls are shown to have a lower core body temperature and absent [$^{18}$F]FDG uptake by BAT, indicating heat acclimation likely due to the hot saunas (63). On the other hand, winter swimmers show enhanced cold-induced thermogenesis and a more pronounced peak in both cortisol and supraclavicular skin temperature before waking (63). Perhaps, by exposing humans to extreme low and high temperatures, the body is being trained to handle different temperatures, similarly as we train our bodies by repeated physical

activity, evoking stronger beneficial effects upon cold exposure (64). Nonetheless, the latter remains speculation and should be investigated in a dedicated clinical trial.

Furthermore, it remains to be elucidated what thermogenic tissues contribute to the transient decrease in circulating TGs after cold exposure in humans. In mice, BAT is the most important thermogenic tissue, as it comprises 0.4-1% of their total body weight and is responsible for most of the cold-induced TG clearance (65). In humans, BAT only comprises approx. 0.02% of total body weight (65) and a large part of circulating glucose and fatty acid clearance can be attributed to skeletal muscles (66, 67). Still, findings from several studies support the significance of BAT activation in the regulation of lipid metabolism in humans. BAT-positive compared to BAT-negative individuals (as discriminated by detectability of 2-[$^{18}$F]fluoro-2-deoxy-D-glucose ([$^{18}$F]FDG) uptake on cold-exposed positron emission tomography-computed tomography (PET-CT) scans) show higher cold-induced fatty acid oxidation (68). Moreover, cold-induced adipose tissue lipolysis is associated with BAT oxidative metabolism and BAT volume, but not with skeletal muscle shivering as measured with electromyography (66, 69). These results are underscored by a large retrospective analysis of more than 50,000 patients in which lower plasma TG and higher HDL-cholesterol levels were reported in BAT-positive individuals than BAT-negative individuals (assessed by detectable [$^{18}$F]FDG uptake on thermoneutral PET-CT scans) (70). Nonetheless, to fully elucidate the exact contribution of BAT in whole-body lipid metabolism, future studies may need to focus on using alternative approaches to activate BAT in humans more specifically than cold exposure and subsequently study its effect on lipoprotein metabolism. Current developments in identifying targets that specifically activate human BAT thermogenesis are discussed in the next section.

**Modulating energy metabolism through activation of BAT**

*Pharmacologically targeting BAT*

Cold activated BAT serves as a metabolic sink for fatty acids and glucose, therewith protecting from insulin resistance and dyslipidemia (68, 69, 71, 72). This makes BAT a promising target to improve cardiometabolic health. However, as stated above, the use of cold exposure as therapeutic intervention has its challenges and may result in low compliance when people find it uncomfortable. Therefore, mimicking the effect of cold exposure on BAT by targeting β-adrenergic receptors represents a promising alternative strategy. In rodents, the β3-adrenergic receptor (ADRB3) is the most potent subtype to activate BAT thermogenesis and improve cardiometabolic health (50, 73, 74). However, in humans the predominant adrenergic receptor subtype on BAT was still controversial until recently. The ADRB3 agonist mirabegron enhances metabolic activity of human BAT (75), but only at a dose highly exceeding the therapeutic dose used to treat hyperactive

bladder (*e.g.*, 200 mg *versus* 50 mg) (76, 77). The findings from a collaborative study of our group showed that human BAT biopsies have high expression of ADRB2 and negligible expression of ADRB3 and that the ADRB2 is responsible for thermogenic activation of human brown adipocytes by noradrenalin (77), thus suggesting that the ADRB2 is predominantly involved in human BAT thermogenesis.

By performing a proof-of-concept study in young healthy male volunteers, we showed in **chapter 9** that a single intravenous bolus of the ADRB2 agonist salbutamol (at a pharmacological dose used to treat a severe asthmatic attack) indeed increases glucose uptake by human BAT (as assessed by a dynamic [$^{18}$F]FDG PET-CT scan), which is prevented by the ADRB1/2 antagonist propranolol. In addition, salbutamol, compared to salbutamol with propranolol, increased heart rate and whole-body energy expenditure, the latter being positively associated to the salbutamol-induced glucose uptake by BAT. Our results are supported by previous studies demonstrating that continuous intravenous salbutamol with and without the ADRB1 blocker atenolol increases energy expenditure, lipolysis, and fat oxidation (78-80). The finding that the ADRB2 is prominently involved in sympathetically activating human BAT thermogenesis provides new opportunities to pharmacologically target human BAT.

Nevertheless, several challenges cannot be overlooked. Salbutamol induces unwanted cardiovascular side effects, including an increase in heart rate (+17 bpm) and blood pressure. This is in contrast to the decrease in heart rate observed after cold exposure (77), illustrating that systemic ADRB2 agonism does not entirely mimic cold exposure. This discrepancy may be explained by the local release of norepinephrine after cold exposure that affects tissue specific beta- as well as alpha-receptors. Importantly, an increase in heart rate of only 5 bpm has been predicted to increase 10-year cardiovascular mortality with 17% (81), which may be even larger in obese individuals with preexisting cardiometabolic risk factors. To overcome cardiovascular side effects, future research may focus on identifying a targetable receptor exclusively expressed by thermogenic adipocytes that could be used to specifically deliver the ADRB2 agonist to the brown adipocyte, similar to inclirisan that has recently bene approved by the Food and Drug Administration (FDA). Inclirisan reduces proprotein convertase subtilisin/kexin type 9 (PCSK9) production through gene silencing specifically in hepatocytes by targeting an antisense molecule to the asialoglycoprotein receptor (ASGPr) that is uniquely expressed on hepatocytes (82). Furthermore, an intriguing finding from our study was that we observed that, even though all participants were healthy and lean, participants with low salbutamol-induced glucose uptake by BAT had a less favorable metabolic phenotype than participants with high glucose uptake by BAT, illustrated by higher body fat mass, waist-hip ratio and LDL-cholesterol levels (**chapter**

**9**). This finding is similar to the negative associations between [$^{18}$F]FDG uptake by cold exposed BAT (83, 84) or thermoneutral BAT (85, 86) and body fat mass percentage and central adiposity that have been shown before. It remains elusive whether these are just associations or in fact causations. Indeed, it is possible that BAT influences the distribution of body fat, as a higher metabolic rate due to more active BAT results in more uptake of energy substrates from the circulation that are derived from WAT. This could additionally result in lower ectopic fat deposition. Indeed, BAT-positive individuals have been shown to have a higher density of the liver as assessed by CT, which reflects lower hepatic fat accumulation (86), indicating less ectopic fat deposition. Alternatively, genetic factors could underlie the association between low [$^{18}$F]FDG uptake by BAT and a less favourable metabolic phenotype. As such, the functionality of the ADRB2 (*e.g.*, due to gene polymorphisms, receptor sensitivity, and/or receptor density) may influence both the metabolic phenotype and the salbutamol-induced glucose-uptake by BAT (80, 87-89). Theoretically, individuals with low ADRB2 functionality may thus benefit less from pharmacological ADRB2 stimulation. Especially for those individuals, pharmacological compounds that activate BAT in a β-adrenergic independent manner may have a higher potential.

β-adrenergic receptors are examples of GPCRs of the $G_s$ subtype. GPCRs represent the largest family of membrane proteins, are present on the cell surface and contain seven transmembrane domains that induce downstream signaling after activation (90). Several hormones (91-95), metabolites (96), and lipids (97) can serve as ligand for GPCRs of the $G_s$ subtype and hold potential to activate BAT thermogenesis, independent of β-adrenergic signaling. Worth mentioning is a novel pathway that was recently revealed, by which brown adipocytes from mice could be activated via a GPCR without an exogenous ligand. Specifically, in this pathway, a cold-activated intracellular lipolytic signal induces the transcription of *GPR3* and therewith intrinsically activate adipocyte thermogenesis. This activation could be augmented by dietary lipids, suggesting a protective role for BAT against lipid overload to maintain metabolic homeostasis, at least in mice (98). Conceivably, this pathway is also enhanced after ADRB2 stimulation, as the latter amplifies the lipolytic signal. On itself or on top of exogenous ligand binding (*e.g.*, by stimulating the ADRB2), increasing GPR3 expression may provide a very selective approach for enhancing thermogenesis as GPR3 is very selectively expressed on thermogenic adipocytes. However, due to technical challenges that come with increasing gene expression, the use of exogenous ligands alone to activate brown adipocytes still holds the largest potential for the near future.

*Visualization of human BAT*

Since accumulating evidence for the presence of metabolically active BAT in human adults appeared in 2007 (99), and was confirmed via [$^{18}$F]FDG PET-CT scans in 2009 (83, 100, 101), BAT research has made an enormous progression. Nonetheless, several methodological challenges exist when studying human BAT. Taking biopsies of human BAT is challenging. The main depots are located deep around the neck, close to important vessels and nerves, causing a safety risk when taking biopsies. In addition, human BAT is highly heterogenic and characterization of the adipocytes (*e.g.*, phenotypically more 'classical' brown *versus* 'beige') can differ dependent on the location of the biopsy taken (102). The most commonly used method and 'gold' standard to assess human BAT activity is therefore still PET-CT scanning with [$^{18}$F]FDG as radiotracer, which we also used in **chapter 9**.

Using this method, the intravenously administered [$^{18}$F]FDG tracer is taken up by the sodium-independent glucose transport family by metabolically active tissues, amongst which BAT, and can be visualized on PET. The CT scan distinguishes tissues based on their radiodensity and provides the anatomical reference. Although many researchers apply static whole-body scanning, we used dynamic [$^{18}$F]FDG PET acquisition to quantify BAT glucose uptake rates after administration of salbutamol without or with propranolol in **chapter 9**. Applying a dynamic approach for image acquisition allows to acquire images with the concentration of radioactivity within the tissues of interest (*e.g.*, BAT) as a function of time, also called time-radioactivity curves (TACs). Using the graphical analysis method 'Patlak linearization', the TACs of the tissue and of arterial plasma can be combined and transformed into a single near-linear plot to determine the net uptake rate of [$^{18}$F]FDG, which is illustrated by the slope of the linear phase of the plot. The net influx rate of [$^{18}$F]FDG together with the circulating glucose level can be used to estimate tissue-specific net uptake of glucose in response to a stimulus such as salbutamol (103). Thus, by applying a dynamic approach, important pharmacokinetic information about the tissue-specific glucose metabolic rate can be obtained. Nevertheless, the use of [$^{18}$F]FDG as a tracer to visualize BAT, even by dynamic PET scan, still has several limitations. Firstly, the use of a radioactive tracer causes a radiation burden, limiting the number of scans that can be taken per individual per year (*e.g.*, typically two in the Netherlands at the required radiation dose for BAT imaging). Secondly, BAT oxidative metabolism is largely driven by intracellular TG utilization (54, 66, 68), whereas glucose is likely mainly taken up for *de novo* lipogenesis, rather than as direct substrate for oxidation (104). As such, BAT glucose uptake does not necessarily reflect BAT oxidative metabolism and assessing glucose uptake alone probably provides limited information about the actual thermogenic function of the tissue. This is nicely illustrated in individuals with T2D that often show a reduced BAT glucose uptake due to their insulin resistance

state (105), whereas BAT oxidative capacity and free fatty acids uptake are similar to individuals without T2D (55). This additionally indicates that [$^{18}$F]FDG uptake by BAT is thus dependent on insulin sensitivity of the tissue, which is particularly a problem when investigating individuals with insulin resistance, including patients with T2D but also elderly patients. Yet, these individuals are usually the target population for BAT-targeted therapy, thus warranting the use of alternative visualization techniques.

Assessing circulating lipid uptake instead of glucose may be an elegant alternative. Currently, the 14 (R,S)-[$^{18}$F]fluoro-6-thia-heptadecanoic acid ([$^{18}$F]FTHA) tracer, which is in fact a long-chain fatty acid tracer, is sparsely used for this purpose. [$^{18}$F]FTHA can be administered either intravenously or orally and visualizes the uptake of circulating free fatty acids by BAT (67). However, as BAT, at least in mice, mainly takes up fatty acids derived from TG-rich lipoproteins, taking advantage of the high expression of the triacylglycerol hydrolase LPL in BAT, rather than circulating free fatty acids (49), the use of this tracer likely highly underestimates the total uptake of fatty acids by BAT. Hence, a tracer that mimics the uptake of TG-derived fatty acids will likely be superior to glucose and/or free fatty acid tracers. Currently, our research group is developing a PET-compatible [$^{18}$F]oleate tracer that is built into a TG and incorporated in TG-rich lipoprotein-like particles (106), which may give a more realistic visualization of the true substrate uptake by active human BAT. However, this tracer still requires optimization and validation in mice, and subsequent use in humans will require synthesis under good manufacturing practice (GMP), and is thus still a future prospect. Apart from visualizing substrate uptake by BAT, it is also possible to assess BAT oxidative capacity and perfusion. BAT oxygen consumption can be visualized using the tracer [$^{15}$O]$O_2$ and BAT perfusion can be visualized using the tracers [$^{15}$O]$H_2O$ and [$^{15}$O]CO, however, the use of those tracers is technically challenging and suboptimal due to the short half-life of [$^{15}$O] and poor image resolution (107). In addition, an [$^{18}$F]FDG PET-CT scan is often still required to identify the BAT depots and define regions-of-interest (108, 109). Alternatively, [$^{11}$C]acetate can be used to assess oxidative metabolism. Compared to [$^{15}$O]$O_2$, [$^{11}$C]acetate has a longer half-life and is cheaper in production, however, the pharmacokinetic model for [$^{11}$C]acetate is technically complex and suitable scanners are generally not available in standard research facilities (110). Moreover, all mentioned radioactive tracers cause radiation burden, limiting its use in longitudinal studies.

A safe and non-invasive alternative may be the use of MRI, that assessing changes in fat fraction during intervention (111), which partly reflects the metabolic activity of BAT. Using this method, previous studies from our group (112) and others (111) have shown that cooling decreases the fat fraction of the supraclavicular BAT depot. An important consideration, however, is that combustion of the intracellular TGs after BAT activation

go along with replenishment of the lipid stores by the uptake of TG-derived fatty acids from the circulation (51), which will result in an underestimation of lipid combustion. Another non-invasive method that may visualize BAT thermogenic activity is infrared thermography, which produces colored heatmaps based on surface temperatures of the skin. By the use of this method in combination with the use of iButtons (*e.g.*, wireless temperature loggers attached to the skin), we showed in **chapter 8** that the supraclavicular skin temperature increases following cold exposure of healthy, lean males and females in the morning. As the cold-induced change in supraclavicular skin temperature has been reported to slightly correlate to [$^{18}$F]FDG uptake by BAT on PET-CT scans (113-115), an increase in supraclavicular skin temperature may point to enhanced BAT activity. However, the main limitation of this method is that all tissues beneath the skin, including arteries and skeletal muscles, contribute to the measured skin temperature, and this cannot be distinguished on the infrared image or with iButtons (116, 117). Moreover, overweight and obese individuals may have thicker subcutaneous adipose tissue that insulate the BAT depots, leading to underestimation of BAT thermogenesis in these individuals (118).

In summary, all currently available visualization techniques to assess BAT metabolic activity have their limitations and a supreme method has not yet been developed. An overview of all methods to visualize BAT activity is given in **Figure 2**. Combining different techniques to measure both oxidative metabolism and substrate uptake, preferably including a TG-based tracer that is packed into a TG-rich lipoprotein-like particle, will likely closest reflect the true metabolic BAT activity in humans. Furthermore, for all visualization methods, protocolized guidelines should be developed to harmonize the performance, analysis, and reporting of BAT imaging studies in humans. An example of such a guideline is the 'brown adipose reporting criteria in imaging studies', BARCIST, 1.0 for static [$^{18}$F]FDG PET imaging published in 2016 (119), which has led to an improvement of the comparison of clinical trials between different research facilities.

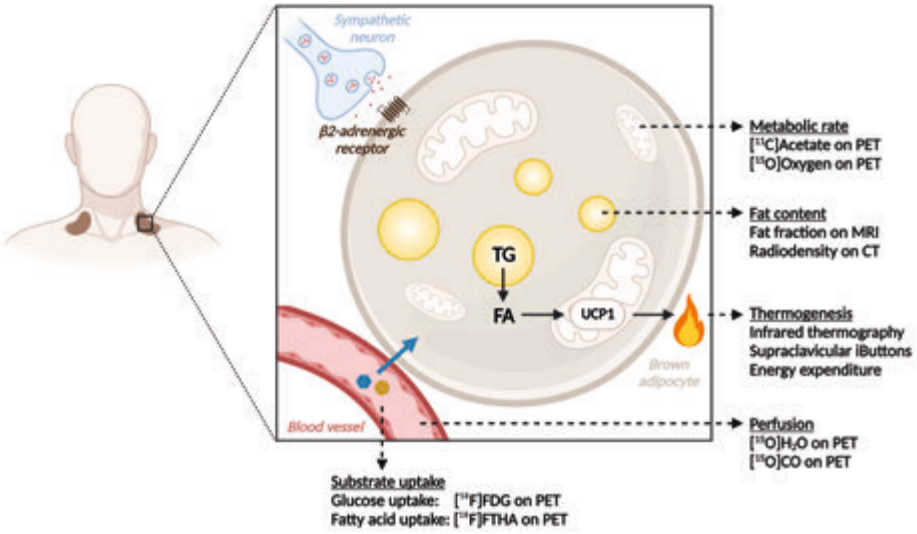


**Figure 2.** Current methods to assess human BAT function in vivo. Global overview of the various available techniques to visualize human BAT in vivo. CO, carbon dioxide; CT, computed tomography; FA, fatty acid; [$^{18}$F]FDG, 2-[$^{18}$F]fluoro-2-deoxy-D-glucose; [$^{18}$F]FTHA, 14 (R,S)-[$^{18}$F]fluoro-6-thia-heptadecanoic acid; $H_2O$, dihydrogen monoxide; MRI, magnetic resonance imaging; PET, positron emission tomography; TG, triglyceride; UCP-1, uncoupling protein-1.

## Novel directions in the field of obesity and cardiometabolic diseases

*Importance of the biological clock*

As described in **chapter 7**, organisms living on earth possess an internal biological clock that regulates a circadian rhythm in physiological processes. Zeitgebers, such as light input, food intake, and temperature changes, can influence the endogenous circadian rhythm, after which the rhythm is formally called a 'diurnal rhythm'. To maintain homeostatic balance during day and night, many metabolic processes are under control of the endogenous circadian rhythm (120, 121) and alignment of the zeitgebers with the circadian rhythm is essential for optimal clock responses.

In **chapter 7**, we described that rodent BAT activity, and likely also human BAT activity, peaks around the onset of the active period and we discussed how diurnal rhythms in BAT are generated. An important remaining question was whether we should consider the circadian rhythm of BAT when measuring its activity and/or therapeutically targeting the tissue. Since cold exposure is the physiological activator of BAT, in **chapter 8** we aimed to investigate whether cold-induced thermogenesis is subjected to a diurnal variation in humans. Indeed, we showed that cold-induced thermogenesis, as assessed by the cold-induced increase in resting energy expenditure and supraclavicular skin temperature, was larger in the morning than in the evening in males. Females, on the other hand, showed a

better cold tolerance in the morning than in the evening and larger increases in circulating TGs, total cholesterol and high-density lipoprotein (HDL) cholesterol concentration upon cold. Although differently regulated between males and females, these findings suggest that the cold responses of thermogenic tissues, such as BAT, are subjected to a diurnal variation and may indicate that applying cold exposure to improve whole-body metabolism has more potential in the morning than in the evening.

To obtain further insight in the diurnal variation in BAT activity, colleagues in our department have recently combined transcriptomics and lipidomics in BAT of mice and found that lipolytic processes seem to define the peak in BAT activity (122). Findings were consistent with a mechanism by which intracellular lipolysis is activated just before the onset of the active phase to provide fatty acids for thermogenesis, whereafter LPL-mediated uptake of TG-derived fatty acids from the circulation is stimulated at the onset of the active phase to subsequently replenish lipid stores (122). LPL activity in BAT played a central role and was shown to be highly rhythmic, with a peak at the start of the active phase and a nadir at the start of the inactive phase. Transcriptional *LPL* expression was shown to be mediated by peroxisome proliferator-activated receptor gamma (PPAR-γ) and posttranscriptional LPL regulation involved angiopoietin-like 4 (Angptl4). Both Angptl4 deficiency and overexpression resulted in blunted rhythms in LPL activity and TG-derived fatty acid uptake by BAT (122). Conceivably, enhanced sympathetic activity in the morning initiates the diurnal variation in BAT activity by stimulating intracellular lipolysis, that in turn endorses the thermogenic program and, via PPAR-γ, the expression of genes involved in the uptake, storage, and intracellular lipolysis of lipids (122). This may also explain previous findings from our group, showing that flattening of corticosterone rhythms in mice results in decreased TG-derived fatty acid uptake by BAT, which seemed mediated by reduced sympathetic outflow towards BAT at waking (123). Although these findings were obtained in mice and remain to be successfully translated to humans, it is plausible that the basal activation state of BAT is important when therapeutically targeting the tissue, explaining a higher effect of cold exposure on thermogenic activity in the morning than in the evening (**chapter 8**). This notion is underlined by the fact that cold-induced thermogenic activity in humans is higher during wintertime when outdoor temperatures are lower (84, 124, 125).

The discovery that many metabolic processes are subjected to a circadian rhythm and that external cues affect or even disturb this rhythm could form a basis for the prevention of cardiometabolic disturbances. This could be especially beneficial for individuals working in shifts, which is strongly associated with metabolic syndrome, T2D and CVD (126-129). The potential benefit of timing interventions in harmony with the natural circadian cycle ('chronotherapy') is illustrated by multiple studies that report

positive effects of timed food intake. Circadian rhythms of key metabolic processes, including lipid oxidation, peak in the morning, suggesting that food intake earlier in the daytime is superior for cardiometabolic health than later in the daytime (130). Indeed, time-restricted eating with an early time-frame (*e.g.*, 6-hours eating period before 3 p.m.) is shown to improve insulin sensitivity and markers of inflammation in men with prediabetes (131) and healthy, lean males and females (132), whereas late time-restricted eating with breakfast skipping (*e.g.*, 8-hours eating period after 12 p.m.) does not show metabolic benefits in overweight-to-obese individuals (133). Importantly, those studies reported no significant differences in energy intake between time-restricted eating *versus* ad libitum eating. Moreover, breakfast skipping and late-night eating are associated with an increased risk of obesity and CVD (134-136). The metabolic effects of meal timing can possibly to a small extent be attributed to the diurnal variation in diet-induced thermogenesis (*i.e.*, the increase in energy expenditure after a meal, also associated to BAT activity (137)) with a higher activity in the morning than in the evening (138-140). Nonetheless, it is important to note that the beneficial effects of time-restricted eating are still under active debate, as a recent clinical trial found no superior effects of time-restricted eating (8-hours eating period from 8 a.m. to 16 p.m.) with caloric restriction *versus* caloric restriction alone (141). Although the time-restricted eating group only reduced their eating window with approx. 2.5 hours and the caloric restriction could have blunted the effects of time-restricted feeding, the results indicate that the importance of caloric restriction should not be overlooked. Whether time-restricted eating does have a superior effect over caloric restriction in individuals with disturbed circadian rhythms, such as shift workers, remains highly interesting for future research.

Thus, by timing an individual's behavior, metabolic processes can be aligned with the circadian rhythm, therewith possibly improving physiological functioning. As such, we propose that the application of cold exposure holds a larger therapeutic potential in the morning than in the evening. Nevertheless, long-term studies with repeated cold and the inclusion of metabolically compromised individuals (*e.g.*, patients with T2D or individuals with obesity) are needed to confirm this. Moreover, future research is essential to elucidate the role of BAT in the diurnal variation in diet- and cold-induced thermogenesis and to investigate whether diurnal variations are of importance when pharmacologically targeting BAT.



*Pharmacological treatment of obesity*

Historically, treatment for obesity focused on implementing lifestyle interventions, by reducing food intake and increasing physical activity. Nonetheless, the majority of patients will regain weight after initial successful weight reduction (142). Only recently,

the combined lifestyle intervention (*gecombineerde leefstijlinterventie*, 'GLI') has been implemented in the obesity standard in the Netherlands. The advantage of the GLI is that next to eating habits and physical activity behavioral changes are also taken into account by the implementation of cognitive behavioral therapy. Nonetheless, participants generally lose approx. 2.5 kg after one year (143, 144), meaning that many patients living with obesity do not reach their target weight using such a GLI alone. It is important to realize that physiological responses to weight loss, including reduced energy expenditure and perturbations in appetite-regulating hormones, complicate the maintenance of reduced body weight (142, 145). For long-term success, combining lifestyle changes with pharmacological anti-obesity therapy will probably provide a more sustained treatment response in part of the patients living with obesity.

One of the current most promising anti-obesity strategies is based on incretin hormones. The two main incretin hormones include glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), which are secreted in response to food intake from the gastrointestinal K- and L-cells, respectively. Through neuronal and endocrine signals, incretins regulate glucose homeostasis by promoting glucose-dependent insulin release and reducing appetite. Therefore, long-acting GLP-1 receptor agonists were in first instance developed to improve glucose homeostasis in T2D (146). Promising enough, GLP-1 receptor agonists additionally induce weight loss in a dose-dependent manner, due to increasing satiety and delaying gastric emptying. As such, in 2015, the European Medicines Agency (EMA) approved the use of the GLP-1 receptor agonist liraglutide (Saxenda; 3 mg injection once daily subcutaneously) for the treatment of obesity, or severe overweight (BMI ≥ 27 kg/m$^2$) with adiposity-associated complications (147, 148). In the Netherlands however, patients only qualify for treatment with liraglutide if they: 1) have followed a registered GLI-program for at least one year, 2) have a BMI ≥ 40 kg/m$^2$ or BMI ≥ 35 kg/m$^2$ with CVD, sleep apnea, or arthrosis, and 3) are not willing to undergo, or not suitable for, bariatric surgery.

While liraglutide 3 mg once daily combined with lifestyle interventions has been reported to induce a mean of 8% weight reduction after 56 weeks (149), more promising results are found with the GLP-1 receptor agonist semaglutide (Wegovy). Semaglutide 2.4 mg only has to be administered once weekly and, combined with lifestyle interventions, results in a mean of 15% body weight reduction after 56 weeks, with 86% of users reaching a weight reduction of 5% or more (150). As a result, EMA approval for semaglutide as treatment of obesity was obtained in 2021, and approval in the Netherlands is still awaited. Despite these positive results on body weight, GLP-1 receptor agonists often give rise to dose-dependent gastrointestinal side effects, including nausea and vomiting, limiting their utility and patient compliance. Moreover,

approximately one third of patients is a so-called 'non-responder' on GLP-1 receptor agonism, as they don't reach the goal of at least 5% body weight loss after 12 weeks of treatment on a stable dose. A solution is likely found in combining GLP-1 receptor agonism with additional targets. For instance, combining GLP-1 receptor agonism with GIP receptor agonism within single peptide reduces nausea and thus increases drug tolerance (151). Moreover, GIP receptor agonism appears to have a synergistic effect with GLP-1 receptor activation and has additional important advantageous effects on adipose tissue, where it increases lipid storage capacity in the postprandial state and reduces pro-inflammatory immune cell infiltration (151). Activating both GLP-1 and GIP receptors (*e.g.*, 'dual' agonism) was thus expected to lead to stronger glucose lowering and improvements in WAT health, resulting in sustained weight loss. Indeed, recent trials combining GIP/GLP-1 receptor agonism (Tirzepatide; once weekly) report superior glycemic control and weight loss compared to GLP-1 receptor agonism alone (152-154). Specifically, GIP/GLP-1 receptor agonism (15 mg once weekly) resulted in a stunning mean 21% body weight reduction after 72 weeks, which was accompanied with improvements in cardiometabolic risk factors such as blood pressure and lipid levels (152). Whilst writing this dissertation, EMA approval for the use of Tirzepatide was given for glycemic control in T2D, but is still pending for obesity. Moreover, preclinical and clinical trials are already investigating the combination of GIP/GLP-1 receptor agonism with glucagon receptor (GCGR) agonim. Such 'triple' agonism shows even more promising results on glycemic control and weight loss in rodent studies (155, 156). In humans, the first phase 1 clinical trial shows that a single dose of the triple agonist LY3437943 ('LY') lowers body weight for at least 43 days after administration, and is safe and well-tolerated (157). Yet, the efficacy and safety of long-term treatment with LY in humans with cardiometabolic diseases still warrant further clinical investigation.

Interestingly, in addition to its effects on glucose metabolism and body weight, treatment with GLP-1 receptor agonists also reduces the risk of non-fatal cardiovascular events in human adults (158-160). Pre-clinical studies have shown that GLP-1 receptor agonism (161-163) and GIP receptor agonism (164, 165) reduce atherosclerosis development via a reduction in inflammation. A recent study from our group now additionally showed that dual GLP-1/GIP receptor agonism significantly attenuated atherosclerotic lesion development via both reduced low-grade inflammation and accelerated VLDL turnover, whereas single GLP-1 or GIP receptor agonism only induced nonsignificant improvements (van Eenige and Ying et al, unpublished). It thus remains interesting for future clinical trials to investigate the long-term effects of dual and triple agonism on inflammatory markers, lipoprotein metabolism, and cardiovascular events. Moreover, research from our department has shown that single GLP-1 receptor agonism increases glucose uptake by human BAT (166), and both single GLP-1 receptor agonism (167)

and dual GLP-1/GIP receptor agonism activate BAT in mice (van Eenige and Ying et al, unpublished). Future research may focus on the effect of dual and triple agonism on human energy metabolism, including BAT thermogenesis. Ultimately, implementing lifestyle changes in combination with a pharmacological therapy that reduces appetite and increases energy expenditure, will support individuals with obesity to achieve long-term sustained weight loss and improve cardiometabolic health.

**Importance of sex and gender**

Two chapters from the current thesis describe studies conducted in both males and females. In **chapter 8**, we studied healthy and young individuals and observed that the diurnal variation in cold-induced thermogenesis unfolded differently in males than in females. In **chapter 3**, we studied overweight-to-obese patients with T2D and found that differences in immune mRNA levels between South Asians and Europids were more pronounced in females than in males. Thus, strikingly, in both studies we observe pronounced differences in the findings between males and females. This underscores the importance of including males and females in clinical trials and considering sex (biological constructs) and/or gender (sociocultural constructs) as determinants of health and disease. The sex and gender differences should be distinguished. Gender is not a binary term, as within one individual characteristics of masculinity and femininity can co-occur in different degrees. Gender constructs influence lifestyle, such as diet, exercise, perceived stress, and smoking, but also access to and the use of healthcare (168). Resulting from the fluidity of gender, most studies simply focus on 'differences between males and females' and as such distinguish two groups based on biological sex. However, it is important to realize that gender constructs may additionally influence results. Differences between males and females found in clinical trials may be largely related to differences in T2D and CVD incidences and exposure to risk factors, including the hormonal transition of the female menopause, across lifespan.

In youth (*e.g.*, <18 years old), T2D incidence is higher among females (169). Although youth-onset T2D is fairly rare, rates are increasing due to increases in childhood obesity. Since diabetes duration is positively associated with a high risk of cardiovascular complications (170), prevention is of high importance and this may especially apply for females. These differences between males and females in young adults seem, at least partly, related to variations in insulin resistance, as females have higher rates of insulin resistance until mid-puberty (169). Males have higher rates of insulin resistance during late puberty and adulthood (169, 171-173). Indeed, around midlife the prevalence of T2D is higher among males (169). A possible explanation includes that males generally develop T2D at a lower BMI than females (174, 175), perhaps due to differences in body composition with more visceral adipose tissue and ectopic liver fat in males and

more subcutaneous adipose tissue in premenopausal females (176). In addition to T2D prevalence, CVD prevalence is generally higher in males than premenopausal females. However, both T2D onset and reaching the postmenopausal age put females at a higher CVD risk (177, 178). It has been proposed that females possess a more prolonged prediabetic state before T2D develops leading to cumulative exposure to cardiovascular risk factors, such as hypertension, dyslipidemia, inflammation, and endothelial dysfunction (179, 180). Moreover, premenopausal females are relatively protected against the development of cardiometabolic diseases by estrogen, whereas after menopause the incidence of metabolic syndrome steeply increases (181, 182). The latter may be of importance in the observed more pronounced ethnic differences (*e.g.*, South Asians *versus* Europids) in immune mRNA levels in female patients with T2D than in male patients with T2D (**chapter 3**). Age at menopause is reported to vary across populations, with lower ages among South Asians (*e.g.*, ranging from 44 to 49 years old), than Europids (*e.g.*, 50 to 54 years old) (183, 184). Hypothetically, in our study more South Asian females have reached the postmenopausal state, leading to a longer disturbed metabolic phenotype. This exemplifies that ethnicity might interfere in the complex interplay between sex and metabolic outcomes. Future studies are warranted to elucidate mechanisms underlying sex and gender differences in cardiometabolic diseases. Results from such studies may lead to the development of precision medicine and ultimately reduce gender disparities in health. Moreover, we propose that for preventive cardiovascular risk management definition of sex- and ethnicity-specific ages are crucial to optimally prevent CVD among the global population.

## CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Obesity is a complex chronic, relapsing, multifactorial disease characterized by excessive fat accumulation as a result of a positive energy balance. Obesity may give rise to serious health problems, including T2D and CVD, that rank among the top causes of death worldwide. Unfortunately, the recognition of obesity as a disease is low and often blamed on poor lifestyle choices of the patient. Increasing the understanding of all facets of energy metabolism, will lead to effective identification of individuals at risk for obesity and cardiometabolic diseases and the development of targeted prevention and treatment strategies. In this thesis, we have addressed two key objectives: 1) to gain more insight in various pathophysiological aspects of cardiometabolic diseases including in the disease-prone South Asian population, and 2) to study the physiological effects of cold exposure and identify a novel pharmacological approach to directly target BAT.



The South Asian population is at especially high risk to develop T2D, partly explained by their unfavorable body composition and higher insulin resistance. Based on results in

this thesis, we propose that a more pro-inflammatory state especially in females might underlie the more severe disease progression and high rates of T2D complications in South Asians compared to Europids. More research is needed to elucidate whether the more pro-inflammatory state results from a longer disease duration or from intrinsic differences between South Asians and Europids. Other proposed underlying mechanisms that need further exploration include perturbations in the stress response and disturbed circadian regulation of physiological metabolic processes. Regardless of the underlying mechanisms, current knowledge indicates that prevention and treatment of cardiometabolic diseases in South Asians require a different approach than in Europids. We propose that preventive cardiovascular risk management should be recommended at a younger age and lower BMI for South Asian individuals. To this end, the previously recommended BMI cutoff for obesity of 23.9 kg/m$^2$ should be considered for South Asians (37). This may additionally implicate that South Asians are at that BMI already eligible for anti-obesity medication, instead of the currently defined threshold of 30 kg/m$^2$ according to the EMA, which seems more applicable for white populations.

With the increasing rate of obesity and its associated cardiometabolic diseases and generally modest long-term efficacy of lifestyle interventions, alternative treatment strategies are highly needed. Since the discovery of the presence of metabolically active BAT in adult humans, increased interest has emerged on cold interventions to activate BAT thermogenesis. In this thesis we provide new insights in and propose how short-term cold exposure affects the immune system and improves lipoprotein metabolism by increasing TG-rich lipoprotein turnover. Furthermore, we propose that cold exposure should be applied in the morning rather than the evening, to optimally use its potential. Future studies investigating the effect of long-term cold exposure on the immune system and lipoprotein metabolism are needed to determine whether cold exposure can be used to reduce inflammation and correct dyslipidemia, and thereby decrease T2D and CVD risk. Moreover, the application of cold exposure in older individuals and patients with CVDs needs further exploration, to assess efficacy and safety. Although the application of cold exposure, using for example cooling vests is feasible at home, it may not be a suitable strategy for everyone. Therefore, directly targeting the thermogenic BAT by pharmacologically mimicking the adrenergic effect of cold exposure may provide a solution. In this thesis we challenge the general belief that the ADRB3 is the primary adrenergic target on human BAT, by showing that specific stimulation of the ADRB2 increases glucose uptake by BAT in healthy volunteers. As clear evidence shows that metabolically active BAT in adult humans is associated with lower T2D and CVD risk, specifically activating BAT by targeting approaches that may be developed in the near future, will provide an opportunity to assess causality and to reveal whether pharmacologically targeting BAT can be used to treat cardiometabolic diseases.

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