Combatting metabolic disease : ethnic aspects, mechanisms and novel treatment strategies

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CHAPTER 8

General discussion and future perspectives

'Diabesity', as the frequently occurring combination of obesity and diabetes is often referred to, is becoming a major global health care problem. Although obesity has just been recently officially recognized as a disease (1), it accounts for a large proportion of total health care costs. The incidence of obesity and of its related diseases, including type 2 diabetes (T2D) and cardiovascular diseases (CVD) keep rising every year. Especially in the South Asian population obesity, T2D and CVD are highly prevalent. To gain more insight into the mechanisms that may underlie their high metabolic risk, we studied different regulatory aspects of the metabolic profile in South Asians, such as the endocannabinoid system and the circulating protein angiopoietin-like 4 (ANGPTL4). In this way, we aimed to identify specific therapeutic targets that might be effective in this vulnerable population. In the second part of this thesis, pharmacological strategies using the β3-adrenergic receptor (β3-AR) agonist mirabegron and the dipeptidyl peptidase-4 (DPP4) inhibitor sitagliptin to combat obesity by activation of brown adipose tissue (BAT) were studied in risk populations, including South Asians and overweight, prediabetic white Caucasians. From this thesis, novel insights into the pathophysiology of metabolic disease in the South Asian population and possible novel therapeutic interventions have arisen to combat metabolic disease in South Asian and the general population. These insights and their implications for clinical practice, as well as future perspectives will be addressed in this final chapter.

1. NOVEL INSIGHTS IN THE METABOLIC PROFILE IN SOUTH ASIANS

The role of the endocannabinoid system

Over-activity of the endocannabinoid system has been associated with obesity (2,3) and T2D (4), and dysregulation of the endocannabinoid system is linked to increased risk for CVD (5). The exact role of circulating endocannabinoids in the regulation of energy metabolism has not been fully elucidated, however, actions of endocannabinoids are thought to contribute to a positive energy balance, *e.g.* by reducing oxidative pathways in skeletal muscle (5,6), and increasing lipogenesis and adipogenesis in white adipose tissue (WAT) (5,9,10). In addition, endocannabinoids are thought to decrease thermogenesis in BAT (7,8). As underlying mechanism, endocannabinoids inhibit presynaptic noradrenalin release and postsynaptic noradrenalin signalling within myocytes and adipocytes (see Figure 2 in the Introduction of this thesis). So far, the role of the endocannabinoid system in relation to BAT activity has been mainly investigated in preclinical studies. In this thesis we aimed to further explore the relationship between the endocannabinoid system and BAT in humans, more specifically in South Asians.

As compared to white Caucasians, South Asians have lower resting energy expenditure (REE) and lower [^{18}F]fluorodeoxyglucose (^{18}[F]FDG) uptake by BAT upon cold exposure,

which is suggestive of less BAT activity (9). Since South Asians also have a lower rise in plasma free fatty acids (FFA) after cold exposure (9), indicative of lower noradrenalin signalling within WAT, we hypothesised that South Asians might have a higher endocannabinoid tone. In **Chapter 2**, we indeed showed that healthy lean South Asians have higher circulating endocannabinoid levels compared to white Caucasians. One of the remaining questions was; what metabolic organ(s) is/are responsible for the elevated endocannabinoid tone in South Asians. In an attempt to answer this question, in **Chapter 3** we investigated gene expression of cannabinoid (CB) receptors and enzymes involved in endocannabinoid synthesis and degradation in WAT and skeletal muscle of middle-aged overweight, prediabetic South Asian and white Caucasian men. We found that South Asian men have lower expression of CB receptors and endocannabinoid degradation enzymes, specifically in skeletal muscle. Collectively, these data may indicate that low expression of degradation enzymes in skeletal muscle of South Asian men might contribute to higher endocannabinoid levels, both locally within skeletal muscles and in the blood by spill-over from skeletal muscles. It should be noted that we did not measure levels of endocannabinoids within the various tissues. Since endocannabinoids have a short half-life and, therefore, are generally believed to act locally at the site of synthesis, it would be relevant to assess differences in tissue-specific endocannabinoid levels in South Asians and white Caucasians in future studies.

Although most mechanistic studies investigating endocannabinoid signalling and BAT activity have been performed in rodents, the potential importance of CB1 receptors for BAT activity was recently confirmed in humans. Using [^{18}F]FMPEP-*d2,* a radiotracer that targets and quantifies CB1 receptor density, it was shown that CB1 receptor density within the brain and BAT is lower in overweight, compared to lean men. In addition, cold exposure increases CB1 receptor density in the brain and BAT in lean men, but not in overweight men (10), possibly reflecting the reduced BAT activity and/or suggesting an impaired regulation of the endocannabinoid system in overweight individuals (10). This underscores the link between the endocannabinoid system and the regulation of BAT activity in humans and opens up novel perspectives to regulate BAT activity via modulating endocannabinoid signalling in humans. For example, it would be interesting to use [^{18}F]FMPEP-*d2* in healthy lean South Asians and white Caucasians to assess CB1 receptor density in the brain and BAT upon cold exposure to reveal potential differences between the ethnicities.

The response to metabolic stressors

Previous studies from our department have shown that South Asians respond differently to dietary stressors compared to white Caucasians. When administrating a 5-day high fat diet, South Asians, in contrast to white Caucasians, rapidly become insulin resistant (11). This suggests that South Asians are less able to handle a lipid overload, which

could make them more prone for ectopic fat accumulation which in turn promotes the development of insulin resistance. Next to a high fat diet, cold exposure also induces a metabolic response. For that reason, we decided to further investigate differences in the response to short-term cooling between South Asians and white Caucasians.

Cold exposure stimulates intracellular lipolysis of WAT resulting in release of FFA into the blood stream, which can be taken up by BAT or skeletal muscle, to fuel metabolic processes that include thermogenesis. Both of these processes are driven by the sympathetic nervous system. Intriguingly, we noticed that the FFA release upon short-term cooling is lower in South Asians compared to white Caucasians (9). FFA activate peroxisome proliferator-activating receptors (PPARs), which are nuclear transcription factors involved in the synthesis of *e.g.* ANGPTL4 that negatively regulates the activity of lipoprotein lipase (LPL) (12). Therefore, we now also investigated the effect of cold exposure on ANGPTL4. In line with the blunted FFA response upon cooling, we also observed a lowered ANGPTL4 response in South Asians upon short-term cold exposure (**Chapter 4**). Unfortunately, we only measured circulating ANGPTL4 levels and were not able to determine tissue-specific levels. To investigate the regulation of LPL by ANGPTL4 within the various metabolic tissues after cold exposure, additional studies are clearly needed, *e.g.* using tissue biopsies, to investigate the tissue-specific changes of ANGPTL4 upon BAT activation in humans.

Aside from ANGPTL4, there are other members of the ANGPTL family that could be of interest in the context of metabolic disease, for example ANGPTL3 and ANGPTL8. Like ANGPLT4, ANGPTL3 and ANGPTL8 individually can inhibit LPL, but when they form a complex the ability of ANGPTL3 and ANGPTL8 to inhibit LPL is greatly increased (13,14). ANGPTL3 is interesting because of its link with CVD (15). ANGPTL8, on the other hand, might be particularly of interest in the context of obesity and T2D, since ANGPTL8 is highly expressed in WAT (16), controlled by insulin (17) and circulating ANGPTL8 levels are elevated in individuals with prediabetes and T2D (18). It would be interesting to study if ANGPTL3 and ANGPTL8 levels differ between South Asians and white Caucasians and what the effect of cold exposure is on (local) ANGPTL3 and ANGPTL8 levels.

Inhibiting ANGPTL3, 4 or 8, *e.g.* with monoclonal antibodies or antisense oligonucleotides, can be a novel strategy to target cardiometabolic disease. Indeed, inhibition of ANGPTL4 or 8 (in rodents and primates) improve insulin sensitivity and the lipid profile (19-21). In addition, inhibition of ANGPL3 reduces plasma TG levels in individuals with hypertriglyceridemia (22,23). Whether ANGPTL inhibitors also improve glucose and lipid metabolism and lowers the risk on CVD in individuals with obesity and/or T2D still needs to be elucidated. The recent observation that genetic ANGPTL3 inactivation associates with decreased odds of atherosclerotic cardiovascular disease is at least promising (22). Since South Asians have an increased risk on the development of cardiometabolic disease, these individuals might possibly particularly benefit from treatment with ANGPTL

inhibitors. Future studies are warranted to reveal the effects of ANGPTL inhibitors in South Asian individuals.

The role of the sympathetic nervous system

To explain the different response to cooling between the two ethnicities, we hypothesize that South Asians have a reduced sympathetic outflow to peripheral organs upon cold exposure. A lower sympathetic output to the periphery, at the same extent of cold exposure, could explain both the blunted FFA response as consequence of less sympathetic activation of WAT and the blunted ANGPTL4 response as a consequence of less FFA release.

When we speculate about the mechanism underlying the reduced sympathetic outflow in South Asians, the endocannabinoid system again comes in play. Sympathetic signalling in adipocytes is induced by the release from nerve terminals of noradrenaline that acts on the β3-AR. However, during cold exposure endocannabinoids are released from the adipocyte to inhibit noradrenalin signalling (7,8), probably to prevent excess sympathetic stimulation of the tissue. Since we showed that South Asians have higher circulating endocannabinoid levels compared to white Caucasians (**Chapter 2**), and assuming they are derived from adipose tissue, this might explain the reduced sympathetic signalling in adipose tissue and thus the blunted FFA and ANGPTL4 response upon cooling in South Asians.

Of note, in **Chapter 6**, we tried to indirectly measure differences in sympathetic outflow between South Asians and white Caucasians by assessing parameters of heart rate variability before and after short-term cooling. There, we did not find differences between South Asians and white Caucasians, which might suggest that there is no difference in parasympathetic or sympathetic outflow upon cooling between the two ethnicities. However, interpretation of heart rate variability is complex, and caution must be applied when interpreting these data.

The influence of body fat

In all study cohorts described in this thesis, despite matching based on age and BMI, South Asians had a significantly higher body fat percentage and a lower lean mass percentage, compared to white Caucasians (**Chapter 2-6**) (24,25). In addition, South Asians in general have more visceral adipose tissue compared to white Caucasians (26,27), which is considered a specifically metabolically unhealthy depot and is associated with higher incidence of metabolic disease and CVD (28-31). The visceral adipose tissue depot, compared to other fat depots, secretes more plasminogen activator inhibitor-1 (32) and interleukin-6 (33), both of which are associated with insulin resistance. In addition, visceral adipose tissue has a higher lipolytic rate, resulting in release of more FFA into the circulation, which can through the portal vein enter the liver thereby inducing hepa-

tosteatosis and promoting insulin resistance (34). Of note, because of the difference in body composition at similar BMI in certain ethnic sub-groups, ethnic-specific BMI cut-off points are already implemented in current guidelines for assessing metabolic risk, including South Asians (35-37).

It is not unlikely that a difference in body composition in itself could already explain some of the metabolic differences between South Asians and white Caucasians that we described in this thesis. For example, since thermoneutral REE is largely determined by lean mass (38) and South Asians have lower lean mass percentage compared to white Caucasians, it is not unexpected that we found lower REE in South Asians. In addition, a high amount of body fat is associated with elevated endocannabinoid levels (**Chapter 2**). As South Asians have a higher fat percentage than white Caucasians this might also, at least in part, explain the fact that we observed higher endocannabinoid levels in South Asians. This hypothesis is supported by the fact that differences between the two ethnicities were lost when we corrected for lean mass (in case or REE) or fat mass (in case of plasma endocannabinoid levels). A follow-up question that arises is; why do South Asians, with similar BMI, have a different body fat distribution? Evidence suggests that South Asians might have a smaller subcutaneous adipose tissue depot compared to white Caucasians (30). Therefore, during the course of obesity development, the maximum storage capacity of the subcutaneous depot may be reached earlier in South Asians, resulting in the necessity for storage in the visceral depots and finally more ectopic fat accumulation.

Taken together, it is likely that a combination of various risk factors in South Asians explain the increased predisposition for metabolic disease and the increased risk for CVD as compared to white Caucasians. Aside from the already identified risk factors as described in the introduction of this thesis (*i.e.* genetics, diet, exercise, obesity, dyslipidemia and endothelial dysfunction), possibly, a combination of increased endocannabinoid tone and reduced sympathetic outflow to peripheral tissues (including WAT and BAT) could also contribute to the risk profile of South Asians. The link between body fat, endocannabinoids and sympathetic outflow is summarized in a hypothetical model (**Figure 1**).

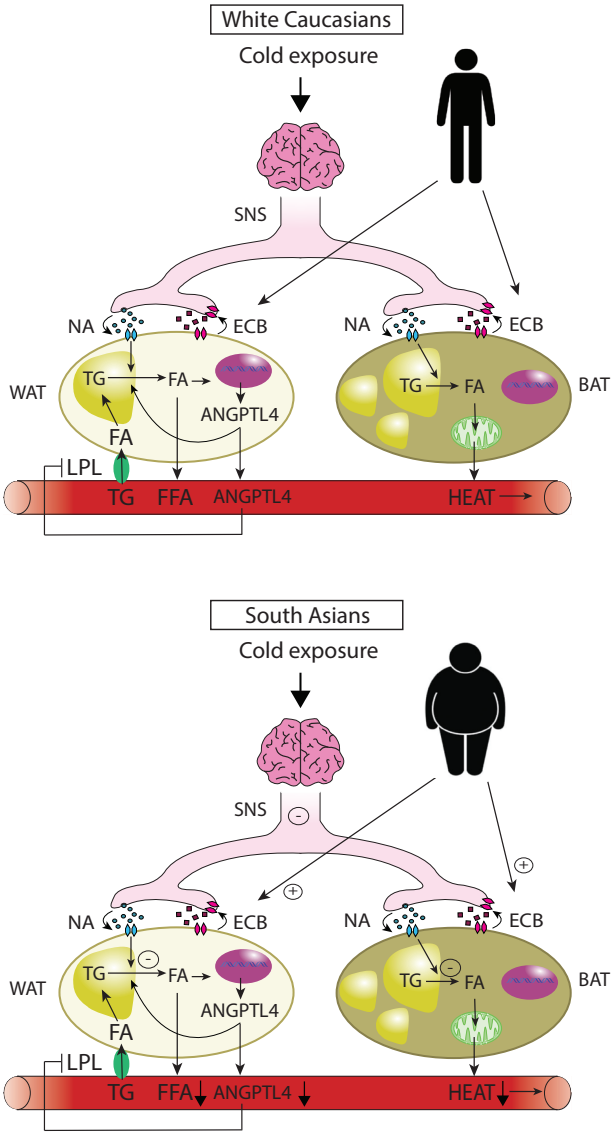

Figure 1. Hypothetical model explaining the link between body fat, endocannabinoids and sympathetic outflow Cold exposure enhances sympathetic outflow to peripheral tissues, including white adipose tissue (WAT) and brown adipose tissue (BAT). In WAT, sympathetic stimulation induces intracellular lipolysis and the subsequent release of free fatty acids (FFA) into the blood stream. In BAT, sympathetic stimulation activates thermogenesis, thereby increasing resting energy expenditure (REE). South Asians have higher endocannabinoid tone compared to white Caucasians as possibly explained by a higher body fat percentage with comparable BMI. As endocannabinoids inhibit noradrenalin signalling, South Asians have a blunted FFA and ANGPTL4 response, less BAT activation and no significant increase in REE compared to white Caucasians upon cold exposure. ANGPTL4, angiopoietin-like 4; BAT, brown adipose tissue; ECB, endocannabinoids; FFA, free fatty acids; NA, noradrenalin; SNS, sympathetic nervous system; TG, triglyceride; WAT, white adipose tissue.

Future perspectives in research into ethnic differences in metabolic disease

When assessing the translational value of our work it is important to note that observational clinical trials, like the ones described in this thesis, have some important limitations. The use of relatively small cohorts in specific subgroups (*e.g.* healthy men) limits the translational value of the findings. Additional studies in different cohorts of subjects (*e.g.* women) and patients are warranted to investigate the clinical value for the general population. In addition, our results might have been confounded by other factors, for example differences in body composition, genetic make-up or diet. Therefore, future dedicated studies are required in cohorts of South Asians and white Caucasians who are matched on body fat percentage instead of BMI. Also, it would be interesting to look into genetics, aiming at identifying gene polymorphisms or mutations in risk susceptibility genes such as the β3-AR, the melanocortin 4 receptor (MC4R) or fat mass and obesity associated genes. Furthermore, it has recently been established that lipid uptake by BAT has a diurnal rhythm, with highest uptake of TG-derived FA at the onset of the active period (39). In this context, the influence of differences in diet and eating habits (*e.g.* timing of eating) on the risk of metabolic disease in South Asians compared to white Caucasians deserves more attention. Since current treatment options for South Asians with metabolic disease are limited and unfocussed, more research on the underlying mechanisms is needed. Identification of specific risk factors in South Asians may eventually result in the discovery and development of potential novel treatment strategies to combat obesity, T2D and CVD in this especially vulnerable population.

2. NOVEL BAT-TARGETED PHARMACOLOGICAL STRATEGIES TO COMBAT METABOLIC DISEASE SPECIFICALLY IN SOUTH ASIANS

Current treatment strategies for obesity and metabolic disease are often focussed on reducing food intake and/ or on increasing energy expenditure. For example, obesity is treated by dietary modifications, *e.g.* low or very low calorie diets, behaviour modifications, *e.g.* psychotherapy, exercise programs, bariatric surgery or a combination. However, compliance to low-calorie diets or intensive exercise programs is difficult. As a consequence, except for bariatric surgery, current treatment strategies are often not effective on the long-term as a high number of individuals regain most of the weight that they initially lost (40). Therefore, novel treatment strategies are warranted. Modulation of adipose tissue, specifically activation of BAT, is considered a promising novel therapeutic strategy to increase energy expenditure (41). In addition, the observation that with the correct stimuli white adipocytes have the ability to transform into a brown adipocyte-like phenotype (42) (a process called 'browning') has further broadened the implications of activating BAT for the treatment of metabolic disease, especially since

humans have a substantial amount of WAT. Beneficial metabolic effects of BAT activation by means of cold exposure have been irrefutably shown in the past years, first in animal models (43-46) and later also in humans (47-51). However, cold exposure does not seem to be a suitable treatment option in the long-term, again because of potential compliance issues. Therefore, more suitable alternative therapeutic modalities, such as pharmacological activation of BAT are currently of interest. In this thesis, we have investigated two potential pharmacological strategies, *i.e.* β3-AR agonism and DPP4 inhibition, which will be further discussed below.

Direct activation of BAT for the treatment of metabolic disease

Targeting the β3-adrenergic receptor

In the past years, β3-AR agonists gained attention as novel therapeutic tools to combat metabolic disease. Although β3-AR agonism stimulates BAT activity, prevents fat accumulation, improves dyslipidemia and insulin sensitivity and attenuates the development of atherosclerosis in rodent studies (46), the effects in human studies, so far, were less conclusive. Mirabegron, a selective β3-AR agonist, was developed and marketed as a drug for hyperactive bladder disease at a daily dose of 50 mg. Interestingly, recently it was shown that a single dose of mirabegron (200 mg) increased [^{18}F]FDG uptake by BAT as determined by PET/CT imaging, and increased REE in healthy lean men, with similar effectiveness as cold exposure (52).

In **Chapter 6**, we were able to confirm that mirabegron increases REE in healthy lean men. In addition, we observed a trend towards lowering of the fat fraction in supraclavicular adipose tissue, which is indicative of combustion of intracellular lipids by BAT. Interestingly, mirabegron is thought to activate both the wild-type β3-AR as well as a less functional form of the β3-AR which contains a missense mutation. This mutated form of the β3-AR is highly prevalent in Pima Indians (53). Like South Asians, they are particularly vulnerable for metabolic disease, and have a lower REE and an earlier onset of T2D than white Caucasians (53). In addition, South Asians with this mutation are, compared to individuals from other ethnical backgrounds, particularly at high risk to develop T2D (54). Therefore, South Asians with this polymorphism might also benefit from treatment with this drug.

Results of trials investigating the long-term effects of mirabegron, for example on lipid levels of body weight, are currently lacking. Whether mirabegron is an effective treatment option for the long-term has to be determined in larger randomized-controlled trials in populations at risk, for example overweight/obese and/or prediabetic individuals and/or South Asians. Recruitment of BAT and browning of WAT should be assessed, as well as chronic effects on glucose tolerance and the lipid profile.

In addition, the side effects of chronic mirabegron treatment should not be overlooked. With one dose of 200 mg we observed a slight increase in heart rate (+8 bpm). Although

rather small, on the long-term, a few beats per minute increase in heart rate can increase the risk of cardiovascular morbidity and mortality (55). This is particularly important in risk populations, including obese individuals, who might already have elevated risk for the development of CVD.

The side effects of mirabegron on the heart are considered to result from actions on β1- and β2-adrenergic receptors. Although mirabegron is a relatively specific β3-AR agonist, at high dose it activates the β1- and β2-adrenergic receptors. Interestingly, [^{18}F]FDG uptake by BAT might also partly be mediated through activation of β1- and/or β2- adrenergic receptors (56,57). Pre-treatment with the nonselective β1/β2-adrenergic receptor antagonist propranolol before performing a PET/CT scan in cancer patients to assess metastasis, largely reduces background [^{18}F]FDG uptake by BAT (56,57). In line, data from rodent studies suggest that an increase in blood flow to BAT through stimulation of β2-adrenergic receptors, already is sufficient to increase metabolic activity of BAT (58). Possibly, the effect of mirabegron on BAT activity, may partly explained by an increased blood flow to BAT through stimulation of β2-adrenergic receptors. If true, this may implicate that modulation of (local) endothelial cells, instead of direct activation of brown adipocytes, might also be an interesting strategy to treat metabolic disease. Clearly, additional studies should be performed to critically assess the beneficial and potentially adverse effects of mirabegron before it can be launched onto the market as a novel drug for the treatment of metabolic disease. In addition, strictly selective β3-adrenergic receptor agonists should be developed, if only to observe whether strict β3-AR agonism activates BAT activity.

Targeting the endocannabinoid system

A few years ago, a systemic CB1 receptor blocker (rimonabant) was brought into the market for the treatment of obesity. It was very effective in humans to induce substantial and long-term maintained weight loss and in addition improved the lipid profile (59-61). However, in 2008, the use of this drug was suspended in Europe due to psychiatric side effects, probably due to off target effects in the brain. Nevertheless, based on the results of our studies described in **Chapter 2 and 3**, modulation of the endocannabinoid system might still be an interesting approach to treat metabolic disease, particularly in the South Asian population. Since South Asians have higher circulating endocannabinoid levels, a drug that inhibits endocannabinoid signalling might be even more effective in South Asians compared to white Caucasians. Possibly, specifically blocking peripheral endocannabinoid receptors, which would eliminate central effects in the brain, might be a promising option. Peripheral blockers can prevent (circulating) endocannabinoids from binding to (and signalling through) their receptors thereby reducing their unwanted metabolic effects on skeletal muscle, WAT and BAT. In addition, strictly peripheral CB receptor blockers would circumvent the off target effects of CB receptor agonism

in the brain, thereby preventing psychiatric side effects. Of note, since some of the weight-reducing effects of rimonabant are induced via the brain, *e.g.* inhibition of food intake, it should be investigated whether strictly peripheral CB1 blockage is as effective in reducing body weight in humans as CB1 receptor agonists that also act centrally. In this respect it is interesting that we previously showed that a strictly peripheral CB1 receptor antagonist activates BAT, enhances REE, and still induces substantial weight loss in mice (62).

Another, more direct approach could be to target the endocannabinoid levels themselves, as high concentrations of local endocannabinoids rather than their receptors are the actual cause of the problem. Lowering of circulating endocannabinoid levels might be achieved by interfering with the activity of enzymes involved in the synthesis and/or degradation of endocannabinoids, *e.g.* using endocannabinoid synthesis inhibitors. However, endocannabinoids are pleiotropic hormones, influencing many different processes in the human body. For example, endocannabinoids can also be beneficial for regulating pain or inflammation. In fact, drugs that increase endocannabinoid tone (*e.g.* inhibitors of the endocannabinoid degradation enzymes FAAH1 and MAGL) are currently studied for the treatment of anxiety disorders and neurodegenerative diseases (63). Therefore, modulating endocannabinoid synthesis and degradation should probably be tissue-specific and may turn out to be a difficult approach to pursue.

Targeting ANGPTL4

In **Chapter 4**, we showed that serum ANGPTL4 levels increase in response to short term mild cooling. ANGPTL4 stimulates intracellular lipolysis (12,64) and inhibits LPL-mediated extracellular lipolysis (65) in WAT. Therefore, with respect to the treatment of obesity, it might be interesting to develop a WAT-specific ANGPTL4 stimulator, possibly based on the C-terminus of the ANGPTL4 molecule which can directly stimulate intracellular lipolysis while preventing LPL-dependent influx of FA, resulting in release of FFA into the circulation (64). To prevent accumulation of FFA in the blood (which is toxic in high concentrations and can leads to steatohepatosis), this drug should preferably be combined with a strategy that stimulates combustion of FFA, for example exercise, a direct activator of BAT or a compound that stimulates browning of WAT. Because we showed that South Asians had a lower ANGPTL4 response upon cooling as compared to white Caucasians, a drug that increases ANGPTL4 levels, specifically in WAT, might particularly effective in this population.

Indirect activation of BAT for the treatment of metabolic disease

Targeting the glucagon-like-peptide-1 receptor

An indirect pathway that may result in BAT activation is the glucagon-like peptide-1 (GLP-1) pathway. This can be achieved by either stimulation of the receptors with GLP-

1 receptor agonists, or by inhibition of degradation of endogenous GLP-1 with DPP4 inhibitors. Both GLP-1 receptor agonists and DPP4 inhibitors are already known for their beneficial effects on glucose metabolism and are, therefore, currently used in the clinic for the treatment on T2D. Interestingly, these drugs also improve lipid metabolism and enhance REE (66-70).

Both GLP-1 receptor agonists and DPP4 inhibitors increase GLP-1 receptor signalling *e.g.* in the pancreas, resulting in increased insulin and reduced glucagon secretion, thereby improving glucose metabolism. However, the mechanism by which these drugs affect lipid metabolism in humans has not yet been clarified. Possibly, part of the effect is mediated through activation of BAT, which has at least been confirmed in mice (71-77). Specifically, activation of central GLP-1 receptors in the hypothalamus results in increased sympathetic outflow towards BAT, mimicking the effect of cold exposure (71). In addition, in **Chapter 7**, we showed that the DDP4 inhibitor sitagliptin improves glucose and lipid metabolism in overweight prediabetic men, concomitant with browning of WAT and/or increased energy metabolism in skeletal muscle. However, an effect of sitagliptin on the activity of classical BAT, as measured with [^{18}F]FDG PET/CT scanning, was not observed, possibly due to insulin resistance of the tissue and a consequently low uptake of the glucose tracer. Future studies should probably use alternative PET/CT tracers (*e.g.* lipid-based tracers or measures of oxidative capacity), to better estimate BAT activity, which would be especially relevant in insulin resistant individuals.

Aside from the beneficial effects on glucose and lipid metabolism, GLP-1 receptor agonists also reduce food intake resulting in weight-loss, an effect that is however not observed for DPP4 inhibitors (**Chapter 7**). In addition, DDP4 inhibitors might have more off target effects because the DPP4 enzyme is involved in the breakdown of many proline (or alanine) containing peptides, including also growth factors and chemokines. Therefore, in my opinion, GLP-1 receptor agonists are more promising than DPP4 inhibitors as a strategy to improve cardiometabolic health.

South Asians have an increased GLP-1 response upon a glucose load (78,79), which is indicative for GLP-1 resistance. Interestingly, GLP-1 analogues lower HbA1c more effectively in South Asians compared to white Caucasians (80). GLP-1 receptor agonists are currently only prescribed and reimbursed by health care insurance companies for patients with T2D and a BMI≥ 35 kg/m^2. This is peculiar since South Asians, already at a lower BMI, have an increased risk on development of T2D and (cardio)metabolic disease (35,81). Dedicated studies are needed to investigate if GLP-1 receptor agonism already improves glucose and lipid metabolism in South Asians with a BMI lower than 35 kg/m^2. If so, as for the assessment of diabetes risk (35), there should probably also be an ethnic-specific threshold for the administration of GLP-1 receptor agonists, in which South Asians should receive the drug already at a lower BMI than white Caucasians. Of note, based on preliminary data from a recently completed study in young healthy lean

South Asians and white Caucasians, 12 weeks of exenatide (Bydureon 2 mg s.c. once a week) indeed decreased serum triglycerides, total cholesterol, low-density lipoprotein and glucose levels and lowered body weight in both ethnicities (Janssen & Nahon, unpublished).

Targeting the melanocortin-4 receptor

A second promising target might be the melanocortin pathway. The MC4R in the hypothalamus is involved in controlling energy intake and expenditure, and mutations in this receptor are associated with morbid obesity (82). Moreover, MC4R deficiency is the most common monogenetic form of childhood obesity (83). Central melanocortins are thought to dampen sympathetic nervous system outflow to WAT and BAT, thereby regulating the function of these tissues (84); Indeed, in mice, MC4R antagonism increases food intake and decreases REE, which collectively contribute to increased body weight (85). On the contrary, treatment with an MC4R agonist decreases body weight and improves insulin sensitivity and cardiovascular function in a diet-induced obese nonhuman primate model (86). In addition, a recent study convincingly showed that chronic treatment with the MC4R agonist setmelanotide induced long-term induced weight loss in individuals with leptin receptor or pro-opiomelanocortin (POMC) deficiencies, both of which are upstream of the MC4R. Whether MC4R agonists also increases BAT activity in humans need to be elucidated.

To conclude, it is undisputable that the development of novel pharmacological approaches to treat obesity and related diseases is warranted, and that targeting BAT activity can be beneficial in this respect. However, it must be noted that promoting a healthy lifestyle should always be the most desirable treatment option, and should be the cornerstone for the prevention and treatment of metabolic disease.

3. METHODOLOGICAL ASPECTS OF CLINICAL TRIALS TARGETING BAT

Research into human BAT has some well-recognized limitations. Not only visualisation of BAT is difficult, also other direct or indirect read-outs of BAT activity are far from straightforward. Whereas in animal models BAT tissue can be easily collected and assessed for histology and expression of genes and proteins, taking BAT biopsies in humans is ethically and practically more challenging. The relatively low amounts of human BAT in combination with its localisation, hided deeper within the body and close to major vessels and nerves, makes it difficult to safely take tissues biopsies, especially in otherwise healthy lean men without indication for a surgical intervention. In addition, some indirect read-outs of BAT, such as increase in lipid oxidation may not be sufficiently specific

for BAT activity. In fact, based on the current available technologies and literature there is no 'ideal' read-out yet.

Visualisation of BAT by [^{18}F]FDG PET/CT as a 'gold' standard

The presence of active BAT in human adults was initially discovered with [^{18}F]FDG PET/CT scanning (87-89). Since no better alternatives are currently available, this technique is currently still considered the 'gold' standard for assessing BAT volume and activity in human research. However, this method has some important limitations. Firstly, this method makes use of a radioactive tracer, which limits the frequency of scans that can be made in one volunteer (typically twice a year). Secondly, the use of a glucose tracer (*i.e.* fluorodeoxyglucose; FDG) is an important limitation as active BAT rather uses FA as the main source to fuel thermogenesis (90), while glucose probably is mainly taken up for *de novo* lipogenesis (91). Moreover, some interventions that activate BAT specifically affect the FA uptake by BAT, while glucose uptake is not necessarily changed (92). This implicates that with a glucose tracer the effects on BAT might, in some cases, be underestimated or missed. In addition, insulin resistance of BAT limits the uptake of glucose. Therefore, it is feasible to assume that in some populations, for example in prediabetic, overweight or ageing individuals, [^{18}F]FDG uptake by BAT reflects insulin resistance of BAT rather than oxidative metabolism of BAT. Of note, the presence of insulin resistance in overweight prediabetic men could also have been a reason why we did not observe an effect of sitagliptin on BAT activity, as described in **Chapter 7**. Thirdly, environmental factors including room temperature influence [^{18}F]FDG uptake, which is the reason why cooling to shivering is required to visualize and quantify BAT with this technique. Finally, until recently there were no consensus guidelines about the standardized uptake values (SUV) and Hounsfield units (HU) thresholds (93,94), which limited the comparison between different studies. Recently, this last issue has recently been solved with publication of the first general recommendation guidelines (BARCIST 1.0) (95). An important aspect of this guideline is the recommendation to use a personalized SUV threshold, which takes into account the lean mass as well (SUVindividualized = 1.2/(lean body mass/body mass)] (95). We used this guideline for the analyses of BAT activity in **Chapter 7**, and, hopefully, this guideline lead to more uniform analysis of BAT on [^{18}F]FDG PET/CT in future studies allowing between-institute comparisons.

Alternative methods for BAT visualisation

As outline above, probably the most important disadvantage of the use of a glucose tracer to trace BAT activity in metabolic research is the problem of insulin resistance that develops during weight gain and ageing. As the ultimate goal of BAT-targeted research is to develop novel treatments for obesity and T2D, these drugs eventually have to be tested in the target population. Since obese individuals and T2D patients are often insu-

lin resistant, leading to decreased translocation of glucose transporter 4 (GLUT4) to the cell membrane, the uptake of the glucose tracer by BAT will be diminished. This does not necessarily mean that they have less active BAT. In fact, a hallmark study demonstrated that ageing and T2D status decrease the uptake of [^{18}F]FDG by BAT, while the FA uptake and oxidative capacity of BAT are not reduced (96,97). Mainly for reason of insulin resistance, the search for alternative tracers are techniques is warranted.

For reasons described in the previous paragraph, 14(R,S)-[^{18}F]fluoro-6-thia-heptadecanoic acid ([^{18}F]FTHA; *i.e.* a fatty acid tracer) or [^{11}C]acetate (*i.e.* a measure of oxidative metabolism) can be used as alternative tracers to visualize BAT activity. However, a main disadvantage of [^{18}F]FTHA is the large background signal, which is due to binding of the radiotracer to albumin and subsequent high uptake of the tracer by other tissues, mainly the liver. In addition, our previous studies in mice showed that BAT takes up preferably TG-derived FA instead of FFA (98), and we have no reason to assume this would not be the case in humans. In addition, at least in mice, under conditions of GPR120 agonism, TG-derived FA uptake by BAT is approx. 10-fold higher than glucose uptake (71). Therefore, a radiolabeled FA molecule that is built into TG which is packed inside lipoprotein-like particles (99) may be a more elegant approach, thereby directly visualizing TG-derived FA uptake by BAT while strongly reducing non-specific uptake by the liver. However, under highly insulin-resistant conditions TG-derived FA uptake is probably also diminished, which might also restrict the application of this tracer in very obese/insulin-resistant volunteers. Future studies should further explore the feasibility of such a tracer, first in rodents and later in human trials.

Alternatively, ^{123}I-meta-iodobenzylguanidine ([^{123}I]MIBG) might be an interesting tracer. [^{123}I]MIBG is a radiolabeled analogue of guanethidine that enters the cell via the noradrenalin transporter and, therefore, visualizes the presynaptic reuptake and storage of noradrenalin within the sympathetic neuron, which occurs during sympathetic BAT activation. Since increased noradrenalin release from the sympathetic nerve results in more stimulation of brown adipocytes, a higher ^{123}I-MIBG uptake would imply increased BAT activity. However, such a tracer may not be relevant in case brown adipocytes are post-synaptically modulated.

Aside from PET/CT, which has the disadvantageous of exposure to radiation, other imaging modalities are currently being investigated. One of these non-invasive techniques is magnetic resonance imaging (MRI), which uses water-fat separation to estimate the fat fraction (FF) of a tissue (100-103). As BAT combusts intracellular lipids (46), intracellular FF of BAT is expected to decrease upon activation. Indeed, in mice intracellular lipolysis of TG is directly related to BAT thermogenesis (43), and BAT activation generally strongly reduced the intracellular lipid content (46). Therefore, quantification of the FF of BAT is likely an appropriate read-out for its activity. An advantage of MRI is that it involves no radioactive burden as with PET-CT and can thus be repeated multiple times in the same

individual without harm. This can be particularly useful when monitoring changes in BAT activity during the day (*i.e.* pre-clinical data point towards differences in BAT activity during the day, with the highest activity at the start of the wakeful period (39)) or during a period of pharmacological intervention. In addition, assessing the intracellular FF of BAT probably gives a more direct estimation of its activity than [^{18}F]FDG uptake since intracellular lipid stores within BAT are considered to be the main source to fuel thermogenesis (90,104). However, a limitation of this technique is that during BAT activation, intracellular lipid stores are concomitantly replenished by uptake of TG-derived FA from the blood (98) and/or the uptake of glucose which can be used for *de novo* lipogenesis. Therefore, during mild BAT activation, when intracellular FA consumption equals FA influx and production, the net FF will not be changed and BAT activity may be underestimated or even missed.

Additionally, MRI can be used to monitor blood flow. Since blood flow to BAT is thought to be increased upon stimulation, monitoring changes in blood flow might give additional information about the activation state of the tissue. Interestingly, next to changes in FF and blood flow, recent evidence suggests changes in glutathione content of BAT upon activation and browning of WAT (105,106). Glutathione acts as an anti-oxidant to protect cells against oxidative stress/damage. Upon activation of BAT, reactive oxygen species (ROS) are generated as a by-product of high metabolic activity in mitochondria (107). It has been suggested that ROS play a critical role in regulating thermogenesis and uncoupling protein 1 activity in BAT (108) and that BAT activation by means of cold exposure reduces intracellular glutathione content (105,109). Therefore, visualisation of glutathione content of BAT could potentially be a novel approach to monitor BAT activation with MRI. However, the relation between glutathione concentration and BAT activity as well as the potential of MRI in clinical research should be further investigated before this technique can replace or be added to [^{18}F]FDG PET/CT scanning.

Another novel non-invasive method to visualize BAT might be infrared thermography (IRT) (110). Supraclavicular skin temperature, as monitored with IRT, increases upon cold exposure in healthy lean men (111) and predicts [^{18}F]FDG uptake by PET/CT (112). In **chapter 6**, we showed that mirabegron treatment of healthy lean also increases the supraclavicular skin temperature, as measured with iButtons, indicative of BAT activation. Because of its non-invasive properties, IRT is also very suitable for BAT research in children. Since children, especially neonates, rely on BAT for their thermogenesis and have more BAT compared to adults, studies in children will result in novel insights on the physiology of BAT. However, although IRT might be feasible in lean participants (as described in (113) and (114)), this technique might be less reliable in overweight or obese individuals with large subcutaneous WAT depots covering the BAT depots due to insulation. As a note of caution, vasodilation related to increased blood flow to BAT upon activation of this tissue might interfere with interpretation of IRT.

Finally, microdialysis has recently been proposed as a novel technique to quantify substrate utilisation by BAT (115). By inserting a catheter into the tissue, interstitial fluid can be collected for analysis. This is particularly interesting because in this way it is possible to measure changes in locally produced hormones and metabolites upon activation of BAT. However, for secure placing of the needle PET/CT imaging modalities are still needed, which has a radiation burden and limits the frequency of the measurements. In addition, this technique is difficult to perform and is invasive, which makes large-scale application in healthy human questionable.

A summary of the above described approaches to analyse BAT function is given in **Figure 2**.

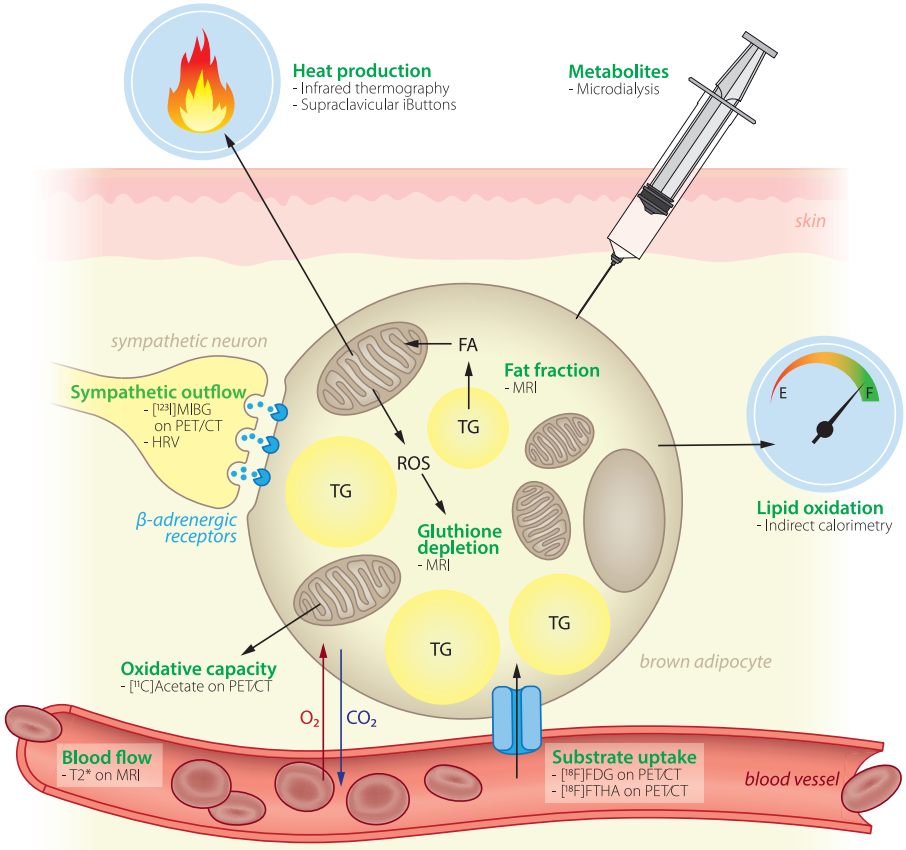

Figure 2. Direct and indirect read-outs of BAT activity. This figure summarises the currently available techniques to assess BAT activity directly and indirectly. CO_2, carbon dioxide; FA, fatty acids; [^{18}F]FDG, [^{18}F] fluorodeoxyglucose; [^{18}F]FTHA, [^{18}F]fluoro-6-thia-heptadecanoic acid; HRV, heart rate variability; [^{123}I]MIBG, [^{123}I]meta-iodobenzylguanidine; MRI, magnetic imaging resonance; O_2, oxygen; ROS, reactive oxygen species; TG, triglycerides.

4. CONCLUDING REMARKS AND FUTURE DIRECTIONS

The emergence of metabolic diseases are rapidly increasing worldwide, leading to high morbidity and mortality and increased health care costs. By identifying risk factors and underlying pathophysiological processes for these diseases novel treatment modalities might be discovered to combat the 'diabesity' epidemic. Here, we specifically focussed on the South Asian population as they are especially recognized for their increased susceptibility to develop (cardio)metabolic disease. Apart from 'classical' risk factors, 'non-classical' risk factors including differences in energy metabolism, endocannabinoid tone and autonomic dysfunction are probably involved. However, additional research is needed to validate these risk factors and assess the translational value for the general population. One of the biggest challenges in this respect is to disentangle the ethnic aspect from the other factors (such as body composition or dietary habits), which are often linked to each other.

Current treatment options for metabolic disease are generalized and mainly focus on food intake while increasing energy expenditure might be a more effective approach on the long-term. During the last few years, it has been recognized that activation and/or recruitment of BAT could be a valuable tool to combust lipids and glucose, thereby improving lipid and glucose metabolism and increasing energy expenditure. Moreover, in some patient groups, *e.g.* patients with T2D, BAT activation by cold exposure has already been shown to reduce fat mass and improve glucose tolerance and insulin sensitivity. Although these results are promising, unfortunately cold exposure is probably not a suitable therapeutic strategy for humans, especially in warm climates. Therefore, identifying pharmacological targets to activate BAT is of high importance.

Based on currently available data the most promising BAT-activating drug to date seems to be the β3-AR agonist mirabegron, which increases serum FFA, REE, lipid oxidation and supraclavicular skin temperature, and tends to decrease the FF of BAT in healthy lean South Asians and white Caucasians. Whether this drug improves the metabolic phenotype in obese or prediabetic individuals and/or prevents the progression into T2D or CVD needs to be shown in the coming years. Furthermore, we recently showed that the GLP-1 receptor agonist exenatide activates BAT in healthy lean South Asians and white Caucasian men (Janssen & Nahon, unpublished). Especially since GLP-1 receptor agonism also reduces food intake, this might be a very interesting drug to further investigate. However, next to pharmacological approaches, lifestyle modifications should always be the cornerstone for the treatment for obesity and related conditions.

In conclusion, with the discovery of energy-combusting BAT in human adults in 2009, research into the pathophysiology and treatment of metabolic disease was put in a novel perspective. The studies described in this thesis underscore the potential of pharmacological BAT activation as a strategy to combat metabolic disease, which might be

particularly beneficial for South Asians, because of their increased risk for the development of metabolic disease. The coming years will reveal the potential therapeutic power of BAT to combat the 'diabesity'-epidemic that is threatening the world.

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