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

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COMBATTING METABOLIC DISEASE

Ethnic aspects, mechanisms and novel treatment strategies

Kimberly Nahon

**Combatting metabolic disease:
Ethnic aspects, mechanisms and novel treatment strategies**

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COMBATTING METABOLIC DISEASE

Ethnic aspects, mechanisms and novel treatment strategies

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CHAPTER 1

General introduction and outline

OBESITY AND RELATED DISORDERS

Obesity is one of the largest global health emergencies of the 21st century. According to the World Health Organisation, obesity is defined as a body mass index (BMI) of $>30 \text{ kg/m}^2$. Since 1980, the prevalence of obesity has doubled worldwide to a number of 603.7 million adults (12.0% of the world population) in 2015 (1). Moreover, this number is expected to further increase in the coming years (2). Obesity deregulates metabolic processes including glucose and lipid handling and results in systemic inflammation. Therefore, obesity is a major risk factor for the development of type 2 diabetes mellitus (T2D), dyslipidemia and cardiovascular diseases (CVD). In line with this, the prevalence of diabetes is rapidly increasing as well, from 425 million adults with diabetes in 2017 to an estimated number of 629 million adults in 2045 (3), of whom 91% will have T2D. CVD are the most common causes of death among people with diabetes, currently accounting for over 2.5 million deaths worldwide (3).

WHITE AND BROWN ADIPOSE TISSUE

Obesity is the consequence of excessive storage of fat in the adipose tissue organ. Under 'lean' conditions, approximately 15% of body weight in men and about 25% of body weight in women is contributed by adipose tissue, while in obesity these percentages can increase up to $>40\%$ (4,5). The most abundant type of adipose tissue is white adipose tissue (WAT), which is located throughout the body and has a function in lipid storage and insulation. Two major WAT depots can be distinguished: subcutaneous WAT, located underneath the skin, and visceral adipose tissue, which surrounds the internal organs and is mainly located in the abdominal cavity (6). WAT stores excessive lipids and sugar in the form of triglycerides. Under conditions of high energy demands (*e.g.* exercise, fasting or cold exposure), WAT can release fatty acids derived from these triglycerides into the blood, via a process known as intracellular lipolysis. These fatty acids can subsequently be taken up by other tissues to fuel metabolism, *e.g.* skeletal muscle in case of exercise. Besides WAT, a different shade of adipose tissue exists, namely brown adipose tissue (BAT). For a long time, BAT was considered to be only present in neonates. However, since 2009, it has been established that human adults still have active BAT as well (7-9). BAT is predominantly located in the supraclavicular and para-aortic regions (8) and combusts fatty acids and to a lesser extent glucose into heat (*i.e.* thermogenesis), thereby maintaining body temperature under conditions below thermoneutrality (10). Although the total amount of WAT largely exceeds that of BAT (with an estimated average of 12-35 kg of WAT versus max. 200-300 g of BAT) (11), BAT has an extremely large capacity to convert energy stored in fatty acids and glucose into heat. It has been

estimated that fully activated BAT can produce up to 300 W/kg, whereas most other tissues produce only 1 W/kg (12).

AETIOLOGY OF INSULIN RESISTANCE AND RELATION WITH DYSLIPIDEMIA

While storage of large amounts of fat in WAT in obesity is not harmful *per se*, the accompanying dyslipidemia and insulin resistance can pose a health risk. Blood glucose levels are tightly regulated within a narrow range by balancing the endogenous glucose production and the uptake by peripheral tissues. Insulin is the most important hormone involved in the glucose homeostasis. Insulin is released by β -cells of the pancreas in response to high blood glucose levels. The main action of insulin is to lower blood glucose levels. This can be established by stimulating the uptake of glucose by peripheral tissues, including muscle and adipose tissue. During the development of obesity, WAT depots expand to increase the storage capacity. In this process adipocytes increase in size, a process called hypertrophy (13). Hypertrophic adipocytes release pro-inflammatory adipokines, including tumor necrosis factor α and interleukin 6 (14,15), that trigger the immune system resulting in infiltration of immune cells including macrophages (16). Pro-inflammatory cytokines can inhibit insulin signalling, which induces insulin resistance of the tissue (17). Eventually, when adipocytes become too large and blood supply is insufficient, hypoxia occurs which triggers cell death, thereby further attracting macrophages. Immune cell infiltration of WAT is one of the main characteristics of dysfunctional WAT in obesity. Moreover, dysfunctional adipocytes become insulin resistant leading to the release fatty acids (FA) into the circulation. High levels of circulating free FA inhibit glucose uptake and combustion (18,19), thereby elevating blood glucose and insulin levels in a process called insulin resistance, which may lead to T2D (18,20,21). In addition, an increased flux of free FA to the liver, combined with hepatic insulin resistance, stimulates hepatic triglyceride synthesis and VLDL secretion which further contributes to the development of dyslipidemia (22). Also, insulin resistance reduces the expression of lipoprotein lipase (LPL) in skeletal muscle and adipose tissue, which reduces VLDL catabolism, thereby further accelerating hypertriglyceridemia and dyslipidemia (23). When the maximum storage capacity of WAT is reached, triglycerides are stored in other organs (e.g. 'ectopic fat accumulation'), including liver, skeletal muscle and pancreas (24,25). In the pancreas local lipid accumulation causes pancreatic dysfunction, and eventually loss of insulin production, which even further exacerbates hyperglycemia and dyslipidemia (25,26). Of note, ectopic fat accumulation is considered one of the most important risk factors for the development of T2D (27,28).

THE SOUTH ASIAN POPULATION, A POPULATION AT INCREASED RISK FOR METABOLIC DISEASE

Obesity rates differ between ethnicities. Particularly in South Asians, originating from the Indian subcontinent and constituting 20% of the world population, obesity prevalence is estimated to reach 50% in urban areas (29), and obesity-associated complications including T2D are highly prevalent (30). Moreover, South Asians develop T2D at a younger age and lower BMI compared to white Caucasians (31,32). In addition, the South Asian ethnicity itself is considered an independent risk factor for CVD (33). The underlying mechanisms of this increased risk for metabolic disease are not completely understood, but cannot fully be explained by their higher prevalence of 'classical' risk factors including obesity and dyslipidemia (33-36). Therefore, it is likely that a combination of many additional risk factors explain the elevated risk on development of metabolic disease in this population (35,37). However, the nature of these additional risk factors have not been elucidated yet.

Obesity manifests differently in South Asians as compared to white Caucasians. With comparable BMI, South Asians have a higher body fat percentage than white Caucasians (38,39). In addition, body fat distribution is different in South Asians, with higher intra-abdominal and truncal subcutaneous adipose tissue dispositions and more ectopic fat disposition, possibly contributing to an increased risk on developing insulin resistance and eventually T2D (40-43). Not only the amount and distribution of body fat differs, it has also been proposed that adipocyte function is disturbed in South Asians (44,45).

Another 'classical' risk factor for metabolic disease, especially for CVD, is dyslipidemia. An unfavourable lipid profile, consisting of high levels of triglycerides and low-density-lipoprotein (LDL)-cholesterol (46) and low levels of high-density-lipoprotein (HDL)-cholesterol (46,47) is frequently present in South Asians. In addition, impaired endothelial function (*i.e.* reduced endothelium-dependent vasodilation and increased vessel stiffness) has been described in South Asians (48-50), which further predisposes to atherosclerosis development and thus CVD. Interestingly, already in cord blood of neonates elevated levels of triglycerides, non-HDL-cholesterol and E-selectin (marker of endothelial dysfunction) are observed (51), which underscores that (some) metabolic disturbances are already present early in life.

However, the above-mentioned 'classical' risk factors probably do not explain the full picture. 'Non-classical' risk factors such as differences in genetic make-up, diet, or differences in energy metabolism could therefore not be overlooked when exploring risk factors for the development of metabolic disease in the South Asian population. With respect to genetic make-up, so far no genetic polymorphisms have been identified that could account for the ethnic difference in predisposition for obesity or T2D (52). Neither do differences in dietary intake or exercise fully explain the increased risk (52). However,

there is increasing evidence that energy metabolism might be differently regulated in South Asians as compared to white Caucasians. We previously showed that healthy lean South Asians have 32% lower resting energy expenditure as compared to BMI-matched white Caucasians (53) and less energy-combusting BAT as assessed with [^{18}F]fluorodeoxyglucose positron emission tomography-computed tomography ([^{18}F]FDG PET/CT). These factors likely contribute to their lower energy metabolism and could thereby, at least in part, contribute to the development of metabolic disease.

Taken together, all of the above-mentioned ‘classical’ and ‘non-classical’ risk factors (summarized **Figure 1**) can potentially contribute to the increased susceptibility for metabolic disease in the South Asian population. However, the list is probably not complete. Additional studies are warranted to gain more insight in the factors that are involved in the increased risk on metabolic disease in this vulnerable population.

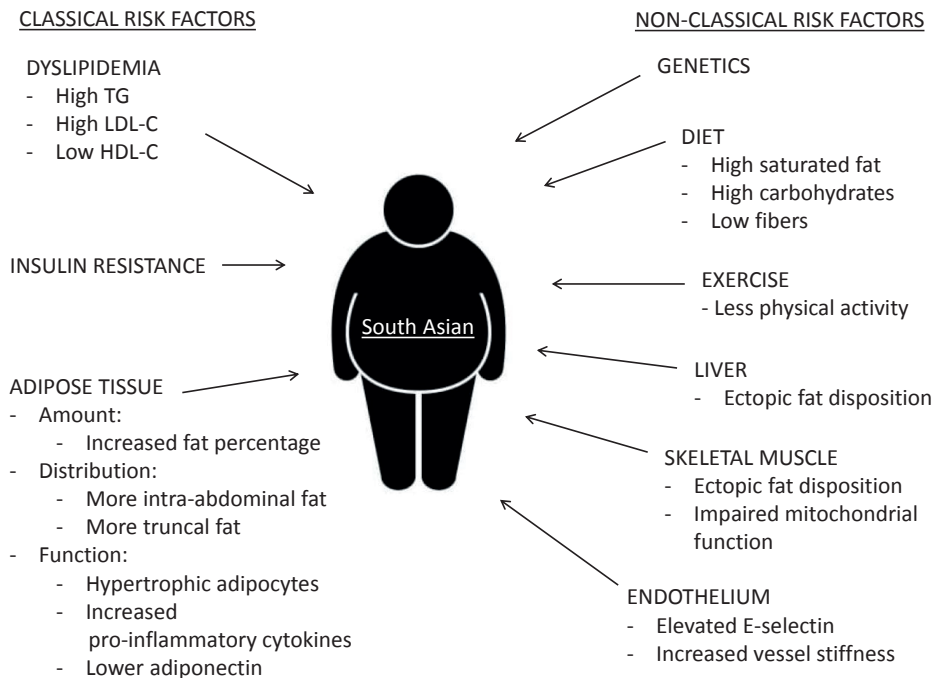


Figure 1. Known ‘classical’ and ‘non-classical’ risk factors for metabolic disease in the South Asian population. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides.

When additional risk factors for the development of metabolic disease will have been identified, specific treatment strategies can be developed to target these diseases. Current treatment strategies for obesity are often focussed on reducing food intake via dieting or on increasing energy expenditure via increased physical activity. However,

these interventions are generally not effective on the long-term. In fact, the only effective anti-obesity intervention thus far is invasive bariatric surgery (54). Therefore, novel treatment strategies are warranted. Since BAT is able to combust lipids and glucose, thereby increasing energy expenditure, modulation of this tissue is considered to be an interesting target to combat obesity and T2D.

PHYSIOLOGICAL ACTIVATION OF BAT

In line with its function to produce heat, the main physiological activator of BAT is cold exposure (10). BAT is highly innervated by sympathetic neurons. Upon cold exposure, the sympathetic outflow from the hypothalamus towards BAT is increased. Sympathetic neurons release norepinephrine that binds to β 3-adrenergic receptors on the brown adipocyte membrane, at least in mice (55-57). In humans it is still under debate what receptor(s) is (are) involved in BAT activation. As a result, the uncoupling protein-1 (UCP-1) in the inner membrane of the mitochondria becomes activated. UCP-1 uncouples mitochondrial respiration from adenosine triphosphate production resulting in the dissipation of chemical energy as heat (58,59). At the same time, BAT releases endocannabinoids, which are believed to act on endocannabinoid (CB) receptors at the presynaptic terminal of sympathetic nerve endings to inhibit noradrenalin signalling (60,61). This sequence of events likely serves as a feedback mechanism to prevent excessive activation of BAT by cold (depicted in **Figure 2**). Interestingly, circulating endocannabinoid levels are elevated in obesity (62-64). It remains to be determined whether circulating endocannabinoid levels also differs between South Asians and white Caucasians and if endocannabinoids could contribute to increased risk on metabolic disease in the South Asian population.

During thermogenesis, BAT combusts FA derived from intracellular lipid stores, thereby lowering intracellular lipid levels. As a consequence, the intracellular lipid stores become depleted and need to be replenished. To this end, BAT takes up free FA and triglyceride (TG)-derived FA from TG-rich lipoproteins in the blood (65). The TG-derived FA uptake by BAT is dependent on LPL, which is present on the vessel wall (66,67). LPL is regulated by many factors including angiopoietin-like 4 (ANGPTL4) (68,69). ANGPTL4 inhibits LPL thereby preventing the uptake of TG-derived FA by metabolic tissues (68) and at the same time stimulates intracellular lipolysis in WAT to further increase circulating free FA levels (70). ANGPTL4 is suggested to be differently regulated in BAT and WAT. Preclinical studies have shown that upon cooling, ANGPTL4 levels increase in WAT while they decrease in BAT in order to facilitate shuttling of FA from WAT to BAT (71). However, the effects of cold exposure on ANGPTL4 in humans is still unknown. In addition, cold exposure increases sympathetic outflow towards WAT which adds to enhanced lipolysis

and more release of FA into the bloodstream (72). These FA can be directly taken up by BAT or travel to the liver, where there are used for the synthesis into triglycerides. Triglycerides can subsequently be secreted into the blood in the form of VLDL. Notably, pre-clinical studies indicate the presence of a feed-forward mechanism in which FA, released by WAT, in the brain, further increases sympathetic outflow to BAT to stimulate the combustion of FA for thermogenesis (73).

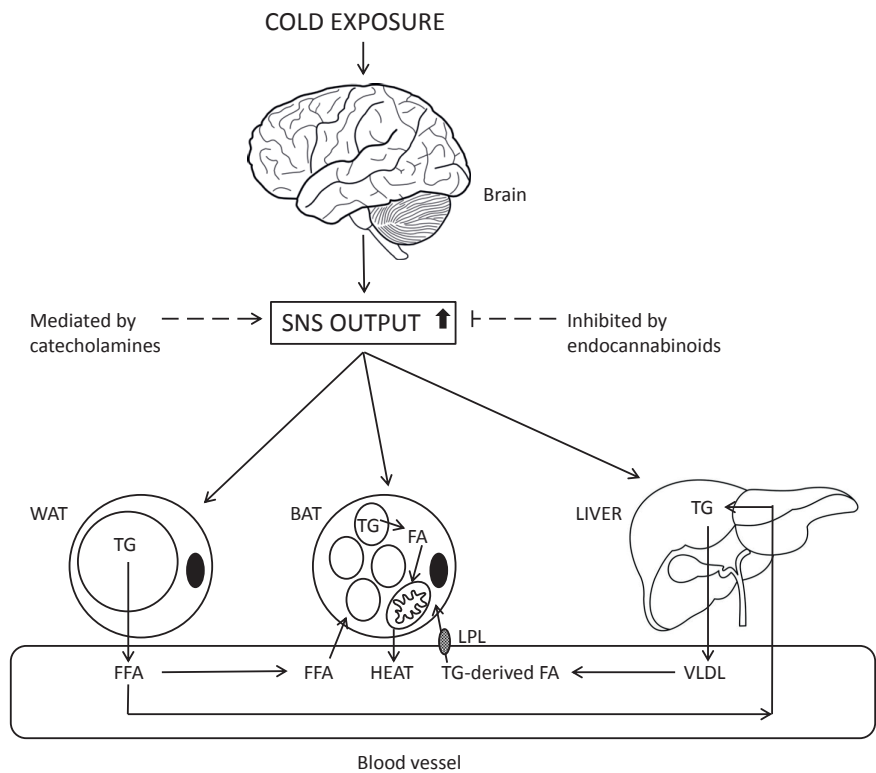


Figure 2. Link between the sympathetic nervous system and the endocannabinoid system during cold exposure. Cold exposure increases output of the sympathetic nervous system to peripheral organs. Sympathetic stimulation of white adipose tissue (WAT) stimulates lipolysis which results in release of free fatty acids (FFA) into the bloodstream. In addition, sympathetic stimulation of the liver increases the production and release of very-low-density lipoproteins (VLDL) into the bloodstream. Sympathetic stimulation of brown adipose tissue (BAT) induces thermogenesis thereby combusting intracellular lipid stores. The intracellular lipid stores can be replenished through uptake of WAT and liver-derived (triglyceride-derived)-fatty acids from the blood. BAT, brown adipose tissue; FFA, free fatty acids; SNS, sympathetic nervous system; TG, triglyceride; VLDL, very low density lipoproteins; WAT, white adipose tissue.

Thus, sympathetic activation of BAT following cold exposure results in combustion of TG-derived FA towards heat. Evidence for the importance of active BAT in energy metabolism comes from pre-clinical and clinical trials. In rodent studies, cold exposure

activates BAT, increases energy expenditure, decreases fat mass and increases glucose tolerance and insulin sensitivity (10,66,74). Human trials have also shown that 10 days (75) or 4 weeks (76) of intermittent cold acclimatisation enhances BAT activity and increases energy expenditure in both lean (75,76) and obese individuals (77). Moreover, intermittent cold exposure increases BAT activity and alleviates peripheral insulin resistance in T2D patients (78), suggesting a pivotal role for BAT in whole-body metabolism. These data underscore that activation of BAT by cold exposure could be a valuable tool to for the treatment of obesity and related diseases such as T2D and CVD.

PHARMACOLOGICAL ACTIVATION OF BAT

Albeit that the metabolic benefits of BAT activation by means of cold exposure have been well established, prolonged cold exposure is not a very convenient treatment option for humans. Therefore, more suitable therapeutic modalities such as pharmaceutical activation of BAT are currently being investigated. Promising therapeutic approaches are either direct modulation of activating receptors on the brown adipocyte itself or indirect modulation of BAT activity by manipulating the sympathetic outflow towards BAT (**Figure 3**). An example of a drug that directly stimulates the β_3 -adrenergic receptor on brown adipocytes is mirabegron. Mirabegron is already on the market for the treatment of overactive bladder disease and clinical studies indicate that one dose of mirabegron (200 mg) activates BAT as effectively as acute cold exposure in young healthy lean men (79). It remains to be determined if long-term administration of this compound indeed improves glucose and lipid profiles and prevents/ reverses obesity and diabetes development. In addition, it would be interesting to investigate if mirabegron is also effective in different risk populations, including South Asians.

A second class of drugs that has the potential to activate BAT are drugs that modulate incretin hormone signalling, such as dipeptidyl peptidase-4 (DPP4) inhibitors and glucagon-like peptide-1 (GLP-1) receptor agonists. DPP4 inhibitors enhance the bioavailability of incretin hormones including GLP-1, while GLP-1 receptor agonists directly stimulate the GLP-1 receptors. GLP-1 receptor stimulation in the hypothalamus increases sympathetic outflow to peripheral metabolic tissues in rodents (80) and is thereby able to modulate BAT activity. Pre-clinical studies indeed show increased BAT activity, increased energy expenditure, an improved glucose and lipid metabolism upon oral administration with the DPP4 inhibitor sitagliptin (81) or central administration of the GLP-1 receptor agonist exenatide (82,83). If this is also the case in humans, and whether different effects would be observed between ethnicities, e.g. between South Asians versus white Caucasians, still needs to be elucidated.

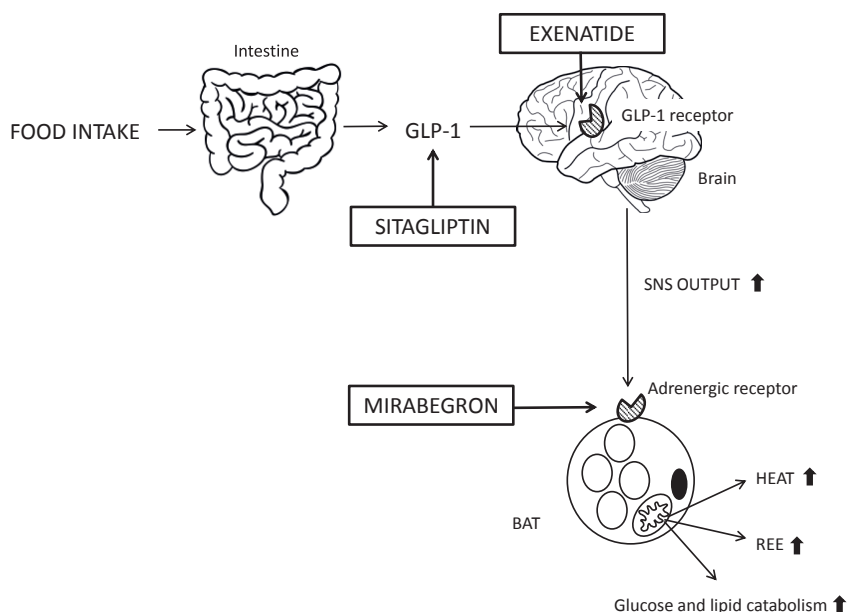


Figure 3. Novel pharmacological approaches to directly and indirectly activate brown adipose tissue. Sitagliptin and exenatide indirectly activate brown adipose tissue through increasing endogenous glucagon-like-peptide-1 and activating central GLP-1 receptors, respectively. Mirabegron directly activates brown adipose tissue through modulation of adrenergic receptors on brown adipocytes. BAT, Brown adipose tissue; GLP-1, glucagon-like-peptide-1; REE, resting energy expenditure; SNS, sympathetic nervous system.

OUTLINE OF THIS THESIS

As is evident from this **chapter**, obesity and related metabolic diseases including T2D and CVD are a growing health care concern. Especially South Asian individuals are at increased risk for the development of metabolic diseases, but the underlying mechanisms are still not fully elucidated. Furthermore, current intervention strategies for the treatment of obesity are not effective on the long-term, apart from bariatric surgery. Although increasing energy expenditure by modulation of BAT seems a promising novel approach, modulation of BAT activation by cold exposure in humans is far from optimal. Therefore, the studies described in this thesis were aimed at 1) unravelling the underlying mechanisms that could explain the increased predisposition for metabolic disease in the South Asian population and 2) identifying novel treatment strategies that activate BAT and increase energy expenditure in risk population, including South Asians and individuals with overweight and prediabetes, with the ultimate goal to combat obesity, T2D and CVD.

In the first part of this thesis, we focused on identification of factors that could, at least in part, explain the enhanced susceptibility for the development of metabolic disease in the South Asian population. First, we focused on the endocannabinoid system. The endocannabinoid system is known to play an important role in energy metabolism by regulating appetite, intracellular lipolysis and energy expenditure and is found to be over-activated in subjects with obesity. In addition, endocannabinoids are thought to act in a negative feedback loop to prevent excessive BAT activity. Theoretically, differences in endocannabinoid signalling between South Asians and white Caucasians can thus contribute to the difference in predisposition for metabolic disease. Therefore, in **Chapter 2**, we first investigated whether endocannabinoid tone is higher in subjects from South Asian descent by studying circulating endocannabinoid levels in young healthy lean white Caucasian and South Asian men. Since endocannabinoid tone is a reflection of local endocannabinoid regulation in metabolically active organs, we next focussed on differences in local endocannabinoid signalling in WAT and skeletal muscle. To this end, we studied, in **Chapter 3**, gene expression of cannabinoid receptors and enzymes involved in endocannabinoid synthesis and degradation in middle-aged, overweight, prediabetic white Caucasian and South Asian individuals. Next, we shifted our focus to other factors that could possibly explain the difference in predisposition for metabolic disease between South Asians and white Caucasians. BAT is a metabolic organ which takes up lipids from the circulation to fuel thermogenesis. Angiopoietin-like (ANGPTL4) inhibits LPL-dependent uptake of TG-derived fatty acids by metabolic tissues. Since differences in substrate uptake by BAT could explain a difference in BAT function, we investigated, in **Chapter 4**, the effect of BAT activation (by means of short-term cold exposure) on circulating ANGPTL4 levels in white Caucasians and South Asians. As combustion of lipids by BAT results in the generation of lipid-associated metabolites, we next explored the effect of short-term cooling on lipid-associated metabolites in blood. In **Chapter 5** we investigated the changes in metabolites upon mild cooling and whether these responses differed between the two ethnicities.

In the second part of this thesis, we focussed on two promising pharmacological strategies to activate BAT. In **Chapter 6**, the effect of the β 3-adrenergic receptor agonist mirabegron was investigated on BAT activity and energy expenditure. To this end, young healthy lean white Caucasian and South Asian men participated in a three day randomized cross-over study in which they were exposed to either short-term cooling, administration of mirabegron or placebo. The acute effects on lipid and glucose metabolism, energy expenditure and BAT activity were studied and potential differences in responses between the two ethnicities were assessed. In **Chapter 7**, we aimed at investigating the effect of chronic administration of the DPP4 inhibitor sitagliptin on lipid and glucose metabolism, energy expenditure and metabolism of BAT, WAT and skeletal muscle in another population at high risk for metabolic disease (e.g. overweight, prediabetic sub-

jects). A double-blinded randomized placebo-controlled trial was performed in which 30 overweight, prediabetic white Caucasian men received either sitagliptin or placebo for a duration of 12 weeks. Pre- and post-treatment blood samples were collected, indirect calorimetry was performed to measure energy expenditure, and an oral bolus of glucose was given to determine glucose tolerance. In addition, a skeletal muscle biopsy was taken to assess effects of sitagliptin on expression of genes involved in glucose and lipid metabolism and mitochondrial function. Furthermore, glucose uptake by BAT was assessed using [^{18}F]FDG PET/CT.

Finally, the results from these studies and their implications are discussed in **Chapter 8**.

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PART 1

**Insights in the metabolic profile of South
Asians versus white Caucasians**

CHAPTER 2

Endocannabinoid tone is higher in healthy lean
South Asian than white Caucasian men

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ABSTRACT

South Asians have a higher risk to develop obesity and related disorders compared to white Caucasians. This is likely in part due to their lower resting energy expenditure (REE) as related with less energy-combusting brown adipose tissue (BAT). Since over-activation of the endocannabinoid system is associated with obesity and low BAT activity, we hypothesized that South Asians have a higher endocannabinoid tone. Healthy lean white Caucasian (n=10) and South Asian (n=10) men were cold-exposed to activate BAT. Before and after cooling, REE was assessed and plasma was collected for analysis of endocannabinoids and lipids. At thermoneutrality, South Asians had higher plasma levels of 2-arachidonoylglycerol (2-AG; 11.36 vs 8.19 pmol/mL, $p<0.05$), N-arachidonylethanolamine (AEA; 1.04 vs 0.89 pmol/mL, $p=0.05$) and arachidonic acid (AA; 23.24 vs 18.22 nmol/mL, $p<0.001$). After pooling of both ethnicities, plasma 2-AG but not AEA positively correlated with triglycerides ($R^2=0.32$, $p<0.05$) and body fat percentage ($R^2=0.18$, $p<0.05$). Interestingly, AA negative correlated with REE ($R^2=0.46$, $p=0.001$) and positively with body fat percentage ($R^2=0.33$, $p<0.01$). Cooling increased endocannabinoids. In conclusion, South Asian compared to white Caucasian men have higher endocannabinoid tone. This suggests that endocannabinoids may, at least in part, underlie the disadvantageous metabolic phenotype of South Asians later in life.

INTRODUCTION

South Asians originally descend from the sub-Indian continent and comprise about 24% of the world population. This population is at higher risk for developing a disadvantageous metabolic phenotype consisting of obesity, dyslipidemia and insulin resistance compared to white Caucasians, making them more prone to develop type 2 diabetes (T2D) at a younger age and lower body mass index (BMI) (1). The underlying mechanism of the increased predisposition for this unfavorable metabolic profile in South Asians is not well understood, but might be related to a disturbed energy metabolism (2).

The endocannabinoid system (ECS) is known to play an important role in energy metabolism by regulating appetite, lipolysis and energy expenditure (3). The ECS is composed of endogenous lipid messengers (endocannabinoids), two distinct G-protein-coupled receptors, i.e. type 1 and type 2 cannabinoid (CB₁ and CB₂) receptors, and enzymes responsible for the synthesis and inactivation of the endocannabinoids. *N*-arachidonylethanolamine (anandamide; AEA) and 2-arachidonoylglycerol (2-AG) are the best-studied endocannabinoids. They are synthesized on demand from the membrane lipid precursors *N*-acylphosphatidylethanoamines and diacylglycerides, respectively. Furthermore, there are endogenous bioactive lipids called *N*-acylethanolamines (NAEs), such as *N*-linoleoylethanolamine (LEA), *N*-palmitoylethanolamine (PEA), *N*-oleoylethanolamine (OEA) and *N*-stearoylethanolamine (SEA), which are produced through the same biosynthetic pathway as AEA. NAEs are able to indirectly modulate cannabinoid receptor activity by interfering with endocannabinoid metabolism. In addition, OEA modulates satiety through its interaction with PPAR α (4,5). The ECS is present in both central and peripheral tissues that are involved in maintaining energy balance. These include the hypothalamus, liver, pancreas, skeletal muscle, white adipose tissue (WAT) and brown adipose tissue (BAT) (6).

High ECS activity has been associated with human obesity (7,8). More specifically, elevated circulating AEA and 2-AG levels have been reported in obese individuals (8-10) and circulating 2-AG levels are positively correlated with different measures of adiposity, including BMI and body fat percentage (8), supporting a causal role of the ECS in energy metabolism. Indeed, reduction in 2-AG formation has been associated with reduced food intake in fasted mice (11,12) and chronic systemic blockade of the CB₁ receptor with the inverse agonist rimonabant leads to long-term maintained weight loss and reduction of dyslipidemia in obese rodents (13,14) and humans (15-17).

Mouse studies have shown that the beneficial metabolic effects of CB₁ receptor blockade are, at least in part, mediated via activation of energy-combusting BAT (18). Moreover, cold mediated BAT activation leads to a tissue-specific upregulation of endocannabinoids in BAT via CB₁ receptors, thereby possibly controlling BAT activity by negative feed-back mechanisms (19). BAT is present and active in human adults and

dissipates triglyceride (TG)-derived fatty acids towards heat, thereby contributing to energy expenditure (20-23). Cold acclimatization, the most important physiological activator of BAT, has been shown to recruit and activate BAT in obese humans (24). The precise role of the ECS in human BAT activation remains to be determined. Interestingly, we have previously shown that South Asian individuals have lower BAT volume and activity, as measured with [^{18}F]fluorodeoxyglucose ([^{18}F]FDG) PET-CT scanning, compared to white Caucasians, which might, at least in part, contribute to their high susceptibility to develop obesity and T2D (25). The underlying cause of the decreased BAT volume in South Asians is still a question that remains.

The main aim of this study was to investigate if healthy lean South Asians men who have not yet developed a disadvantageous metabolic phenotype have a higher endocannabinoid tone as compared to white Caucasians. Furthermore, we investigated whether cold mediated BAT activation leads to upregulation of endocannabinoid levels in humans. In addition, we aimed to assess whether plasma endocannabinoid levels correlate with BAT function, energy expenditure and serum lipid levels.

MATERIALS AND METHODS

Ethics

Venous blood samples were collected as part of a previously conducted observational study aimed at investigating BAT activity and volume in Dutch South Asian and white Caucasian individuals (25). This study was approved by the Medical Ethical Committee of the Leiden University Medical Center (LUMC) and undertaken in accordance with the principles of the revised Declaration of Helsinki. All volunteers provided written informed consent. Trial registration number: Netherlands Trial Register 2473.

Participants and study design

We enrolled twenty-four Dutch healthy lean men, between 18-28 years of age, with a BMI 18-25 kg/m². Twelve men were of Dutch South Asian descent and twelve of Dutch white Caucasian descent (25). This study was conducted at the Alrijne Hospital, Leiderdorp (the Netherlands) between March 2013 and June 2013 (25). In short, subjects underwent medical screening including their medical history, a physical examination, blood chemistry tests and an oral glucose tolerance test to exclude individuals with type 2 diabetes according to the American Diabetes Association 2010 criteria. Other exclusion criteria were rigorous exercise, smoking and recent body weight change up to 3 months prior to the start of the study.

Study procedures

Subjects were studied after overnight fasting and 24 hours without heavy exercise. We determined body composition using a Dual Energy X-ray Absorptiometry (DEXA) scan (iDXA, GE healthcare, UK or Discovery A, Hologic, Bedford, MA, USA). Next, an intravenous cannula was inserted in the antecubital vein for blood collection and injection of [^{18}F]FDG. To assess BAT volume and activity, subjects were exposed to an individualized water cooling protocol to maximally activate BAT. In short, subjects were cooled for approximately 2 hours until just above shivering temperature, to ensure maximum non-shivering thermogenesis, followed by an [^{18}F]FDG PET-CT scan (Gemini TF PET-CT, Philips Healthcare, Best, the Netherlands). For the individual cooling protocol, subjects lay between two water perfused cooling mattresses (Blanketrol III, Cincinnati Sub-Zero Products, Cincinnati, OH, USA or ThermoWrap Universal 3166, MTRE Advanced Technologies, Yavne, Israel). After 60 minutes at 32°C (thermoneutral temperature), the temperature of the mattresses were gradually decreased with 5°C every 10 minutes until shivering occurred. At this point, the temperature was raised by 3°C and the official cooling period of 2 hours was started. After 1 hour of cooling, 2 MBq/kg [^{18}F]FDG was administered intravenously. Both in thermoneutral and cold-induced condition, indirect calorimetry was performed with a ventilated hood (Oxycon Pro™, CareFusion, Heidelberg, Germany). Venous blood samples were collected in a fasted state at the end of the thermoneutral period and at the end of the cooling period. After 2 hours of cooling, an [^{18}F]FDG PET-CT scan was acquired starting with a low-dose CT scan followed by PET scanning.

Brown adipose tissue measurements and energy expenditure

BAT volume (in mL) and BAT activity (in standardized uptake value (SUV)) in the region of interest were determined from the [^{18}F]FDG PET-CT scans (Gemini TF PET CT, Philips, Best, the Netherlands) by a blinded nuclear physician and a researcher. Resting energy expenditure (REE), respiratory quotient and rates of lipid and glucose oxidation were measured with indirect calorimetry using a ventilated hood system (Oxycon Pro, CareFusion, Heidelberg, Germany).

Serum lipid measurements

Serum TG and free fatty acid concentrations were determined in the blood samples with the use of commercially available enzymatic kits 11488872 and 91096 (Roche Molecular Biochemicals, Indianapolis, USA) as described previously (25).

Endocannabinoid measurements

Plasma sample of only 20 subjects could be studied since four subjects were excluded due to insufficient volume of plasma sample left available for the analysis in 2 individuals of each ethnicity. In total, endocannabinoids and their congeners were quantified in 40

plasma samples using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). From the pool of individual study samples, quality controls (QCs) were used to generate calibration curves. Additionally, all samples were randomized and each batch included calibration samples and an even distribution of QC samples and blanks.

Endocannabinoid sample extraction

Endocannabinoid extraction was performed on ice. Briefly, 50 μ L of plasma was transferred into 1.5 mL Eppendorf tubes, spiked with 10 μ L of deuterated internal standard mix (**Supplementary Table S1**) followed by addition of 50 μ L of 0.1 M ammonium acetate buffer (pH 4). After two times extraction with 400 μ L of methyl tert-butyl ether (MTBE) (443808, Sigma Aldrich, Zwijndrecht, the Netherlands), the tubes were thoroughly mixed for 4 min using a bullet blender (Next Advance, Inc., Averill park, NY, USA) at medium speed, followed by a centrifugation step (4°C, 5,000 g, 12 min). Then 750 μ L (combined from two extractions) of the upper MTBE layer was transferred into clean 1.5 mL Eppendorf tubes. Samples were dried in a speed vac followed by reconstitution with 50 μ L of acetonitrile/water (90/10, v/v). The reconstituted samples were centrifuged (4°C, 14,000 g, 3 min) before transferring into LC-MS vials. 5 μ L of each sample was injected into the LC-MS/MS system.

LC-MS/MS Analysis

A targeted analysis of 22 compounds, including endocannabinoids and related *N*-acylethanolamines (NAEs) along with their precursor molecule and metabolite arachidonic acid (AA) (**Supplementary Table S1**), was measured using an Acquity UPLC I class Binary solvent manager pump (Waters, Milford, USA) in conjugation with AB SCIEX 6500 quadrupole-ion trap (QTRAP) (AB Sciex, Massachusetts, USA). The separation was performed with an Acquity HSS T3 column (1.2x100 mm, 1.8 μ m) maintained at 45°C. The aqueous mobile phase A consisted of 2 mM ammonium formate and 10 mM formic acid, and the organic mobile phase B was acetonitrile. The flow rate was set to 0.4 mL/min; initial gradient conditions were 55% B held for 2 minutes and linearly ramped to 100% B over 6 minutes and held for 2 minutes; after 10 seconds the system returned to initial conditions and held 2 minutes before next injection. Electrospray ionization-MS and a selective Multiple Reaction Mode (MRM) was used for endocannabinoid quantification. Individually optimized MRM transitions using their synthetic standards for target compounds and internal standards are described in **Supplementary Table S1**. Relative standard deviations (RSD) of QC samples were calculated to monitor the data quality and RSD less than 15% was observed for 90% the target compounds. Peak area integration was performed with MultiQuant (AB Sciex, Version 3.0.2) data analysis software. The obtained peak areas of targets were corrected by appropriate internal standards. Calculated response ratios, determined as the peak area ratios of the target analyte to

the respective internal standard, were used to obtain absolute concentrations from their respective calibration curves. The response ratios were further corrected using in-house QC tool (26).

Statistical analysis

As previously mentioned, four participants (2 white Caucasians and 2 South Asians) were excluded from the analyses due to insufficient sample for the endocannabinoid measurements. Data were collected and analysed using IBM SPSS statistics version 23.0. Baseline characteristics were compared using unpaired student t-tests. Differences in plasma endocannabinoid levels in (between) groups were analysed using two tailed paired (unpaired) t-tests. Furthermore, linear regression analysis computed by Pearson's correlation was used to determine correlations between plasma endocannabinoid levels and different metabolic parameters. Regression analysis was performed both with and without correction for the effect of ethnicity, by respectively including/excluding ethnicity as a covariate. P values < 0.05 were considered as statistical significant. Data are presented as mean $\pm$ SEM.

RESULTS

Clinical characteristics

The characteristics of the participants are partly previously described in (25). Twenty-four healthy lean men were included, however, sufficient plasma to analyse the endocannabinoids of only twenty participants (10 white Caucasians and 10 South Asians) was available. In this cohort of 20 participants, mean age was comparable between South Asians and white Caucasians (23.7 vs 25.2 years, respectively) as was BMI (21.4 vs 22.3 kg/m²), body fat percentage (23.4 vs 19.4%) and serum TG concentration (0.91 vs 0.82 mmol/L). South Asians had lower REE during thermoneutral conditions (1304 vs 1671 kcal/day, $p < 0.01$) and cold conditions (1480 vs 2063 kcal/day, $p < 0.01$), also after correction for lean body mass. Cold exposure increased REE significantly only in white Caucasians (+23.4%, $p < 0.01$). Furthermore, BAT volume was lower in South Asians as compared to white Caucasians (185 mL vs 303 mL), which was borderline significant ($p = 0.052$) while the difference was significant in the original study (25) due to larger sample size.

Circulating endocannabinoid levels are higher in South Asians and are increased after cooling

At thermoneutrality, South Asians had higher mean levels of plasma 2-AG compared to white Caucasians (11.36 vs 8.19 pmol/mL, $p < 0.05$) (**Figure 1A**). In addition, there was a trend towards higher mean levels of AEA in South Asians (1.04 vs 0.89 pmol/mL,

$p=0.05$) (**Figure 1B**). Levels of AA were also higher in South Asians (23.24 vs 18.22 nmol/mL, $p<0.001$) (**Figure 1C**). No significant differences were observed for other NAEs and mono- and di-acyl glycerols measured, except LEA (*N*-linoleoylethanolamide), of which mean plasma levels were higher in South Asians compared to white Caucasians (5.88 vs 3.99 pmol/mL, $p<0.005$) (**Supplementary Table S1**). Collectively, these results suggest that circulating endocannabinoid levels (2-AG and AEA) are higher in lean healthy male South Asians as compared to white Caucasians. We next assessed the endocannabinoid levels in plasma samples collected after short-term mild cold exposure. Interestingly, after cooling circulating levels of 2-AG were higher in South Asians (+41%, $p<0.05$) as well as white Caucasians (+32%, $p<0.05$) (**Figure 1A**). Furthermore, AEA levels were significantly elevated after mild cold exposure in white Caucasians only (+16%, $p<0.05$) (**Figure 1B**). Plasma AA significantly increased after cold exposure in both ethnic groups (South Asians (+22%, $p<0.01$) and white Caucasians (+23%, $p<0.01$) (**Figure 1C**). The relative increases in endocannabinoid levels upon cooling were not significantly different comparable between the two ethnicities.

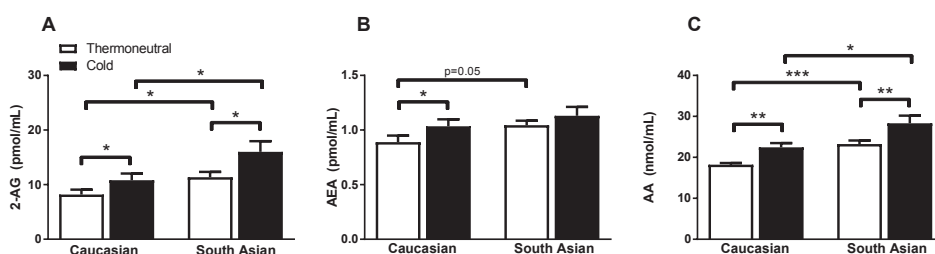


Figure 1. Circulating endocannabinoid levels are higher in South Asians and increased after short-term cooling. Blood was collected from healthy young South Asian ($n=10$) and matched white Caucasian ($n=10$) individuals before (thermoneutral) and after short-term mild cooling (Cold). Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used to measure plasma concentrations of 2-AG (**A**), AEA (**B**), and AA (**C**) concentrations. Values are mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. P-values are based on paired t-tests (effect of cold) or unpaired t-tests (effect of ethnicity).

Circulating endocannabinoid levels correlate with serum triglycerides and body fat

As the ECS is known to play an important role in energy metabolism, we investigated whether circulating endocannabinoid levels correlated with different metabolic parameters in our study cohort. For these analyses we used combined data of South Asians and white Caucasians. Thermoneutral 2-AG levels positively correlated with TG levels ($R^2=0.32$, $p<0.05$) (**Figure 2A**) and total body fat percentage ($R^2=0.27$, $p<0.05$) (**Figure 2B**), but not with visceral fat percentage (data not shown). In contrast, AEA levels did not correlate with any of these metabolic parameters (data not shown). Additionally, 2-AG positively

correlated with AA under thermoneutral ($R^2=0.45$, $p=0.001$) and cold conditions ($R^2=0.28$, $p<0.05$) (**Figure 3**). AA also positively correlated with body fat percentage ($R^2=0.34$, $p<0.01$) (**Figure 4A**). Interestingly, a strong negative correlation was observed with AA and REE ($R^2=0.46$, $p<0.001$) (**Figure 4B**), with AA levels being higher in South Asians compared to white Caucasians (23.24 vs 18.22 nmol/mL, $p<0.001$). AA did not correlate with either fat or glucose oxidation (data not shown). We found no significant correlations between circulating endocannabinoids and other metabolic parameters, including (systolic and diastolic) blood pressure, heart rate, fasting serum glucose or C-reactive protein (CRP) levels (data not shown). After cold exposure, there was no correlation between circulating endocannabinoids and REE (data not shown). Both thermoneutral and cold-induced REE positively correlated with BAT volume, but not activity (data not shown) (25). To further understand the inter-relationship between endocannabinoids and BAT metabolism, we performed several regression analyses. Notably, no correlation was found between circulating endocannabinoid levels and BAT volume (2-AG: $R^2=0.016$, $p=0.611$; AEA: $R^2=0.024$, $p=0.531$; and AA: $R^2=0.066$, $p=0.288$) or activity (2-AG: $R^2=0.008$, $p=0.714$; AEA: $R^2=0.132$, $p=0.126$; and AA: $R^2=0.031$, $p=0.473$), nor was there a correlation between cold-induced or delta (cold minus thermoneutral) endocannabinoid levels upon cooling and BAT activity or volume (data not shown). To test whether the effects could be attributed to ethnicity, we also performed the regression analysis including ethnicity as covariate. Correcting for the effect of ethnicity, plasma 2-AG levels still correlated with serum TG levels ($p<0.05$) and tended to correlate with body fat percentage ($p=0.08$). In addition, thermoneutral 2-AG levels still correlated with thermoneutral AA levels ($p<0.05$), but not under cold conditions ($p=0.12$). Also, after correction for ethnicity the negative correlation between AA and REE was not significant anymore ($p=0.41$).

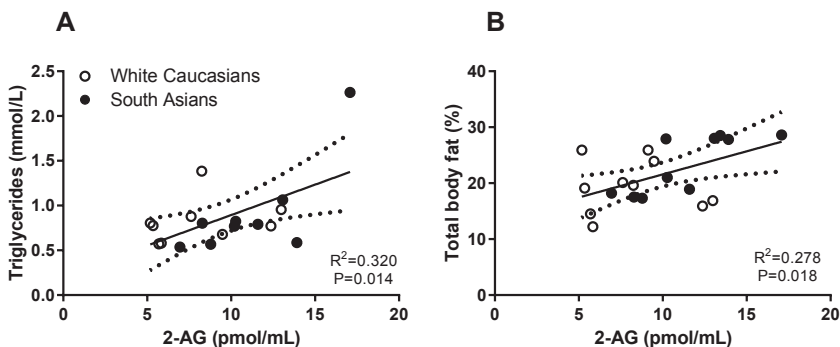


Figure 2. Thermoneutral plasma 2-AG levels positively correlate with serum triglyceride levels and total body fat percentage. Scatterplot of the correlations between 2-AG levels and serum TG (A) or total body fat percentage (B). Correlations are shown for the total group combined ($n=20$), white circles are white Caucasian individuals ($n=10$) and black circles are South Asian individuals ($n=10$), with 95% confidence limits. Correlations were analysed using linear regression analysis.

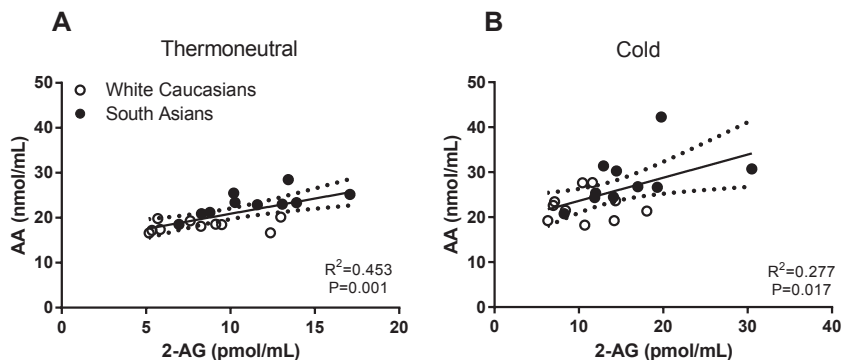


Figure 3. Plasma 2-AG levels correlate with plasma AA levels. Scatterplot of the correlations between plasma 2-AG and AA in both Caucasian and South Asian individuals (n=40). Correlation is shown for the total group combined under thermoneutral (**A**) and cold conditions (**B**), black circles are South Asian individuals and white circles are white Caucasian individuals, with 95% confidence limits. Correlations were analysed using linear regression analysis.

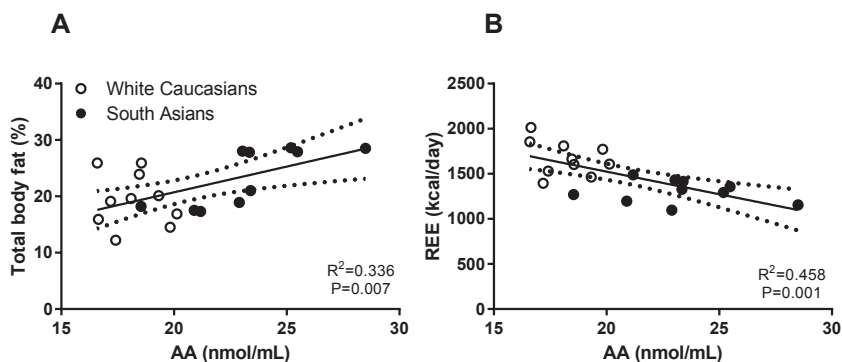


Figure 4. Thermoneutral plasma AA levels positively correlate with body fat percentage and negatively correlate with resting energy expenditure. Scatterplot of the correlations between plasma AA levels measured at thermoneutrality and total body fat percentage (**A**) or thermoneutral resting energy expenditure (REE) (**B**) (n=20). Correlations are shown for the total group combined, black circles are South Asian individuals and white circles are white Caucasian individuals, with 95% confidence limits. Correlations were analysed using linear regression analysis.

DISCUSSION

The contribution of the ECS in energy metabolism has been well established in humans and research has focused on manipulating this system for the treatment of metabolic disease, such as obesity and T2D. The South Asian population is very prone to develop metabolic disease; however, the underlying mechanism remains to be elusive. In the

present study, we showed that healthy lean South Asian men without an apparent disadvantageous metabolic phenotype have higher circulating levels of endocannabinoids (2-AG and AEA) and their metabolite AA compared to matched white Caucasian men. In addition, plasma 2-AG levels positively correlate with serum TG and body fat percentage, and AA levels negatively correlate with REE and positively with body fat percentage.

To the best of our knowledge, this is the first study that shows higher plasma 2-AG, AEA and AA levels in healthy South Asians compared to white Caucasians. Jumpertz et al. (27) previously showed higher 2-AG levels in cerebrospinal fluid (CSF) of American Indians compared to white Caucasians. However, they did not observe differences in 2-AG or AEA levels in plasma of these individuals. This might be due to differences in the study population or a difference in the sensitivity of the assay. For example, Jumpertz et al. included young (± 29 year), overweight individuals, while in the current study young (± 24 year), healthy individuals were included. Furthermore, in South Asians we found higher levels of AA, the breakdown product of endocannabinoids. Although AA can also be synthesized independent of endocannabinoids, these data support the notion that the endocannabinoid tone is higher in healthy young male individuals of South Asian compared to white Caucasian origin.

High circulating endocannabinoid levels in South Asians can result in overstimulation of cannabinoid receptors on tissues such as liver, WAT, skeletal muscle and pancreas. This in turn can lead to several disadvantageous metabolic effects. In mice, a high endocannabinoid tone in the liver, for example, contributes to the development of fatty liver disease (28-30). In addition, in WAT CB₁ receptor activation promotes adipogenesis, lipogenesis and energy storage instead of combustion (3). Moreover, CB receptor stimulation in WAT and skeletal muscle has shown to disrupt insulin signaling thereby promoting insulin resistance, which in combination with reduced insulin secretion from the pancreas (also induced by CB receptor stimulation on pancreatic islet cells) might promote development of T2D (3,31). Taken together this indicates that overall high endocannabinoid tone can deteriorate/ induce metabolic disease. As South Asians have already been shown to have more hepatic steatosis (32), adipocyte hypertrophy (32), disturbed muscle insulin signaling (2) and lower REE (25) (i.e. high endocannabinoid tone reduces REE (18) and high plasma AA levels negatively correlate with REE (**Figure 4B**)) compared to white Caucasians, they are possibly at even greater risk for the negative metabolic effects of high endocannabinoid tone.

In addition, we observed positive correlations between plasma 2-AG and serum TG and body fat percentage, albeit the correlation with TG was enforced by a biological outlier. These results are in line with results from Bluher et al. (8) who showed positive correlations between circulating 2-AG levels and both body fat percentage and serum TG in lean and obese individuals. However, they also showed positive correlations between 2-AG and BMI and free fatty acids which could not be reproduced in our study

population (8). Jumper et al. (27) showed positive correlations between AEA, but not 2-AG, and BMI and body fat percentage. However, they studied overweight individuals whereas we studied healthy lean individuals. Consistent with our data, they also found no correlation between plasma 2-AG or AEA levels and REE (27).

Furthermore, we found that plasma 2-AG and AA levels in both ethnicities are increased after short-term mild cooling, the physiological stimulator of BAT (33). This observation is consistent with a study in mice showing that BAT activation increases expression and protein levels of endocannabinoids in BAT (19). The authors hypothesized that increase in endocannabinoids in BAT represents a negative feedback mechanism, as they could locally act on the presynaptic nerve terminal to inhibit noradrenalin release, thereby preventing excessive activation of BAT (19). Possibly, also in humans the increase in 2-AG and AEA could be derived from BAT, however in our observational study the contribution of other tissues to the plasma endocannabinoid levels could not be excluded. Another explanation for the increased endocannabinoid levels upon cooling could be a higher availability of biosynthetic precursors such as diacylglycerols (DAGs) or *N*-acyl-phosphatidyl-ethanolamines (NAPEs), which are generated during lipolysis of WAT and/or BAT during cooling (19). In addition, it has been shown that cold exposure reduces the mRNA expression of the breakdown enzyme *FAAH* in BAT, which might also in part explain higher plasma endocannabinoid levels upon cooling (19). Alternative explanations can also not be excluded at present. For example, 2-AG has a circadian rhythm in humans with the lowest plasma concentration peak around 5:00 am and the highest peak concentration around 13:00 pm (34). Since the blood samples in the current study were withdrawn around 9:30 (thermoneutrality) and 12:30 (cold), a small increase, of approx. 15%, in plasma 2-AG could already be expected from its circadian rhythm. However, we found a larger 37% average increase in 2-AG when combining the South Asians and white Caucasians. In addition, cooling elicits a stress response in the body, and stress, via glucocorticoids, has been shown to increase the peripheral ECS tone (35,36).

Previous mouse studies showed that global blockade of the CB₁ receptor by rimonabant activates BAT, markedly enhances energy expenditure and leads to a tissue-specific upregulation of endocannabinoids (18,19). In fact, human studies showed that taranabant, another inverse agonist of the CB₁ receptor also reduced food intake, induced weight loss and increased REE and fat oxidation in obese individuals (37). Therefore, we hypothesized that these effects may have been due to BAT activation. However, in our study there was no significant correlation between plasma endocannabinoids and BAT volume/activity, fatty acid or glucose oxidation. Possibly our sample size was too small to reliably assess this, as the variation in BAT between subjects was rather large.

A strength of this study is that we investigate differences in circulating endocannabinoid levels in lean healthy South Asians and white Caucasians individuals before they

develop metabolic diseases. This allowed us to investigate in more detail whether ethnic differences in endocannabinoid tone are already present before the onset of the disease rather than being a consequence of the metabolic disease. However, a limitation of our study is that we could only measure circulating endocannabinoid levels which do not necessarily reflect endocannabinoid signalling within peripheral organs and thus the endocannabinoid function. For future studies, it would be interesting to measure endocannabinoid levels and levels of endocannabinoid synthesis and breakdown enzymes in specific tissues, such as WAT and skeletal muscle. In addition, we cannot exclude that 'thermoneutrality' might have been experienced differently between the two ethnicities, which might have biased our results. Furthermore, the small sample size might have limited the number of correlations we could detect. However, despite this we were able to reproduce some correlations shown previously by others underscoring the robustness of these findings. Future studies in larger cohorts should verify the translational value of these results for the general population.

In conclusion, our data demonstrate that healthy lean South Asian men have a higher endocannabinoid tone compared to white Caucasian men. Provided that our cohort is representative for the general population, this might, at least in part, contribute to the development of a disadvantageous metabolic phenotype later in life. These results are in line with the hypothesis that the ECS functions as a negative feedback system on BAT. Future research should focus on elucidating the underlying cause of the high endocannabinoid tone in this vulnerable population and verify potential causal relationships. This knowledge might lead to the development of novel treatment strategies to combat metabolic disease.

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SUPPLEMENTAL APPENDIX

Supplementary Table S1. MPM (Multiple reaction monitoring) parameters and endogenous concentrations of the target list

S.No	ID	Metabolite Name	Q1	Q3	DP, CE	Polarity	Concentration (pmol/mL)		
							Caucasians	South Asians	P-Value
Standards									
1	1-1OG (18:1)	1-Oleoyl Glycerol	357	265	80, 20	+	1055±100	954±135	0.554
2	2-OG (18:1)	2-Oleoyl Glycerol	357	265	80, 20	+	97.6±7.2	83.0±9.9	0.247
3	1-LG (18:2)	1-Linoleoyl Glycerol	355	263	80, 15	+	338.0±39.4	436.4±54.3	0.160
4	2-LG (18:2)	2-Linoleoyl Glycerol	355	263	80, 20	+	59.6±8.1	74.5±9.3	0.244
5	2&1-AG (20:4)	2&1-Arachidonoylglycerol	379	287	56, 19	+	8.19±0.89	11.36±0.97	0.028*
6	2AGE (20:4)	2-Arachidonyl Glycerol ether	365	273	72, 25	+	ND	ND	-
7	PDEA (15:0)	N-Pentadecanoyl ethanolamide	286	62	80, 18	+	0.23±0.13	0.10±0.01	0.312
8	PEA (16:0)	N-Palmitoyl ethanolamide	300	62	78, 36	+	9.28±0.71	10.60±0.26	0.099
9	SEA (18:0)	N-Stearoyl ethanolamide	328	62	72, 31	+	7.24±0.33	7.41±0.19	0.677
10	POEA (16:1)	N-Palmitoleoyl ethanolamide	298	62	86, 32	+	1.03±0.15	1.02±0.12	0.990
11	OEA (18:1)	N-Oleoyl ethanolamide	326	62	72, 30	+	6.57±0.43	7.39±0.38	0.175
12	LEA (18:2)	N-Linoleoyl ethanolamide	324	62	74, 36	+	3.99±0.39	5.88±0.39	0.003*
13	Alpha-LEA (18:2)	N-α-Linolenyl ethanolamide	322	62	80, 20	+	0.01±0.01	0.01±0.01	0.408
14	DGLEA (18:3)	Dihomo-γ-Linolenoyl Ethanolamide	350	62	70, 20	+	0.21±0.02	0.20±0.02	0.796
15	ETAEA (20:3)	Eicosatrienoic Acid Ethanolamide	350	62	80, 46	+	ND	ND	-
16	AEA (20:4)	Anandamide	348	62	70, 42	+	0.89±0.06	1.04±0.04	0.050
17	O-AEA (20:4)	O-Arachidonoyl ethanolamine	348	62	70, 25	+	ND	ND	-
18	EPEA (20:5)	Eicosapentaenoyl Ethanolamide	346	62	80, 20	+	0.05±0.01	0.07±0.01	0.191
19	DEA (22:4)	N-Docosatetraenoyl ethanolamide	376	62	72, 20	+	0.27±0.02	0.28±0.03	0.916
20	DHEA (22:6)	N-Docosahexaenoyl ethanolamide	372	62	70, 25	+	5.14±0.44	5.61±0.55	0.522

Supplementary Table S1. MRM (Multiple reaction monitoring) parameters and endogenous concentrations of the target list (continued)

S.No	ID	Metabolite Name	Q1	Q3	DP,CE	Polarity	Concentration (pmol/mL)		P-Value
							Caucasians	South Asians	
21	NADA (28:4)	N-Arachidonoyl dopamine	440	137	75, 24	+	ND	ND	-
22	Arachadonic Acid (20:4)	5,8,11,14-Eicosatetraenoic acid	303	259	-80, -18	-	18223±401	23244±872	0.001 ***
Internal standards									
1	2-AG (20:4)-d8	2-Arachidonoylglycerol-d8	387	294	56, 20	+	-	-	-
2	PEA (16:0)-d4	Palmitoyl ethanolamide-d4	304	62	78, 36	+	-	-	-
3	SEA (18:0)-d3	Stearoyl ethanolamide-d3	331	62	72, 31	+	-	-	-
4	OEA (18:1)-d4	Oleoyl Ethanolamide-d4	330	66	72, 30	+	-	-	-
5	LEA (18:2)-d4	Linoleoyl ethanolamide-d4	328	66	74, 36	+	-	-	-
6	AEA (20:4)-d8	Arachidonoyl Ethanolamide-d8	356	62	70, 42	+	-	-	-
7	DHEA (22:6)-d4	Docosahexaenoyl Ethanolamide-d4	376	66	70, 25	+	-	-	-
8	NADA (28:4)-d8	N-Arachidonoyl dopamine-d8	448	137	75, 34	+	-	-	-

The target list includes endocannabinoids and N-acyl ethanolamines (NAEs) and deuterated (d) labelled internal standards. The compound ID is the abbreviation of metabolite name along with number of carbon atoms and number of double bonds in the fatty acid chain of the molecule, respectively. All compounds are analysed in positive mode except arachidonic acid in negative mode. Q1 and Q3 are optimized precursor ion and product ion, expressed as *m/z*. DP and CE are declustering potential (Volts) and collision energy (volts). Endogenous concentrations are denoted as mean ± SEM (standard error of mean). **P*<0.05, ****P*<0.001. *P*-values are based on unpaired *t*-tests. ND: Not detected in human plasma.

CHAPTER 3

Gene expression of endocannabinoid system components in skeletal muscle and adipose tissue of South Asians and white Caucasians with overweight

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ABSTRACT

The study aimed to investigate whether markers of endocannabinoid signaling differed between overweight men of South Asian and white Caucasian descent. We included overweight, prediabetic South Asian (n=10) and white Caucasian (n=10) men aged 35-50 years. Plasma samples were analyzed for endocannabinoids, their congeners and lipids. In white adipose tissue (WAT) and skeletal muscle biopsies, mRNA expression of genes involved in the endocannabinoid system (ECS) was assessed using qRT-PCR. Fasting lipid oxidation and glucose oxidation were determined with indirect calorimetry. Compared to white Caucasians, South Asians had higher levels of plasma 2-linoleoyl glycerol ($p<0.01$) and *N*-linoleoylethanolamine ($p<0.05$). Interestingly, in skeletal muscle of South Asians, expression of cannabinoid receptors CB₁ and CB₂ was 10-fold lower ($p<0.001$) and that of the endocannabinoid degradation enzyme FAAH2 was 5-fold lower ($p<0.001$) compared to white Caucasians. Expression of genes involved in the ECS in WAT were not different between the two ethnicities. After pooling of both ethnicities, plasma 2-AG positively correlated with plasma triglycerides ($R=0.77$, $p<0.001$) and lipid oxidation ($R=0.55$, $p<0.05$). Overweight South Asian men have higher plasma 2-linoleoyl glycerol and *N*-linoleoylethanolamine levels and lower expression of CB receptors and the endocannabinoid degradation enzyme FAAH2 in skeletal muscle compared to white Caucasians.

INTRODUCTION

The prevalence of obesity and related diseases, such as type 2 diabetes (T2D), is rapidly increasing worldwide. Particularly in South Asians, constituting nearly one-fourth of the world's population (1), an unfavorable metabolic profile consisting of obesity, dyslipidaemia and T2D, is highly prevalent (2). Moreover, South Asians have a higher risk of developing cardiovascular disease, resulting in high morbidity and mortality (3). It is generally thought that the increased susceptibility to metabolic disease of South Asians might be due to a disturbed energy metabolism (4).

The endocannabinoid system (ECS) is involved in maintaining energy balance by impacting on energy intake and expenditure as well as lipolysis (5). The ECS is present both centrally and in peripheral metabolic tissues, including the liver, pancreas, skeletal muscle, white adipose tissue (WAT) and brown adipose tissue (6). In skeletal muscle, endocannabinoids reduce glucose uptake and oxidative pathways (7, 8), thereby reducing energy expenditure. In WAT, endocannabinoids stimulate energy storage by increasing lipogenesis and adipogenesis (5, 9, 10). Thus endocannabinoid stimulation of skeletal muscle and WAT collectively results in a positive energy balance.

The ECS consists of G-protein-coupled cannabinoid receptors, i.e. type 1 and type 2 (CB₁ and CB₂), the endogenous lipid ligands (endocannabinoids) and the enzymatic machinery for their synthesis and degradation. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the most studied endocannabinoids and are synthesized on demand from their membrane lipids through the action of the biosynthetic enzymes N-acylphosphatidyl-ethanolamine phospholipase D (NAPE-PLD) and diacylglycerol lipases (DAGL), respectively. Endocannabinoids are degraded by specific enzymes. Fatty acid amide hydrolase (FAAH1 and FAAH2) inactivates AEA and monoglycerol lipase (MAGL) hydrolyzes 2-AG. Arachidonic acid is a precursor molecule and metabolite for endocannabinoids. AEA belongs to a family of bioactive *N*-acylethanolamines, which include *N*-linoleoylethanolamine, *N*-palmitoylethanolamine, *N*-oleoylethanolamine, *N*-stearoylethanolamine and *N*-docosatetraenylethanolamide. These *N*-acylethanolamines arise from the same biosynthetic pathway as AEA and are capable to indirectly modulate cannabinoid receptor activity by interfering with endocannabinoid metabolism, known as an "entourage effect" (11). Palmitoylethanolamine and, especially, *N*-oleoylethanolamine interact with nuclear receptor peroxisome proliferator-activated receptor α , stimulate lipolysis in the liver and adipocytes and act as a satiety factor (9, 12).

We previously showed that young healthy lean South Asian men have lower resting energy expenditure (13) and higher circulating endocannabinoid levels compared to matched white Caucasians (14). High plasma endocannabinoid levels are associated with obesity (15) and T2D (6) and affect peripheral metabolic organs, including adipose tissue and skeletal muscle (5). In addition, obese individuals have reduced expression

of endocannabinoid degradation enzymes and CB₁ receptor in adipose tissue (16, 17). Elevated circulating endocannabinoid levels might be a result of decreased enzymatic degradation (16). As little is known about ECS in overweight, prediabetic South Asian compared with white Caucasian men, we investigated circulating endocannabinoid levels and gene expression of CB receptors and enzymes involved in endocannabinoid synthesis and degradation in WAT and skeletal muscle of middle-aged men of South Asian and white Caucasian descent. We hypothesized that overweight, prediabetic South Asian men would have elevated plasma endocannabinoid levels and consequently lower expression of CB receptors on peripheral tissues compared to overweight, prediabetic Caucasian men.

MATERIALS AND METHODS

Ethics

Blood and tissue samples were collected as part of a previously conducted cross-over study to investigate the effect of L-arginine on brown adipose tissue activity and resting energy expenditure in overweight, prediabetic South Asian and white Caucasian men (18). This study was approved by the Ethics Committee of Maastricht University Medical Center (MUMC) and the Leiden University Medical Center (LUMC) and undertaken in accordance with the principles of the revised Declaration of Helsinki. All volunteers provided written informed consent. Trial registration number and date: NCT02291458, 14 November 2014.

Participants and study design

Twenty Dutch overweight (BMI 25-35 kg/m²) prediabetic men of South Asian (n=10) and white Caucasian (n=10) descent (aged 35-55 years) were enrolled. South Asians and white Caucasians were matched for BMI. This study was conducted at the LUMC (The Netherlands) between November 2014 and August 2015 (18). Subjects underwent medical screening including an oral glucose tolerance test to identify individuals with impaired glucose tolerance according to the American Diabetes Association 2010 (19) and/or WHO criteria. In line with the ADA criteria (19), prediabetes was defined as having either fasting plasma glucose levels between 5.6 and 6.9 mmol/L or plasma glucose levels 2 h after an oral glucose tolerance test between 7.8 and 11.1 mmol/L. Exclusion criteria included the presence of chronic disease including T2D, smoking and recent body weight change up to 3 months prior to the start of the study.

Study procedures

The study procedures were described previously (18). In short, subjects were treated for 6 weeks with either L-arginine (9 g/day) (Argimax, Hankintatukku Oy, Karkkila, Finland) or placebo supplements (containing 70% microcrystalline cellulose, 29% maize starch and 1% magnesium stearate) in a randomized double-blind cross-over design, with a 4-week washout period. Each treatment period was followed by two consecutive study days, which consisted of several measurements. During the first study day lipid oxidation and glucose oxidation were determined for 30 minutes with indirect calorimetry using a face-mask (EZcal, IDEE, Maastricht, the Netherlands) and subsequently fasted venous blood samples were collected to quantify plasma triglycerides (using commercially available enzymatic colorimetric kits and an ABX Pentra 400 autoanalyzer) and endocannabinoids (liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)) as described previously (14). The next day, a fasted WAT (abdominal subcutaneous adipose tissue) and skeletal muscle (vastus lateralis) biopsy were collected as described previously (20). The tissues were instantly placed in ice-cold preservation medium (BIOPS, OROBOROS Instruments, Innsbruck, Austria) and stored at -80°C. Thereafter, body composition was determined by means of dual x-ray absorptiometry (DEXA; Discovery A, Hologic, Bedford, MA, USA). From one white Caucasian no DEXA scan data was available due to technical problems. For the purpose of this study only the fasted blood samples and WAT and skeletal muscle biopsies taken after placebo treatment were analyzed. All samples were taken at the same time of the day in all volunteers. Blood samples were obtained during the first study day at 11 A.M. after a 4-hour fast. WAT and skeletal muscle biopsies were obtained during the second study day at 8:00 A.M. after an overnight fast.

Endocannabinoid measurements

Plasma endocannabinoids and their congeners were quantified with LC-MS/MS. From the pool of individual study samples, quality controls were used to generate calibration curves. Additionally, all samples were randomized and each batch included calibration samples and an even distribution of quality control samples and blanks. The sample extraction procedure and method is as described in detail previously (14).

WAT and skeletal muscle gene expression

WAT and skeletal muscle biopsies of South Asians and white Caucasians after placebo treatment were analyzed. RNA was extracted using Tripure RNA isolation reagents (Roche, Molecular Biochemicals, Indianapolis, USA). Total RNA was reverse transcribed using Promega M1705 Reverse transcriptase for RT-PCR according to the instructions by the manufacturer to produce cDNA. Expression levels of enzymes involved in endocannabinoid synthesis (*DAGLA* and *DAGLB*, *NAPEPLD* and *PLA2G4E*) and degradation (*MGLL*,

FAAH1 and *FAAH2*) and CB receptors (*CNR1* and *CNR2*) were determined in a 96-well PCR plate by real-time PCR (Bio-Rad CFX96), using gene-specific primers (**Table 1**) and IQ SYBR-Green super mix (PromegaA6002). For analysis and quantification, Bio-Rad CFX Manager software version 3.1 was used. Gene expression was normalized by using *18S* (for WAT) or *GAPDH* (for skeletal muscle) mRNA content and expressed as fold difference compared to white Caucasian using the $\Delta\Delta CT$ method.

Table 1. Sequences of forward and reverse primers

Gene	Forward primer	Reverse primer
<i>18S</i>	AGGATCCATTGGAGGCAAGT	TCCAACTACGAGCTTTTAACTGCA
<i>CNR1</i>	GATGTACTTGCCCTGACCATA	AACATTCTAGGACTGATTCATCATG
<i>CNR2</i>	AAGATTGGCAGCGTGACTATG	CAGGCAGAGGTATCGGTCAA
<i>DAGLA</i>	CCATCTTCCTTTCTCCT	CTCGTGCGGGTTATAGAC
<i>DAGLB</i>	GTCTCCAGGGTTCTTC	TGAGGACGATCAAGTAAC
<i>FAAH1</i>	GGGCCGTACGTACACTATGC	ATGTTCCATCTGGGCCTCGTC
<i>FAAH2</i>	CGCTAGGCTTCTCATAGGC	CCGAAAGCAGAAGCAATGGTT
<i>GAPDH</i>	TTGCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG
<i>MGLL</i>	TCTCTCTTCTGGGCCACTCCA	GGATTGGCAAGAACCAGAGG
<i>NAPEPLD</i>	CCCTCTATTCCAATGTTCT	CATCCATTCCACCATTAC
<i>PLA2G4E</i>	GAGCCCAAAACGGATGAAG	GTCTGTCTGGCTCAGCATATCA

Statistical analysis

Data were collected and analyzed using IBM SPSS statistics version 23.0. Baseline characteristics and differences in plasma endocannabinoid levels between ethnicities were compared using unpaired student t-tests. Furthermore, linear regression analysis computed by Pearson's correlation was used to determine correlations between plasma endocannabinoid levels and different metabolic parameters. Regression analysis was performed both with and without correction for the effect of ethnicity, by respectively including/excluding ethnicity as a covariate. P values < 0.05 were considered as statistically significant. Data are presented as mean $\pm$ SEM. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

RESULTS

Clinical characteristics

The clinical characteristics of the participants were partly described previously (18). In brief, twenty overweight, prediabetic men of South Asian (n=10) and white Caucasian

($n=10$) descent were included. Mean age (46.5 ± 2.8 vs 47.5 ± 2.0 years) and BMI (30.1 ± 1.1 vs 30.7 ± 1.2 kg/m²) were comparable between South Asians and white Caucasians, respectively, as were body fat percentage ($31.0 \pm 1.1\%$ vs $30.1 \pm 1.0\%$), plasma triglyceride concentration (1.6 ± 0.2 vs 1.5 ± 0.2 mmol/L) and fasting glucose level (5.6 ± 0.5 vs 5.7 ± 0.7 mmol/L). Resting energy expenditure was lower in South Asians as compared to white Caucasians (4.9 ± 0.2 vs. 5.7 ± 0.2 kJ/min; $p < 0.05$), while lipid and glucose oxidation did not differ significantly.

South Asians have higher plasma levels of some endocannabinoids

First, we studied circulating endocannabinoid levels in both ethnicities. Plasma 2-AG and AEA levels were comparable between South Asians and white Caucasians (12.8 ± 1.5 vs 10.6 ± 1.2 pmol/mL; **Figure 1A**, and 1.0 ± 0.1 vs 0.9 ± 0.1 pmol/mL; **Figure 1B**, respectively). Plasma arachidonic acid tended to be higher in South Asians (18.3 ± 1.1 vs 14.6 ± 1.6 nmol/mL, $p = 0.07$; **Figure 1C**). In addition, plasma 2-linoleoyl glycerol (208 ± 23 vs 114 ± 17 pmol/mL, $p < 0.01$; **Figure 1D**) and *N*-linoleoylethanolamine (3.0 ± 0.2 vs 2.2 ± 0.2 pmol/mL, $p < 0.05$; **Figure 1E**) were higher in South Asians compared to white Caucasians. Plasma 1-linoleoyl glycerol (243 ± 28 vs 169 ± 30 pmol/mL, $p = 0.09$; **Table S1**), and *N*-docosatetraenylethanolamide (6.3 ± 0.4 vs 5.3 ± 0.4 pmol/mL, $p = 0.08$; **Table S1**) both tended to be higher in South Asians. No significant differences were observed for other *N*-acylethanolamines and mono- and di-acyl glycerols measured.

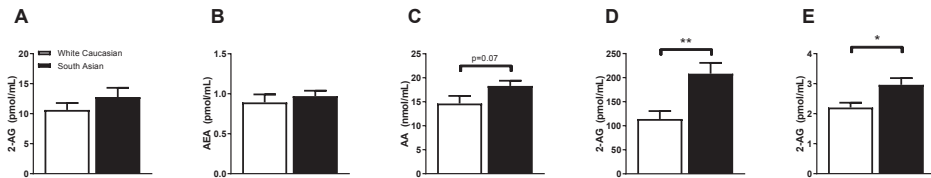


Figure 1. Circulating endocannabinoid levels in South Asian and white Caucasian men. Blood was collected from overweight, prediabetic South Asian ($n=10$; black bars) and matched white Caucasian ($n=10$; white bars) men. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used to measure plasma concentrations of 2-AG (**A**), AEA (**B**), arachidonic acid (AA) (**C**), 2-linoleoyl glycerol (2-LG) (**D**) and *N*-linoleoylethanolamine (LEA) (**E**) concentrations. Values are mean $\pm$ SEM. P-values are based on unpaired t-tests. * $p < 0.05$, ** $p < 0.01$.

Circulating endocannabinoid levels correlate with plasma triglycerides and lipid oxidation

We next investigated whether circulating endocannabinoid levels correlated with metabolic parameters in our study by pooling endocannabinoid levels of the two ethnicities. Plasma 2-AG levels positively correlated with plasma triglyceride levels ($R=0.77$, $p < 0.001$; **Figure 2A**) and lipid oxidation ($R=0.55$, $p < 0.05$; **Figure 2B**). In contrast, AEA levels did

not correlate with any of these parameters (data not shown). To test whether the effects could be attributed to ethnicity, we repeated the regression analysis including ethnicity as covariate and found that this did not change the results.

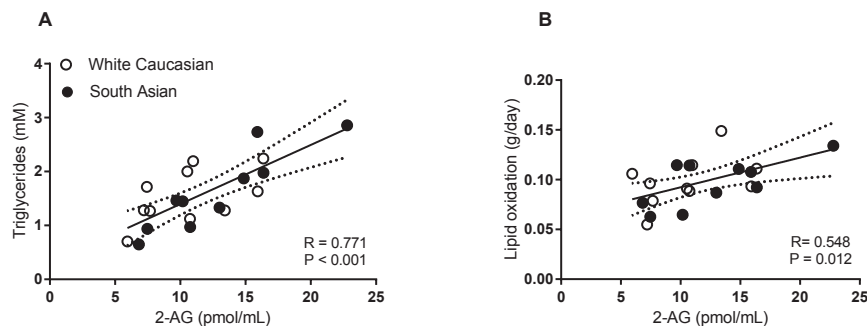


Figure 2. Plasma 2-AG levels positively correlate with plasma triglyceride levels and with lipid oxidation in South Asian and white Caucasian men. Scatterplot of the correlations between 2-AG levels and plasma triglycerides (A) or lipid oxidation (B). Correlations are shown for the total group combined ($n=20$), white circles represent white Caucasian men ($n=10$) and black circles represent South Asian men ($n=10$), with 95% confidence limits. Correlations were analyzed using linear regression analysis.

South Asians have lower mRNA expression of CB receptors and endocannabinoid degradation enzymes in skeletal muscle

Since circulating endocannabinoid levels not necessarily reflect tissue-specific signaling, we next assessed mRNA expression of CB receptors and enzymes involved in endocannabinoid synthesis and degradation in WAT and skeletal muscle biopsies of South Asian and white Caucasian men.

In WAT, expression levels of all measured genes were not different between the two ethnicities (**Figure 3A**). However, in skeletal muscle, the relative expression of the degradation enzyme *FAAH2* (*FAAH2*) was 5-fold lower in South Asians compared to white Caucasians (-81%, $p < 0.001$; **Figure 3B**). In addition, expression of the other degradation enzymes, *MAGL* (*MGLL*) and *FAAH1* (*FAAH1*) tended to be lower in South Asians (-22%, $p = 0.05$ and -18%, $p = 0.07$, respectively; **Figure 3B**). Moreover, expression of both the CB_1 (*CNR1*) and CB_2 (*CNR2*) receptor was approx. 10-fold lower in South Asians as compared to white Caucasians (-93%, $p < 0.001$ and -90%, $p < 0.001$; **Figure 3B**). Expression of endocannabinoid synthesis enzymes was comparable (**Figure 3B**) between the two ethnicities.

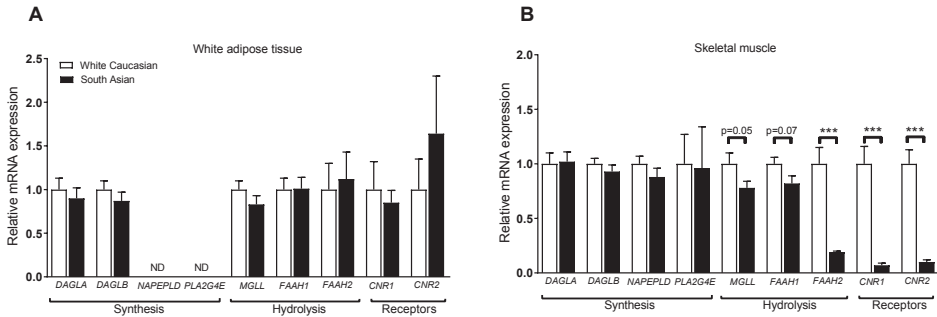


Figure 3. Relative expression of synthesis and degradation enzymes of endocannabinoids and mRNA expression of cannabinoid (CB) receptors in South Asian and white Caucasian men. Abdominal white adipose tissue and skeletal muscle tissue biopsies from the vastus lateralis muscle were taken after an overnight fasting period. Gene expression in white adipose tissue (A) and skeletal muscle (B) was determined by qPCR. Gene expression was normalized by using *18S* (white adipose tissue) or *GAPDH* (skeletal muscle) mRNA content and expressed as fold change compared to white Caucasians using the $\Delta\Delta CT$ method. ND is not detectable. Values are presented as mean $\pm$ SEM. p-values are based on an unpaired student t-test. *** $p < 0.001$.

DISCUSSION

The incidence of obesity and T2D is particularly high in individuals of South Asian descent. In the present study, we investigated different markers of endocannabinoid signaling in overweight, prediabetic South Asian and white Caucasian men. Here we report that, plasma 2-linoleoyl glycerol and *N*-linoleoylethanolamine were higher in South Asians compared to white Caucasians. In addition, gene expression of the enzymes involved in endocannabinoid degradation as well as CB receptors in skeletal muscle was markedly lower in South Asians as compared to white Caucasians. In addition, plasma 2-AG correlated positively with plasma triglycerides and lipid oxidation.

We observed higher levels of the endocannabinoids 2-linoleoylglycerol and *N*-linoleoylethanolamine in overweight, prediabetic South Asian compared to white Caucasian men. In our previous study in healthy lean South Asians and white Caucasians we also observed higher endocannabinoid levels in South Asians, however, in that study other endocannabinoids (*i.e.* 2-AG and AEA) were higher (14). If we compare the effect sizes between the two different cohorts we observe that except for *N*-palmitoylethanolamide, all effects are in the same direction albeit that the effect sizes differ for most endocannabinoids between the two cohorts. Furthermore, there is no clear pattern in the effect size with respect to whether it is larger in either the lean or the overweight cohorts. This is likely due to the different phenotype of the subjects in both cohorts. Of note, circulating endocannabinoid levels in this cohort of overweight, prediabetic men were not significantly higher compared to endocannabinoid levels measured in our previous cohort of healthy lean men. Probably, the difference in BMI between the two cohorts

was insufficiently large (average BMI 22 kg/m² in lean versus BMI 30 kg/m² in overweight men) to observe differences in circulating endocannabinoid levels between the two cohorts.

We also found a strong correlation between 2-AG and plasma triglyceride levels. This correlation was also present in our previous cohort of healthy lean South Asian and white Caucasian men (14) and is in line with results of Bluher et al. (21). The biological explanation for this correlation is currently unknown. Possibly, it can at least partly be explained by the fact that both 2-AG and triglycerides are lipid molecules with common lipid intermediates as precursors (e.g. diacylglycerol), resulting in production and secretion into blood of 2-AG concomitant with VLDL-triglyceride.

To investigate potential differences in endocannabinoid signaling in metabolically active tissues we examined WAT and skeletal muscle biopsies from these overweight, prediabetic men. We did not observe differences in expression of genes involved in endocannabinoid signaling in WAT between the two ethnicities. Interestingly, in skeletal muscle, we found that mRNA expression of the endocannabinoid degradation enzymes and CB receptors was lower in South Asians as compared to white Caucasians. Possibly, low expression of degradation enzymes in skeletal muscle of the South Asian men might have contributed to higher local endocannabinoid levels within skeletal muscle thereby possibly inducing CB receptor downregulation in this tissue (15). Alternatively, the dietary intake between South Asians and white Caucasians may have been different, as South Asian diets often contain low n-3 PUFA which has been shown to modulate the expression of endocannabinoid synthesis and degradation enzymes and CB receptors (22). Although all subjects used a standardized meal the evening before the study day, we cannot exclude that differences in diet might have influenced our results. Based on our study, we can thus only speculate about the underlying mechanisms, which is an interesting subject of future studies. Interestingly, the CB1 receptor regulates metabolic processes including insulin signaling, glucose uptake, and fatty acid oxidation in skeletal muscle (7,8). Moreover, overstimulation of CB receptors in skeletal muscle can disrupt insulin signaling thereby promoting insulin resistance which could eventually lead to development of (pre)diabetes (5). Since South Asians are known to have an increased risk for the development of metabolic disease, including T2D (2), dysregulation of ECS in skeletal muscle might thus contribute to diabetes development in this population.

Our study has several limitations. Firstly, we had a small sample size of ten South Asian and ten white Caucasian men. Secondly, all individuals were already metabolically deregulated as they are overweight and have prediabetes. This may have limited the differences we could observe between the two ethnic groups. In addition, we can only speculate about the mechanisms underlying the differences in ECS signaling between South Asians and white Caucasians. Future studies in larger cohort should investigate if these results also apply to women and lean, non-prediabetic individuals to determine

if these results could be translated to the general population. A strength of our study is that we measured both circulating endocannabinoids and gene expression of the ECS in WAT and skeletal muscle in the same individuals.

Taken together, our data show that, plasma 2-linoleoyl glycerol and *N*-linoleoylethanolamine were higher in overweight, prediabetic South Asians compared to white Caucasians. In addition, South Asian individuals had lower expression of enzymes involved in endocannabinoid degradation and CB receptors in skeletal muscle. Although it remains speculative, high endocannabinoid levels may deteriorate endocannabinoid signaling in metabolic organs, including skeletal muscle and thereby may contribute to the development and/or progression of obesity and possibly even T2D, both of which are highly prevalent in South Asians. Further studies are required to show if therapeutic interventions that target the ECS will improve the metabolic profile of these individuals.

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SUPPLEMENTAL APPENDIX

Supplementary Table S1. MPM (Multiple reaction monitoring) parameters and endogenous concentrations of the target list

S.No	ID	Metabolite Name	HMDB ID	Q1	Q3	DP, CE	Concentration (pmol/mL)		
							White Caucasians	South Asians	p-Value
1	1-1OG (18:1)	1-Oleoyl Glycerol	HMDB11567	357	265	80, 20	2441±353	2372±261	0.876
2	2-OG (18:1)	2-Oleoyl Glycerol	HMDB11537	357	265	80, 20	248±42	250±36	0.978
3	1-LG (18:2)	1-Linoleoyl Glycerol	HMDB11568	355	263	80, 15	169±30	243±28	0.086
4	2-LG (18:2)	2-Linoleoyl Glycerol	HMDB11538	355	263	80, 20	114±17	208±23	0.004
5	2&1-AG (20:4)	2&1-Arachidonoyl Glycerol	HMDB04666	379	287	56, 19	11±1	13±2	0.276
6	2AGE (20:4)	2-Arachidonyl Glycerol ether	HMDB13657	365	273	72, 25	ND	ND	
7	PDEA (15:0)	N-Pentadecanoyl Ethanolamide	53832-58-9a	286	62	80, 18	0.09±0.01	0.08±0.01	0.440
8	PEA (16:0)	N-Palmitoyl Ethanolamide	HMDB02100	300	62	78, 36	9.3±0.7	9.1±0.5	0.847
9	SEA (18:0)	N-Stearoyl Ethanolamide	HMDB13078	328	62	72, 31	6.3±0.5	6.1±0.2	0.672
10	POEA (16:1)	N-Palmitoleoyl Ethanolamide	HMDB13648	298	62	86, 32	0.53±0.06	0.41±0.04	0.145
11	OEA (18:1)	N-Oleylethanolamide	HMDB02088	326	62	72, 30	5.9±0.7	5.5±0.3	0.602
12	LEA (18:2)	N-Linoleoyl Ethanolamide	HMDB12252	324	62	74, 36	2.2±0.2	3.0±0.2	0.014
13	Alpha-LEA (18:2)	N-α-Linolenyl Ethanolamide	HMDB13624	322	62	80, 20	0.013±0.001	0.011±0.001	0.339
14	DGLEA (18:3)	Dihomo-γ-Linolenoyl ethanolamide	HMDB13625	350	62	70, 20	0.24±0.03	0.24±0.02	0.904
15	ETAEA (20:3)	Eicosatrienoic Acid Ethanolamide	169232-04-6a	350	62	80, 46	ND	ND	
16	AEA (20:4)	Anandamide	HMDB04080	348	62	70, 42	0.89±0.10	0.97±0.07	0.522

Supplementary Table S1. MRM (Multiple reaction monitoring) parameters and endogenous concentrations of the target list (continued)

S.No	ID	Metabolite Name	HMBD ID	Q1	Q3	DP, CE	Concentration (pmol/mL)		
							White Caucasians	South Asians	p-Value
17	O-AEA (20:4)	O-Arachidonoyl ethanolamine	HMDB13655	348	62	70, 25	ND	ND	
18	EPEA (20:5)	Eicosapentaenoyl Ethanolamide	HMDB13649	346	62	80, 20	0.06±0.01	0.06±0.01	0.910
19	DEA (22:4)	N-Docosatetraenoyl Ethanolamide	HMDB13626	376	62	72, 20	0.24±0.03	0.25±0.02	0.843
20	DHEA (22:6)	N-Docosahexaenoyl Ethanolamide	HMDB13658	372	62	70, 25	5.3±0.4	6.3±0.4	0.078
21	NADA (28:4)	N-Arachidonoyl dopamine	199875-69-9a	440	137	75, 24	ND	ND	
22	Arachidonic Acid (20:4)	5,8,11,14-Eicosatetraenoic acid	HMDB01043	303	259	-80, -18	14639±1578	18300±1076	0.071

The target list includes endocannabinoids and N-acyl ethanolamines (NAEs). The compound ID is the abbreviation of metabolite name along with number of carbon atoms and number of double bonds in the fatty acid chain of the molecule, respectively. All compounds are analyzed in positive mode except arachidonic acid in negative mode. Q1 and Q3 are optimized precursor ion and product ion, expressed as m/z. DP and CE are declustering potential (Volts) and collision energy (volts). Endogenous concentrations are denoted as mean ± SEM, n=10 in each group. P-values are based on unpaired t-tests. ND: Not detected in human plasma.

CHAPTER 4

Short-term cooling increases serum
angiopoietin-like-4 levels in healthy lean men

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ABSTRACT

Cold exposure enhances sympathetic outflow to peripheral tissues, thereby stimulating intracellular lipolysis in white adipose tissue and increasing the lipoprotein lipase (LPL)-dependent uptake and combustion of triglyceride-derived fatty acids (FA) by brown adipose tissue. Angiopoietin-like 4 (ANGPTL4) inhibits LPL and can be regulated by cold exposure, at least in mice. In the current study we examined the effect of short-term mild cooling on serum ANGPTL4 levels in healthy lean men of white Caucasian and South Asian descent. Healthy, lean white Caucasian (n=12) and South Asian (n=12) men were exposed to an individualized cooling protocol for 2 hours. Serum ANGPTL4 levels were measured before and after cooling and its relation with previously measured parameters (*i.e.* FFA levels, body fat percentage and resting energy expenditure) was determined. Short-term cooling increased ANGPTL4 levels (+17%, $p<0.001$). Thermoneutral ANGPTL4 levels positively correlated with FFA levels ($R^2=0.250$, $p<0.05$) and body fat percentage ($R^2=0.338$, $p<0.05$). Furthermore, ANGPTL4 negatively correlated with resting energy expenditure ($R^2=0.235$, $p<0.05$). The relative increase in ANGPTL4 levels was higher in white Caucasians compared to South Asians (25 ± 4 vs $9\pm4\%$, $p<0.05$). Short-term cooling increases ANGPTL4 levels in healthy lean men. We anticipate that FFA liberated from white adipose tissue during cooling increases ANGPTL4 to limit uptake of triglyceride-derived FA by this tissue.

INTRODUCTION

Cold exposure enhances the sympathetic outflow towards peripheral metabolic organs, including white adipose tissue (WAT) and brown adipose tissue (BAT) (1,2). In WAT, sympathetic stimulation increases intracellular lipolysis and subsequent release of free fatty acids (FFA) into the bloodstream (3). In contrast, sympathetic stimulation of BAT increases the uptake and combustion of triglyceride (TG)-derived FA (4,5).

Uptake of TG-derived FA from TG-rich lipoproteins by metabolic organs is determined by the activity of lipoprotein lipase (LPL). LPL is tightly regulated by many factors including angiopoietin-like 4 (ANGPTL4). ANGPTL4 is expressed by metabolic tissues (*i.e.* liver, WAT and BAT (6,7)) and inhibits LPL resulting in limited uptake of TG-derived FA uptake by tissues (8,9). ANGPTL4 expression is stimulated by elevated intracellular FFA levels (10), through activation of peroxisome proliferator-activated receptors (PPARs) (9). In mice we recently showed that cold exposure decreases ANGPTL4 protein content in BAT while increasing ANGPTL4 protein content in WAT (6,11). This opposite regulation of ANGPTL4, and thus LPL activity, in BAT and WAT makes physiological sense to secure the shuttling of FA towards BAT in order to meet the increased energy demand of activated BAT for heat production (11).

Although the regulation of ANGPTL4 upon cooling is well described in mice, the effect of short-term cooling on circulating ANGPTL4 levels in humans remained to be elucidated. Moreover, ANGPTL4 levels may be differentially regulated in subjects of South Asian descent, in which we previously showed lower resting energy expenditure (REE) and BAT volume upon cold exposure as compared to white Caucasians (12). The aim of the current study was, therefore, to determine the effect of short-term mild cooling on serum ANGPTL4 levels in healthy lean men of white Caucasian and South Asian descent.

MATERIALS AND METHODS

For the current study, blood samples were used from a previous clinical study (12) that was approved by the Medical Ethical Committee of the Leiden University Medical Center and undertaken in accordance with the principles of the Declaration of Helsinki. All volunteers provided written informed consent. Netherlands Trial Register 2473.

An extensive description of the materials and methods has been described previously (12). In short, twenty-four healthy, young (18-28 years), lean ($BMI < 25 \text{ kg/m}^2$) white Caucasian ($n=12$) and South Asian ($n=12$) men, participated in a prospective case-controlled study. Body composition was assessed by bioelectrical impedance analysis (BIA). An individualized water cooling protocol (2 hours mild cooling) followed by [^{18}F] FDG-PET-CT imaging was performed to quantify BAT volume and activity. Before and

after cooling, REE was measured (Oxycon) and fasted blood samples were collected. Pre- and post-cooling blood samples were analysed for ANGPTL4 by ELISA (10) and related to previously measured FFA levels, lipoprotein constituent levels (triglycerides (TG), low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C)), body fat percentage and REE. The effects of cooling on ANGPTL4 and FFA, TG, LDL-C and HDL-C were assessed using paired (effect of cold) and unpaired (effect of ethnicity) student t-tests with the SPSS 20 software. Correlations were analysed using linear regression analysis, both with and without correction for the effect of ethnicity, by respectively including/excluding ethnicity as a covariate. For lipid and lipoprotein measurements one South Asian and one white Caucasian individual were excluded because of familial hypercholesterolemia and hyperventilation, respectively. Data on body fat is missing for four South Asians and two white Caucasians because BIA measurements could not be performed. Values of $P < 0.05$ were considered statistically significant. Data are presented as mean $\pm$ SEM.

RESULTS

Clinical characteristics

Clinical characteristics of the participants and REE, BAT volume and lipid and lipoprotein levels were described previously (12,13). In short, we observed lower thermoneutral REE in South Asians as compared to white Caucasians (1297 ± 36 vs 1689 ± 56 kcal/day, $p < 0.001$). Short-term cooling significantly increased REE in white Caucasians (2027 ± 136 kcal/day, +20%, $p < 0.01$) but not in South Asians (1462 ± 37 kcal/day, +13%, ns). In addition, body fat percentage was significantly higher in South Asians compared to white Caucasians (16 ± 1 vs 12 ± 1 %, $p < 0.05$) and BAT volume was lower in South Asians as compared to white Caucasians (188 ± 23 vs 287 ± 49 mL, $p < 0.05$). Thermoneutral serum FFA levels were not different between white Caucasians and South Asians (0.66 ± 0.09 vs 0.88 ± 0.12 mmol/L, n.s.). However, cooling strongly increased FFA levels in white Caucasians (0.99 ± 0.09 mmol/L, +49%, $p < 0.001$), but not in South Asians (0.97 ± 0.11 mmol/L, +10%, n.s.). In addition, South Asians had comparable thermoneutral serum TG levels (1.33 ± 0.77 vs 1.27 ± 0.12 mmol/L) as well as HDL-C levels (0.86 ± 0.04 vs 0.92 ± 0.07 mmol/L), while South Asians tended to have higher thermoneutral LDL-C levels (2.39 ± 0.14 vs 2.01 ± 0.13 mmol/L, $p = 0.06$), as compared to white Caucasians. Cooling significantly increased serum TG in both ethnicities (+18%, $p < 0.05$ in white Caucasians and +24%, $p < 0.05$ in South Asians), while HDL-C (+9%, $p < 0.05$) and LDL-C (+8%, $p < 0.01$) were only increased in white Caucasians upon cooling.

Short-term cooling increases serum ANGPTL4 levels and ANGPTL4 correlates with FFA, body fat percentage and REE

We first evaluated the effect of short-term cooling on ANGPTL4 levels in the entire study population and assessed how ANGPTL4 levels correlated with previously measured metabolic parameters, including serum lipid and lipoprotein levels, fat mass and REE (12,13). Short-term cooling increased ANGPTL4 levels (+17%, $p<0.001$; **Figure 1A**). Moreover, thermoneutral ANGPTL4 levels correlated positively with FFA levels ($R^2=0.250$, $p<0.05$; **Figure 1B**) as well as total body fat percentage ($R^2=0.338$, $p<0.05$; **Figure 1C**), and correlated negatively with REE ($R^2=0.235$, $p<0.05$; **Figure 1D**). To assess whether ethnicity may have influenced these correlations, we re-performed the analysis with correction for ethnicity. After correction for ethnicity, serum ANGPTL4 levels still correlated with FFA levels ($p<0.05$) and tended to correlate with REE ($p=0.06$). The correlation between ANGPTL4 and body fat percentage was not significant anymore ($p=0.25$). No correlation was present between ANGPTL4 levels and serum TG, HDL-C, LDL-C, or BAT volume or activity (not shown).

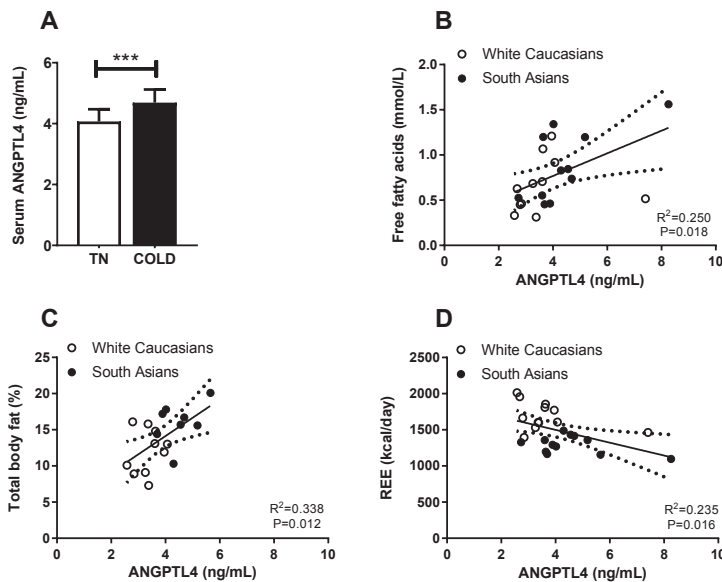


Figure 1. Short-term mild cooling increases serum ANGPTL4 levels in healthy lean men and correlates with metabolic parameters. Blood was collected from healthy, young lean white Caucasian ($n=12$) and matched South Asian ($n=12$) individuals before (Thermoneutral; TN) and after short-term cooling (COLD). Serum ANGPTL4 levels were measured using ELISA. **(A)** Grouped data of thermoneutral (TN) and cold (COLD) measurement ($n=24$). Scatterplot of the correlations between serum ANGPTL4 levels measured at thermoneutrality and serum free fatty acid levels at thermoneutrality (white Caucasians, $n=11$; South Asians, $n=11$) **(B)**, and body fat percentage (white Caucasians, $n=10$; South Asians, $n=8$) **(C)** and thermoneutral resting energy expenditure (REE) (white Caucasians, $n=12$; South Asians, $n=12$) **(D)**. Correlations are shown for the total group combined, white circles are white Caucasian individuals and black circles are South Asian individuals, with 95% confidence limits. Values are mean $\pm$ SEM. *** $P<0.001$. P-values are based on paired t-tests (effect of cold). Correlations were analysed using linear regression analysis.

ANGPTL4 response upon cooling is more pronounced in white Caucasian compared to South Asian men

We previously showed lower REE and BAT volume upon cold exposure in South Asians compared to white Caucasians (12). Therefore, we hypothesized that ANGPTL4 levels may be differentially regulated in subjects of South Asian descent and thus explored the effect of short-term cooling on ANGPTL4 levels in the two ethnicities separately. At thermoneutrality, ANGPTL4 levels were not different between white Caucasians and South Asians (3.63 ± 0.37 vs 4.52 ± 0.41 ng/mL, $p=0.122$, **Figure 2A, B**). In contrast, cooling markedly increased ANGPTL4 levels in white Caucasians ($+25\%$, $p<0.001$; **Figure 2A**), while ANGPTL4 levels only showed a tendency towards an increase in South Asians ($+9\%$, $p=0.09$; **Figure 2B**). After excluding two 'biological outliers', *i.e.* the white Caucasian and South Asian individual with the highest ANGPTL4 levels at thermoneutrality, short-term cooling significantly increased ANGPTL4 levels in South Asians as well ($+10\%$, $p<0.05$; data not shown). Nevertheless, the relative increase in ANGPTL4 levels was higher in white Caucasian men compared to South Asian men ($+25 \pm 4$ vs $+9 \pm 4\%$, $p<0.05$; **Figure 2C**). Also after excluding the two 'biological outliers' the relative increase was still larger in white Caucasians as compared to South Asians ($+24 \pm 5$ vs $+10 \pm 4\%$, $p<0.05$; data not shown).

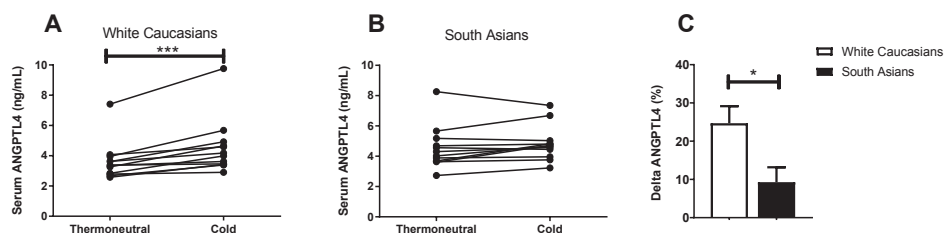


Figure 2. Short-term mild cooling increases serum ANGPTL4 levels in white Caucasians, but only tended to increase in South Asians. Blood was collected from healthy, young lean white Caucasian ($n=12$) and matched South Asian ($n=12$) individuals before (Thermoneutral) and after short-term cooling (Cold). Serum ANGPTL4 levels were measured using ELISA. Individual changes in ANGPTL4 levels upon cooling in white Caucasian (**A**) and South Asian (**B**) individuals, respectively. Percentage increase in ANGPTL4 levels upon cooling in white Caucasians (white bar) and South Asians (black bar) (**C**). Values are mean $\pm$ SEM. * $P<0.05$, *** $P<0.001$. P-values are based on paired t-tests (effect of cold) or unpaired t-tests (effect of ethnicity).

DISCUSSION

ANGPTL4 is an important regulator of LPL activity and thereby controls lipid availability. In the current study we examined the effect of short-term mild cooling on serum ANGPTL4 levels in healthy lean men of white Caucasian and South Asian descent. We show

that cooling increased ANGPTL4 levels and that ANGPTL4 levels positively correlated with FFA levels and total body fat percentage and negatively correlated with REE. The relative increase in ANGPTL4 levels was higher in white Caucasians compared to South Asians.

The finding that cooling increased ANGPTL4 levels in healthy lean men is in line with previous work that showed increased plasma ANGPTL4 levels after administration of the β_3 -receptor agonist Salbutamol (10). β_3 -receptor agonism causes FFA liberation from WAT and TG-derived FA uptake by BAT, thus mimicking the effect of cold exposure (4). In addition, we previously showed that cooling increases FFA levels in white Caucasians (12), and now show that FFA levels at thermoneutrality correlate positively with ANGPTL4 levels. This is in concordance with other studies showing increased FFA levels after cooling (14,15) and β_3 -receptor activation (16), and a positive correlation between FFA levels and ANGPTL4 (10,17,18). Similarly, the increase in FFA levels that is induced by fasting is also accompanied by an increase in ANGPTL4 levels (10). Together, these data indicate that circulating FFA levels are either an important driver or predictor of ANGPTL4 levels. A likely explanation is that liberation of FFA from white adipocytes by intracellular lipolysis, as induced by cold exposure, β_3 -receptor activation and fasting (3), activates PPAR γ leading to increased ANGPTL4 expression (9) (see hypothetical model **Figure 3**).

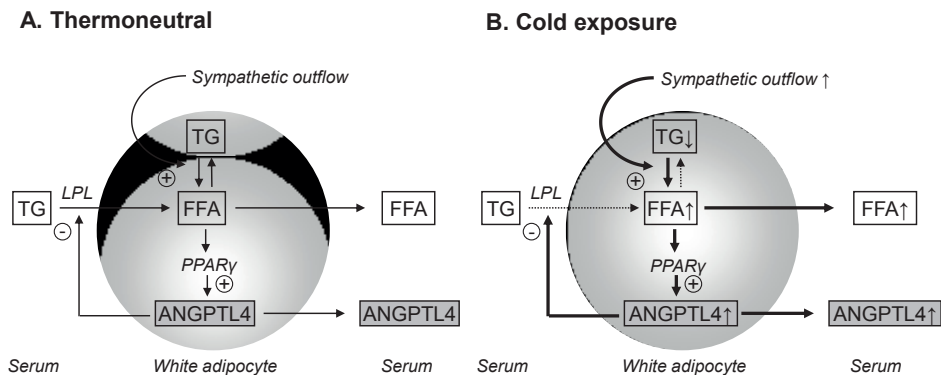


Figure 3. Hypothetical mechanism explaining that cold increases ANGPTL4 and FFA. Compared to thermoneutrality (panel A), cold exposure (panel B) activates the sympathetic nervous system (SNS) to stimulate intracellular lipolysis in white adipose tissue (WAT) and release of fatty acids (FA). The liberated FA is secreted as free FA (FFA) in serum, and activates PPAR γ to increase expression of ANGPTL4 that is subsequently released into the circulation where it can inhibit LPL to prevent cellular FA entry.

The fact that we found a positive correlation between thermoneutral ANGPTL4 levels and body fat percentage may support the notion that WAT is a main organ that contributes to circulating ANGPTL4 levels (10). In mice, cold exposure increases ANGPTL4

in WAT and decreases ANGPTL4 protein levels specifically in BAT in order to shuttle TG-derived FA to BAT for combustion (11). It can be speculated that this mechanism also occurs in humans during cooling to guarantee lipid availability for BAT during cold exposure. We could only measure systemic ANGPTL4 levels, which is the net effect of whole-body ANGPTL4 regulation. Given that the total volume of WAT exceeds that of BAT by far, an overall increase in serum ANGPTL4 levels is indeed expected. We showed that thermoneutral ANGPTL4 levels correlated negatively with REE, which suggests that ANGPTL4 is a negative regulator of energy expenditure. This is line with the function of ANGPTL4 to inhibit LPL and thus the uptake of TG-derived FA uptake by metabolic tissues, especially under conditions where BAT activity is considered negligible (8,9).

When exploring the ANGPTL4 response within the different ethnicities we found that cooling significantly increased serum ANGPTL4 in white Caucasians but not in South Asians. Previously, we showed that in South Asians also the FFA response is blunted and that they have lower REE and BAT volume upon short-term cooling (12). Collectively, these data may suggest that South Asian individuals have lower cooling-induced sympathetic outflow towards peripheral organs. Interesting, we recently showed that South Asians, compared to white Caucasians, have higher levels of circulating endocannabinoid levels (19), which are thought to inhibit sympathetic signalling (20,21). Future studies are necessary to further elucidate the possibility that South Asians have lower sympathetic signalling, for example by measuring the effect of cooling on heart rate variability and ^{123}I -metaiodobenzylguanidine uptake using single-photon emission computed tomography.

Our study has several limitations. First, we cannot exclude that the higher body fat percentage in the South Asians compared to the white Caucasians might also have contributed to the difference in ANGPTL4 response that we observed between the two ethnicities. Future studies in cohorts with similar body fat percentages at baseline should be performed to elucidate this possibility. Furthermore, we had a relatively small sample size of twelve South Asian and twelve white Caucasian men. Based on an unpaired student t-test we did not observe a significant difference in baseline ANGPTL4 levels between both ethnicities. In addition, when in both ethnical groups the individual with the highest baseline ANGPTL4 level was removed from the analysis, short-term cooling significantly increased ANGPTL4 levels in the South Asians as well, although the relative increase in ANGPTL4 levels was still larger in the white Caucasians. Whether ANGPTL4 responses are truly different between the two ethnicities should be further investigated in studies with larger sample sizes. Furthermore, we only investigated a group of healthy lean men. Future studies in larger cohorts should investigate if baseline levels of ANGPTL4 are indeed not different between the two ethnicities and if these results also apply to the general population.

In conclusion, our data demonstrate that short-term mild cooling increases circulating ANGPTL4 levels in healthy lean men, with a significantly higher response in white Caucasian compared to South Asian men. As underlying mechanism we speculate that cold exposure causes intracellular lipolysis in white adipocytes to liberate FFA that, via activation of PPAR γ , increase ANGPTL4 synthesis. Possibly, this sequence of events is blunted in South Asians because of reduced sympathetic signalling, which would an interesting topic for future research.

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CHAPTER 5

Physiological changes due to mild cooling in healthy lean males of white Caucasian and South Asian descent: a metabolomics study.

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ABSTRACT

During mild cold exposure, non-shivering thermogenesis increases to maintain core body temperature by increasing utilization of substrates, especially fatty acids (FA), ultimately affecting lipid-associated metabolites. We aimed to investigate whether mild cooling induces changes in other metabolites and whether this response differs between white Caucasians and South Asians, who have a disadvantageous metabolic phenotype. 12 lean male Dutch white Caucasians and 12 matched Dutch South Asians were exposed to mild cold. Before and after 100 minutes exposure, serum samples were collected for analysis of 163 metabolites and 27 derived parameters using high throughput metabolomics. The overall response to mild cooling between both ethnicities was not different, therefore the data were pooled. After Bonferroni correction, mild cooling significantly changed 44 of 190 (23%) metabolic parameters. Specifically, cooling increased 19 phosphatidylcholine (PC) species, only those containing very long chain FAs, and increased the total class of PC containing mono-unsaturated FAs (+12.5%). Furthermore, cooling increased 10 sphingomyelin species as well as the amino acids glutamine (+18.7%), glycine (+11.6%) and histidine (+10.6%), and decreased short-chain (C3 and C4) acylcarnitines (-17.1% and -19.4%, respectively). In conclusion, mild cooling elicits substantial effects on serum metabolites in healthy males, irrespective of white Caucasian or South Asian ethnicity.

INTRODUCTION

Mammals are programmed to tightly maintain their core body temperature, during high as well as low environmental temperatures. Cold exposure results in a fast physiological response that includes minimizing heat loss by peripheral vasoconstriction and enhancing heat production by thermogenesis (*e.g.* 'cold-induced thermogenesis'). Cold-induced thermogenesis is a tightly regulated process in which a coordinated action of many organs is involved (1). It can be divided in non-shivering thermogenesis (NST) and shivering thermogenesis. NST is quickly activated upon cold exposure and is largely mediated by activation of UCP1 in brown adipose tissue (BAT), which uncouples the electron transport chain from ATP synthesis resulting in production of heat (2). Shivering thermogenesis is induced when NST is insufficient to maintain core body temperature and occurs as a consequence of muscle contractions. Shivering thermogenesis can increase human basal metabolic rate (BMR) by as much as 3-5 fold, however, it is generally experienced as uncomfortable. NST can contribute to BMR up to a more modest +30% but can be sustained without appreciable discomfort. Therefore, enhancing NST is generally considered an attractive target to fight obesity (2).

Exposure to mild cold exposure, generally resulting in enhanced NST, has large metabolic implications, for instance it results in high demand of extra fuel, of which fatty acids (FAs) are likely the most important. FAs are released from white adipose tissue (WAT) as a consequence of lipolysis and are subsequently redirected to several metabolic organs, including BAT, for oxidation and subsequent production of ATP or heat (3,4). Interestingly, we recently showed that the increase in FA upon mild cold-induced thermogenesis shows an ethnic variation in healthy lean men. When exposed to mild cold, serum FA levels significantly increased in white Caucasians but not in South Asians (5).

Metabolomics is an efficient approach to study many separate metabolic pathways in a high-throughput fashion (6). Therefore, the objective of the current study was to study changes in metabolites upon mild cooling and to assess ethnic differences in this response in white Caucasians versus South Asians.

MATERIALS AND METHODS

Ethics

Blood samples were collected as part of a clinical study aimed at investigating the activity and volume of BAT in Dutch white Caucasian and Dutch South Asian individuals (5). The study was approved by the Medical Ethical Committee of the Leiden University Medical Center (LUMC) and undertaken in accordance with the principles of the revised Declaration of Helsinki. All volunteers provided written informed consent.

Participants and study design

Twenty four Dutch healthy, lean ($BMI < 25 \text{ kg/m}^2$) males of white Caucasian ($n=12$) and South Asian ($n=12$) origin between 18 and 28 years of age were included. The study was conducted in The Rijnland Hospital, Leiderdorp (The Netherlands).

Subjects were studied in the morning after a 10-hour overnight fast and subjects were not allowed to exercise 24 hours prior to the study. Subjects wore standardized clothing, consisting of a T-shirt and boxer shorts. Upon arrival, subjects ingested a telemetric capsule (Jonah™, BMedical, Australia) to measure core body temperature at 1-minute intervals. An intravenous cannula was inserted for blood collection.

To establish maximum NST, an individualized cooling protocol was used in which a subject was placed between two water perfused cooling mattresses (Blanketrol® III, Cincinnati Sub-Zero (CSZ) Products, Inc). During the procedure subjects stayed in a clinical examination room (temperature approx. 24°C) in a semi-supine position. The protocol started with a baseline period of one hour in thermoneutral condition (water temperature cooling mattresses 32°C), after which subjects were exposed to mild cold. Since the onset temperature of shivering shows a high interindividual variation (e.g. due to differences in body composition), an individualized cooling protocol was used to ensure maximum NST for each subject. For the cooling protocol, we gradually decreased the water temperature of the cooling mattresses until shivering occurred. In short, we first decreased the temperature with steps of 5°C every 5 minutes. After we reached a water temperature of 17°C, we decreased the temperature with 2°C every 10 minutes. When a water temperature of 11°C was reached (but not all subjects reached this temperature), we decreased temperature with 1°C every 10 minutes. This was continued until shivering occurred. Shivering was detected visually and by asking the subject if he experienced shivering. When the shivering temperature had been reached, the subject was warmed for 3 minutes with a bathrobe so that shivering stopped after which he was cooled with a water temperature 3°C higher than the temperature at which shivering occurred. From that moment, a cooling period of two hours was started (defined as $t=0 \text{ min}$). In case of shivering, temperature was raised by steps of 1°C until shivering just stopped. In this manner NST was maximized for each individual without shivering. At the end of the thermoneutral period and during the stable cooling period ($t=110 \text{ min}$) venous blood was drawn. For metabolomics, we used serum samples from the 22 participants that were BAT positive on the basis of the ^{18}F -FDG PET-CT scan and therefore excluded the 2 (white Caucasian) subjects that exhibited virtually no BAT. These two excluded subjects were both from white Caucasian descent. We measured 190 metabolic parameters (of which 163 small molecule metabolites) in these samples.

Metabolomics

Metabolomic measurements were performed at the Genome Analysis Center of the Helmholtz Zentrum München, Germany, using the Biocrates Absolute/DQ™ p150 kit (BIOCRATES Life Science AG, Innsbruck, Austria) and ESI-FIA-MS/MS measurements (7). This assay allows simultaneous quantification of 163 small molecule metabolites out of 10 µL serum. The metabolite panel consists of free carnitine, 40 acylcarnitines, 14 amino acids (13 proteinogenic + ornithine), hexoses (of which 90-95% glucose), 92 glycerophospholipids (of which 15 lysophosphatidylcholines; LysoPCs) and 77 phosphatidylcholines; PCs), and 15 sphingolipids (sphingomyelins). This assay does not measure triglyceride (TG), cholesterol or free FA levels. Quantification of the metabolites was achieved by reference to appropriate internal standards. The assay has been previously described in detail (7,8). One metabolite, PCae38:1, was only detected in one participant and therefore excluded from the analyses, leaving 162 metabolites for analyses. Furthermore, 27 additional derived metabolic parameters (metabolite classes and metabolite ratios) as provided by Biocrates were examined.

Statistical analyses

First, all metabolites were Z-score transformed to make effect estimates between metabolites comparable. Then, we assessed the effect of mild cooling on metabolite concentrations or metabolic parameters using paired t-tests. The percentage change was calculated by dividing the mean change of a metabolite or metabolic parameter by the mean baseline concentration. Subsequently, we examined whether the metabolic response to mild cooling differed between white Caucasians and South Asians using linear regression analysis. No adjustments (for e.g. BMI or age) were made, since these subjects were already matched for these factors. After Bonferroni correction, a p-value of 3.1×10^{-4} (= 0.05/162 metabolites) was considered statistically significant. All statistical analyses were performed using SPSS 20 (SPSS Inc, Chicago, IL, USA).

RESULTS

Clinical characteristics

The characteristics of the participants and the effects of mild cold exposure on cardio-metabolic parameters have been described previously and are shown in **Table 1** (5). Subjects were matched for age and BMI. In both ethnic groups, diastolic but not systolic blood pressure significantly increased in response to cold (+7 mmHg in white Caucasians, $P < 0.01$; +6 mmHg in South Asians, $P < 0.01$), while heart rate decreased (-5 beats/min in white Caucasians, $P < 0.05$; -8 beats/min in South Asians, $P < 0.01$). Mean inlet water temperature of the cooling mattresses at which shivering started was lower for white Caucasians than for South Asians (8.9°C vs 10.9°C; $P < 0.01$). Mean core body temperature

was not affected by cold exposure (data not shown) (5,9). Mild cold exposure increased energy expenditure, and thus thermogenesis, in white Caucasians (+21%; $P<0.01$) and a tendency towards an increase was observed in South Asians (+13%; $P=0.07$) (5).

Table 1. Participants' characteristics.

	White Caucasians	South Asians
Age (years)	24.6 (2.8)	23.6 (2.8)
Number of participants (% males)	10 (100%)	12 (100%)
Body mass index (kg/m^2)	22.0 (1.6)	21.5 (2.0)
Shivering temperature ($^{\circ}\text{C}$)	8.9 (1.5)	10.9 (1.8) ^{**}
Systolic blood pressure (mmHg)		
Prior to cooling	135 (11)	126 (18)
After cooling	143 (13)	125 (13)
Diastolic blood pressure (mmHg)		
Prior to cooling	77 (6)	73 (9)
After cooling	84 (6) ^{**}	79 (8) ^{**}
Heart rate (beats/min)		
Prior to cooling	64 (10)	62 (8)
After cooling	57 (10) [*]	54 (8) ^{**}
Resting energy expenditure (kcal/day)		
Prior to cooling	1727 (183)	1297 (123) ^{***}
After cooling	2084 (456) ^{**}	1462 (127) ^{***}

Values represent number of participants (%) or mean (standard deviation). ^{*} $P<0.05$, ^{**} $P<0.01$ and ^{***} $P<0.001$ vs prior to cooling. ^{**} $P<0.01$; ^{***} $P<0.001$ vs white Caucasians. All P values are based on a mixed model.

Mild cooling increases plasma free fatty acids

After mild cooling plasma free fatty acid concentrations increased significantly in white Caucasians (+44%, $p<0.001$) but not in South Asians, which was previously published (5).

Mild cold exposure markedly affects several serum metabolites

First, we demonstrated using linear regression that the overall response to the mild cold challenge with respect to changes in metabolic parameters did not differ between white Caucasians and South Asians (**Supplementary table 2**, last column). Therefore, metabolic changes will be presented per ethnicity and as pooled results, and results will be described per metabolite class in the sections. After mild cold exposure, a marked 116 out of 189 (61%) metabolic parameters (metabolites and classes/ratios) were changed in serum at a nominal significance level. After correction for multiple testing, 44 of the 189 (23%) measured metabolic parameters remained significant (see **Table 2**). The complete listings of abbreviations are shown in **Supplementary Table 1**. A list of the full results is given in **Supplemental Table 2**.

Table 2. Effect of cooling on metabolite or metabolite groups.

	white Caucasians (n=10)		South Asians (n=12)		All subjects (n=22)	
	% change (SD) ¹	p-value	% change (SD)	p-value	% change (SD)	p-value
Acylcarnitines						
C3	-21.3% (0.0)	4.25E-04	-14.4% (0.0)	1.46E-03	-17.1 (0.0)	1.17E-06
C4	-18.7% (0.0)	6.78E-03	-19.9% (0.0)	9.25E-03	-19.4% (0.0)	1.33E-04
Amino acids						
Gln	+20.0% (66.4)	2.22E-03	+17.6% (50.5)	2.60E-04	+18.7% (57.1)	1.09E-06
Gly	+9.8% (29.1)	3.84E-02	+13.3% (24.6)	2.59E-03	+11.6% (26.2)	1.97E-04
His	+10.5% (9.4)	3.38E-02	+10.6% (5.9)	2.02E-03	+10.6% (7.5)	2.18E-04
Phosphatidylcholines						
PC aa C34:1	+14.2% (23.7)	3.21E-03	+11.0% (25.4)	1.51E-02	+12.5% (24.5)	9.57E-05
PC aa C36:1	+13.3% (3.9)	4.97E-03	+11.3% (4.3)	1.45E-02	+12.2% (4.0)	1.33E-04
PC aa C38:3	+17.7% (4.3)	1.02E-03	+12.7% (4.5)	5.17E-03	+15.0% (4.4)	1.07E-05
PC aa C38:4	+19.4% (9.7)	1.01E-03	+14.5% (13.5)	2.42E-03	+16.3% (11.6)	5.59E-06
PC aa C38:5	+19.4% (4.7)	8.73E-04	+15.4% (5.0)	1.39E-03	+17.2% (4.8)	1.92E-06
PC aa C38:6	+22.6% (6.8)	2.63E-04	+12.8% (7.1)	7.43E-04	+16.5% (6.9)	3.55E-07
PC aa C40:4	+18.4% (0.4)	3.00E-03	+19.0% (0.6)	4.17E-03	+18.8% (0.5)	2.75E-05
PC aa C40:5	+22.4% (1.1)	2.66E-04	+13.1% (1.0)	1.04E-03	+17.3% (1.1)	8.31E-07
PC aa C40:6	+21.9% (1.8)	2.07E-04	+14.8% (2.5)	9.75E-04	+17.5% (2.2)	4.33E-07
PC ae C38:4	+18.2% (1.7)	4.35E-03	+15.4% (1.8)	5.93E-03	+16.6% (1.7)	4.25E-05
PC ae C38:5	+15.5% (2.2)	3.93E-03	+11.7% (2.2)	6.57E-03	+13.4% (2.2)	4.29E-05
PC ae C38:6	+15.7% (0.6)	7.79E-04	+7.4% (0.8)	4.38E-02	+10.9% (0.8)	1.58E-04
PC ae C40:0	+15.7% (0.5)	5.61E-04	+10.3% (0.6)	1.06E-02	+12.7% (0.5)	1.77E-05
PC ae C40:2	+13.6% (0.1)	5.10E-03	+19.6% (0.2)	3.74E-03	+16.8% (0.2)	5.15E-05
PC ae C40:4	+15.9% (0.3)	5.02E-03	+19.8% (0.5)	1.09E-02	+18.0% (0.4)	1.74E-04
PC ae C40:5	+16.9% (0.6)	5.93E-03	+23.5% (0.8)	3.16E-03	+20.4% (0.7)	4.10E-05
PC ae C40:6	+19.7% (0.5)	7.62E-04	+15.9% (0.5)	6.14E-04	+17.6% (0.5)	7.02E-07
PC ae C42:5	+16.6% (0.3)	9.70E-03	+14.3% (0.2)	8.45E-04	+15.5% (0.2)	3.45E-05
PC ae C44:5	+18.9% (0.1)	2.64E-03	+13.7% (0.1)	4.06E-03	+16.4% (0.1)	3.17E-05
Sphingomyelins						
SM (OH) C14:1	+14.1% (0.6)	3.15E-03	+14.2% (0.7)	3.07E-03	+14.2% (0.6)	1.65E-05
SM (OH) C16:1	+16.0% (0.3)	7.48E-03	+12.2% (0.3)	1.73E-02	+13.8% (0.3)	2.33E-04
SM (OH) C22:1	+21.7% (0.6)	2.03E-04	+17.2% (0.9)	1.07E-03	+19.0% (0.8)	5.77E-07
SM (OH) C22:2	+21.5% (0.5)	2.37E-04	+18.5% (0.7)	1.19E-03	+19.8% (0.6)	7.00E-07
SM C16:0	+13.5% (10.0)	5.26E-03	+12.1% (13.2)	7.40E-03	+12.7% (11.6)	8.01E-05
SM C16:1	+13.5% (1.4)	5.37E-03	+11.6% (1.9)	8.21E-03	+12.4% (1.6)	9.29E-05
SM C18:0	+16.1% (1.5)	1.21E-03	+13.3% (2.1)	4.33E-03	+14.5% (1.8)	1.29E-05
SM C18:1	+17.1% (0.7)	6.89E-04	+15.4% (1.0)	1.07E-03	+16.1% (1.2)	1.72E-06

Table 2. Effect of cooling on metabolite or metabolite groups. (continued)

	white Caucasians (n=10)		South Asians (n=12)		All subjects (n=22)	
	% change (SD) ¹	p-value	% change (SD)	p-value	% change (SD)	p-value
SM C24:0	+21.4% (1.2)	2.08E-04	+20.5% (1.9)	2.66E-04	+20.8% (1.6)	1.87E-07
SM C24:1	+20.6% (3.3)	5.45E-04	+18.9% (4.4)	8.82E-04	+19.6% (3.8)	9.49E-07
Metabolite classes/ ratios						
MUFA (PC)	+13.7% (31.5)	3.81E-03	+10.86% (33.7)	1.62E-02	+12.2% (32.4)	1.19E-04
MUFA (PC) / SFA (PC)	+6.6% (2.1)	1.36E-03	+4.1% (2.2)	2.67E-03	+5.4% (2.2)	1.42E-05
PUFA (PC) / MUFA (PC)	-3.6% (0.1)	3.18E-04	-2.8% (0.1)	3.22E-04	-3.1% (0.1)	1.50E-07
Total SM	+16.1% (19.7)	1.62E-03	+14.3% (27.0)	2.96E-03	+15.0% (23.5)	1.05E-05
Total SM / Total (SM+PC)	+4.0% (0.0)	1.56E-02	+6.1% (0.0)	7.93E-04	+5.2% (0.0)	2.65E-05
Total SM / Total PC	+4.3% (0.0)	1.50E-02	+6.7% (0.0)	8.10E-04	+5.7% (0.0)	2.74E-05
Total SM-non OH	+15.7% (17.8)	1.86E-03	+14.1% (24.4)	3.19E-03	+14.8% (21.1)	1.30E-05
Total SM-OH	+18.8% (2.0)	5.38E-04	+16.3% (2.7)	1.75E-03	+17.3% (2.4)	2.06E-06
Total SM-OH / Total SM-non OH	+2.6% (0.0)	1.93E-03	+2.1% (0.0)	6.08E-04	+2.3% (0.0)	2.51E-06
Tyr / Phe	+12.9% (0.1)	6.30E-05	+9.9% (0.1)	1.15E-04	+11.3% (0.1)	1.42E-08

Shown metabolites are those that reached Bonferroni significance ($p < 0.05/162$, i.e. $p < 3.1 \times 10^{-4}$). The complete listings of abbreviations are shown in Supplemental Table 1. The complete table of 190 metabolites and related metabolic parameters is shown in Supplemental Table 2. ¹Values represent % change after cooling with SD. P-values were calculated using paired t-tests.

Effect of cooling on serum acylcarnitines

Of the acylcarnitines, C3 (Propionyl-L-carnitine) and C4 (Butyryl-L-carnitine) were significantly decreased upon mild cooling (-17.1% and -19.4%, respectively). Interestingly, these were the only two individual metabolites that showed a decrease rather than an increase after cooling.

Effect of cooling on amino acids

Of the fourteen measured amino acids, only glutamine (+18.7%), glycine (+11.6%) and histidine (+10.6%) increased significantly after mild cooling.

Effect of cooling on glycerophospholipids

None of the lysoPCs were changed significantly after Bonferroni correction upon mild cooling. In contrast, of the 76 measured PCs, 19 (25%) increased significantly and these all contained at least 34 carbon atoms. Of the increased PCs, only one was saturated (PCaeC40:0), two were mono-unsaturated (PCaaC34:1 and PCaaC36:1) and as many as 16 were poly-unsaturated.

Effect of cooling on sphingomyelins (SMs)

Of the fifteen SMs, ten SMs (63%) significantly increased after mild cooling.

Effect of cooling on metabolite classes/ratios

We also assessed the effect of mild cooling on 27 additional derived metabolic parameters (metabolite classes and metabolite ratios), and found several changes. The total concentration of mono-unsaturated FAs (contained in PCs) significantly increased (+12.2%), while saturated and poly-unsaturated FAs in PC did not change. Subsequently, the mono-unsaturated-to-saturated FA ratio increased (+5.4%), while the poly-unsaturated-to-mono-unsaturated ratio decreased after mild cooling (-3.1%). Again, as determined from linear regression, no differences between white Caucasians and South Asians were observed.

DISCUSSION

Mild cold exposure increases NST and FA utilization (10) ultimately affecting lipid-associated metabolites. In the present study, we used metabolomics to investigate the effect of mild cooling on serum levels of metabolites, specifically those related to lipid metabolism, by using the Biocrates platform. We found that 44 of the 189 (23%) measured metabolites and derived metabolic parameters (metabolite classes and metabolite ratios) were significantly changed. Interestingly, the PCs and SMs were significantly increased upon mild cooling, as were the amino acids glutamine, glycine and histidine. In contrast, the acylcarnitines C3 and C4 were significantly decreased. The overall response to mild cold between white Caucasian and South Asians subjects was not different.

The increased thermogenesis that is induced upon mild cold exposure requires enhanced availability of FA which can subsequently be used for thermogenesis. In our study subjects, we previously showed a marked increase in serum FA and triglyceride (TG) levels upon mild cooling (5). The enhanced FA levels are likely the consequence of enhanced WAT lipolysis, possibly due to increased sympathetic outflow towards the tissue. In our metabolomics assay, we did not measure serum FA or TG levels, but instead assessed several other metabolites related to lipid metabolism, including PCs, lysoPCs and SMs. PCs and SMs are both abundantly present in all lipoprotein classes (*i.e.* VLDL, LDL and HDL) as a component of the surface shell (11,12). We found that, upon mild cooling, both serum PC and SM levels were increased. This may point to either enhanced lipoprotein synthesis or decreased breakdown. Considering the large capacity of BAT to take up FA from TG-rich lipoproteins, the latter mechanism seems unlikely. In human serum most of the SMs are present in LDL and VLDL (approximately 63-75% of all serum

SM), and a minority in HDL (13). Taken together with the elevated serum TG levels seen upon mild cooling, these data strongly suggest that the increases in PCs and SMs are a result of increased hepatic VLDL production.

Interestingly, when the PCs that increased upon mild cooling were studied in more detail, it was evident that almost all contained unsaturated FAs. In fact, 16 of the 19 increased PCs belonged to the poly-unsaturated PCs. In addition, all contained very long chain FAs, containing at least 34 carbon atoms. A clear explanation for this phenomenon is not yet known but may be related to desaturation and elongation of PCs synthesized in the liver.

Mild cold exposure may not only affect lipid-associated but also other metabolites. We, therefore, also assessed serum levels of amino acids and acylcarnitines. The amino acids glutamine, glycine and histidine were found to be increased upon two hours of mild cooling. This is likely to be explained by enhanced release by organs with concomitant decreased clearance, although the underlying biological mechanism remains to be determined. Surprisingly, during mild cooling, serum levels of the short-chain acylcarnitines C3 and C4 decreased. Acylcarnitines are involved in FA oxidation, as they are part of the carnitine shuttle which shuttles FA into mitochondria (14-16). In case of inefficient FA oxidation, *e.g.* when FA supply exceeds FA oxidation, acylcarnitines accumulate in the serum. During mild cold exposure BAT becomes activated, which increases the need for FAs. Possibly the carnitine shuttle becomes more efficient during mild cooling to facilitate the increased need for FAs thereby reducing acylcarnitines in plasma. Apparently, despite increasing the supply of FA from WAT, mild cooling does not result in inefficient FA oxidation, which is likely to be explained by enhanced β oxidation, *e.g.* due to enhanced BAT activity. Interestingly, recent research suggests an association between elevated levels of serum acylcarnitines and insulin resistance, possibly due to the fact that acylcarnitines activate pro-inflammatory pathways thereby facilitating the development of insulin resistance (17-19). In this respect, the decreased serum levels of C3 and C4 upon cold exposure may thus be considered beneficial.

We found no changes between white Caucasian and South Asian subjects in their response of serum metabolite levels upon mild cooling. A priori, we anticipated that the metabolic response would differ since we previously found in this study population that upon mild cold exposure the increase of serum FA was lower in South Asian as compared to white Caucasian subjects. Moreover, we found that mild cold exposure did not significantly increase energy expenditure in South Asians while it did in white Caucasians. However, mean water inlet temperature differed 2°C upon start of shivering between South Asians and white Caucasians and this might at least in part have influenced our results. However, we chose to use the method of a personalized cooling protocol to equalize NST, since muscle shivering markedly affects serum metabolites. Interestingly, these differences in mild-cold induced thermogenesis were not reflected

in differential changes in serum metabolites as measured by the Biocrates platform. Therefore, it seems that changes in these metabolites are, at least in part, independent of the extent of the increase in energy expenditure.

In conclusion, we found that mild cooling elicits substantial effects on serum metabolite levels in healthy male subjects, which might provide novel insights into the contribution of mild-cold induced thermogenesis on whole body substrate release and utilization. These findings were irrespective of white Caucasian versus South Asian ethnicity. Future studies should investigate if these data also apply to other populations, *e.g.* subjects with obesity or type 2 diabetes.

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SUPPLEMENTAL APPENDIX

Supplementary table S1. The complete listings of abbreviations of all measured metabolic parameters can be found at:

<https://ars.els-cdn.com/content/image/1-s2.0-S0003986115300527-mmc1.pdf>

Supplementary table S2. The complete listings of results for all measured metabolic parameters can be found at:

<https://www.sciencedirect.com/science/article/pii/S0003986115300527?via%3Dihub>

PART 2

**Novel BAT-targeted pharmacological
strategies to combat metabolic disease**

CHAPTER 6

The effect of mirabegron on energy expenditure and brown adipose tissue in healthy lean South Asian and white Caucasian men

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ABSTRACT

Activation of brown adipose tissue (BAT), either indirectly via sympathetic nervous system activation through cold exposure or directly by β 3-adrenergic receptor agonism, is considered a promising treatment strategy to combat metabolic disease. Metabolic disease is more prevalent in South Asians compared to white Caucasians, possibly linked to lower sympathetic outflow. Our aim was to compare the effects of cold exposure and mirabegron on BAT activation and energy expenditure in South Asians versus white Caucasians. Ten healthy lean Dutch South Asian (aged 18–30 years; BMI 18–25 kg/m²) and ten age- and BMI-matched white Caucasian men participated in a randomized, double-blinded, cross-over study consisting of three interventions; cold exposure, mirabegron (200 mg one dose p.o.) and placebo. Before and after each intervention, fasting serum lipids, glucose and insulin levels were measured. Additionally resting energy expenditure (REE), substrate utilisation, skin temperature and supraclavicular adipose tissue volume and fat fraction (FF) were assessed. Cold exposure and mirabegron increased free fatty acids, REE, lipid oxidation and supraclavicular skin temperature and tended to decrease FF of BAT. Aside from a larger cold-induced increase in free fatty acids in white Caucasians, all other effects were similar for South Asians and white Caucasians. Cold exposure and mirabegron both induce beneficial metabolic effects that are associated with increased BAT activity. There are no clear differences in response between South Asians and White Caucasians.

INTRODUCTION

Obesity and related diseases, including type 2 diabetes (T2D) and cardiovascular disease, are a major public health problem worldwide (1). Certain ethnic subgroups, such as the South Asian population, (2-4), are particularly vulnerable to develop cardiometabolic disease. This is likely, at least in part, due to their disadvantageous metabolic profile, consisting of susceptibility to develop abdominal obesity, dyslipidaemia and insulin resistance (2,3). The underlying mechanisms that explain this susceptibility are not fully understood. As a result, treatment options to improve the metabolic profile of the South Asian population are limited and unfocussed, and specific strategies are needed.

Activation of brown adipose tissue (BAT) could be an interesting therapeutic target to improve metabolism. BAT takes up triglyceride (TG)-derived fatty acids (FA) (5) and glucose from the systemic blood supply for combustion into heat, thereby increasing energy expenditure and improving lipid and glucose metabolism (6,7). BAT is strongly innervated by the sympathetic nervous system. Cold exposure, resulting in sympathetic nervous system activation, is the most potent physiological activator of BAT, resulting in release of noradrenalin from sympathetic nerve endings (8) that subsequently acts on β 3-adrenergic receptors (β 3-AR) on brown adipocytes thereby enhancing thermogenesis (9-11). Simultaneously, BAT releases endocannabinoids, which are believed to inhibit noradrenalin signalling, thereby preventing excessive activation of BAT by cold (12,13). Circulating endocannabinoid levels are elevated in obesity (14-16) and, interestingly, South Asians have higher basal circulating endocannabinoid levels compared to white Caucasians (17). This might, at least partly, explain the reduced ^{18}F -fluorodeoxyglucose (^{18}F -FDG) uptake by BAT that was shown in one PET study and lower resting energy expenditure (REE) during cold exposure (18). In addition, South Asians have a lower cold-induced increase in free fatty acid (FFA) levels, which results from lipolysis induced by sympathetic stimulation of white adipose tissue (18). Taken together, these data suggest that South Asians have a lower sympathetic outflow upon cold exposure compared to white Caucasians.

Repetitive cold exposure is an effective strategy to enhance BAT metabolism, as cold acclimation increases BAT volume and reduces fat mass in both lean and obese men (19). However, since a treatment involving prolonged cold exposure may be hard to adhere to, current research is focussed on pharmacological compounds that can activate BAT. As BAT activation by cold is considered to occur via sympathetic stimulation of the β 3-AR, agonists of this receptor might be of interest. Indeed, pre-clinical studies have shown that treatment with the selective β 3-AR agonist CL316,243 strongly stimulates BAT activity, prevents fat accumulation, improves dyslipidemia and insulin sensitivity, and attenuates the development of atherosclerosis (20). Likewise, in humans, the β 3-AR agonist mirabegron increased ^{18}F -FDG uptake by BAT as well as increased REE (21) in

healthy young men to a similar extent as cold exposure (22). In addition, a single dose of 200 mg mirabegron increased plasma FFA levels, which is suggestive for stimulation of lipolysis in white adipose tissue (21). Therefore, β 3-AR agonism could be a promising treatment option to activate BAT and enhance REE, especially in South Asians, because it directly activates the β 3-AR, thereby circumventing sympathetic outflow.

The aim of the current study was to study the effect of the β 3-AR agonist mirabegron and cold exposure on REE and BAT activity in healthy lean South Asian versus white Caucasian men. We hypothesized that South Asians would respond better to direct activation of BAT by targeting the β 3-AR using mirabegron, compared to indirect activation through the sympathetic nervous system by cold exposure.

MATERIALS AND METHODS

Participants

Ten healthy, young (aged 18–30 years), lean (BMI 18–25 kg/m²) Dutch South Asian men and ten age- and BMI-matched white Caucasian men were included in the study. South Asian descent was defined as born in the Netherlands with both grandparents originating from the Indian subcontinent. Exclusion criteria were smoking, recent weight change (> 3 kg within the last 3 months), rigorous exercise, use of any medication known to influence glucose and/or lipid metabolism, BAT activity, cardiac function or QT interval time (*e.g.* beta blockers, thyroid medication, calcium channel blockers, monoamine oxidase inhibitors, or systemic corticosteroids), the presence of a chronic disease (including, but not limited to T2D, thyroid disease, renal disease, or liver dysfunction), and/or contraindications for magnetic resonance imaging (MRI). Contraindications for undergoing an MRI scan were the presence of non-MR safe metal implants or objects in the body (*i.e.* a pacemaker, neurostimulator, hydrocephalus or drug pump, non-removable hearing aid, or large recent tattoos), a history of claustrophobia, tinnitus, or hyperacusis. The study was approved by the Medical Ethical Committee of the Leiden University Medical Center (LUMC) and performed in accordance with the principles of the revised Declaration of Helsinki (23). Written informed consent was obtained from all volunteers prior to participation. Trial register clinicaltrial.gov number NCT03012113.

Study design

Participants were enrolled in a randomized, double-blinded, placebo-controlled cross-over study consisting of three different interventions that were each performed on a different study day. During the first occasion, participants were exposed to an individualized water-cooling protocol to activate BAT as previously described (18). Supraclavicular fat volume and fat fraction (FF) were assessed with MRI. As BAT combusts intracellular

lipids (20), intracellular FF of BAT is expected to decrease upon activation. Therefore, quantification of the FF of BAT is considered to be a read-out for its activity. Only if BAT could be detected on MRI after cold exposure, participants continued with drug and placebo treatment. Thereafter, participants were randomised to receive first either 200 mg mirabegron (Betmiga®, Astellas BV, the Netherlands) in one oral dose or placebo. Mirabegron tablets were repacked into capsules by the LUMC pharmacy to resemble the placebo. Randomisation was performed by the LUMC pharmacy. After the cold exposure, there was a wash-out period of at least one week, and the wash-out period between the mirabegron and placebo occasion was at least two weeks. An overview of the study design is depicted in **Supplementary Figure 1**. Before all study days, subjects were fasted for 10 hours overnight and remained fasted until the end of the experiment. A standardized dinner was consumed the evening before and participants wore standardized clothing consisting of a boxer short and a thin pair of MRI-compatible trousers and hospital gown.

Occasion 1: Cold exposure

During the first visit, a medical screening consisting of a medical questionnaire, measurement of length, weight and blood pressure, and a blood draw was performed to assess if participants met the inclusion criteria. In case of eligibility, the study day continued with insertion of an intravenous cannula and a measurement of body composition by bio-electrical impedance analysis (Bodystat 1500, Bodystat, UK). Pre-cooling (thermoneutral conditions), a fasted blood sample was collected, REE, lipid and glucose oxidation were measured via indirect calorimetry (Oxycon Pro, CareFusion, Germany), and cardiovascular parameters (including heart rate, blood pressure, and heart rate variability (HRV)) were assessed with Finapres Nova (Finapres Medical Systems BV, the Netherlands). Thereafter, a pre-cooling MRI scan (3T MRI, Philips Ingenia, Philips Healthcare, Best, the Netherlands) was performed to assess supraclavicular adipose tissue volume and FF. Participants were placed in supine position head-first in the scanner, with a 16-channel anterior array on the pelvis and their head in the 16-channel head and neck coil. After being placed on the scanner table, participants were asked to reach as far as possible with their fingers towards their feet and to relax their shoulders afterwards to ensure reproducibility of subject positioning. The left supraclavicular BAT depot was assessed using a three-dimensional six-point chemical-shift encoded gradient-echo acquisition with the following parameters: repetition time TR=15 ms, first echo time TE=1.98 ms, echo time separation $\Delta TE=1.75$ ms, flip angle=8°, field-of-view of 480 mm × 300 mm × 90 mm (Right-Left, Foot-Head, Anterior-Posterior), 1.1 mm isotropic resolution, four retrospectively averaged signal averages. Next, 18 wireless iButtons were placed to monitor skin temperature (iButton®, Maxim Integrated Products, USA), and an individualized water cooling protocol was applied to activate BAT (as described previously (18)).

Participants were placed in a bed in semi-supine position between two water-perfused mattresses (BlanketRol® III, Cincinnati Sub-Zero Products, USA). The water temperature was set at 32°C and gradually decreased with 5°C every 10 minutes, until the participants started to shiver or until they reached the minimum water temperature of 9°C. Shivering was reported by the participants and confirmed visually by researchers. At that point, the water temperature was increased with 3°C to ensure maximal non-shivering thermogenesis. This cold exposure continued for 60 more minutes. Thereafter, a blood sample was obtained and cold-induced REE, lipid and glucose oxidation, and cardiovascular parameters were measured again. Lastly, a second MRI scan was performed to assess changes in supraclavicular adipose tissue after cold exposure.

Occasion 2 and 3: Mirabegron and placebo treatment

During these study days, all measurements were performed under thermoneutral conditions. After insertion of an intravenous cannula and measurement of body composition, a fasted blood sample was collected and REE, lipid and glucose oxidation, and cardiovascular parameters were assessed. Next, mirabegron or placebo was ingested. One hour ($t=60$), two hours ($t=120$), and three hours ($t=180$) after administration, REE, lipid and glucose oxidation, and cardiovascular parameters were again assessed. At three hours and a half ($t=210$), when reaching the maximum plasma concentration of mirabegron (*i.e.* $T_{max} \sim 3\text{--}4$ h), another blood sample was drawn and an MRI scan was performed to assess changes in supraclavicular adipose tissue.

Serum measurements

Commercially available enzymatic kits were used to measure serum concentrations of TG and total cholesterol (TC) (Roche Diagnostics, the Netherlands), high density lipoprotein-cholesterol (HDL-C) (Roche Diagnostics), FFA (Wako Chemicals, Germany), and glucose (Instruchemie, the Netherlands). Insulin concentrations were measured using ELISA (Crystal Chem Inc., IL, USA). Low density lipoprotein-cholesterol (LDL-C) was calculated using the Friedewald equation (24). Data were analysed using SoftMaxPro 5.4.1 software.

Skin temperature

18 wireless iButton temperature sensors were placed at 14 prescribed ISO-defined positions (25) and 4 additional positions (right hand, left lower leg, left elbow, and right armpit) (26). Data were analysed using Temperatus software (<http://profith.ugr.es/temperatus>). Armpit temperature was estimated as a proxy of core body temperature (27). Supraclavicular skin temperature was estimated from an iButton placed above the left clavicle. Distal skin temperature was calculated as the average temperature of the left hand and right feet (28). Proximal skin temperature was defined as the average

of the iButtons on the chest, abdomen, scapula, and lower back (29). The difference in skin temperature between the right forearm and the right fingertip was calculated as an estimate for vasoconstriction (30).

Indirect calorimetry

Indirect calorimetry was performed in time-frames of 30 minutes. Participants were instructed to lie still and were not allowed to talk. Oxygen consumption and carbon dioxide production were determined every minute. REE and the respiratory quotient (RQ) were calculated and substrate utilization was assessed by calculation of lipid and glucose oxidation after correction for protein oxidation, as described previously (31).

MRI analysis

An in-house water-fat separation algorithm based on the known frequencies of the multi-peak fat spectrum and assuming monoexponential effective transverse relaxation time ($T2^*$) was used to reconstruct FF maps, combined with a region-growing scheme to mitigate main field inhomogeneity effects (32–35). Registration was performed using Elastix registration software (36,37). For calculating the deformation field, the first echoes of the pre- and post-cooling image stacks were co-registered using a three-dimensional B-spline transform with a $10 \times 10 \times 10 \text{ mm}^3$ grid, adaptive stochastic gradient descent with two resolutions for optimization and Mattes mutual information as the similarity measure (the registration parameter file can be downloaded from <http://elastix.bigr.nl/wiki/index.php/Par0048>). Subsequently, FF and $T2^*$ maps were first transformed using the calculated deformation field and then smoothed using the 3×3 mean filter. Regions of interest (ROIs) encompassing the known location of the left supraclavicular BAT depot (38) were drawn manually by one observer (**Supplementary Figure 2**). Semi-automated segmentation rejecting voxels below the 3% FF was performed, and a FF threshold of 50–100% was used for data analysis. In this work, we set the lower threshold to 50% as it was shown to segment more non-fat areas compared with a lower threshold of 40% (39), which is commonly used in other studies. The change in volume and FF after cold exposure was estimated as the difference between before and after cooling. The change in volume and FF induced by mirabegron was estimated as the difference between post-placebo and post-mirabegron. One participant was excluded from the MRI analysis because of failure to reconstruct the scan due to excessive movement.

Heart rate variability, blood pressure and heart rate

Heart rate, blood pressure, and HRV were continuously non-invasively assessed with Finapres Nova (Finapres Medical Systems BV, the Netherlands) in time-frames of 30 minutes. A small cuff was connected to the intermediate phalanges of the middle-finger of the non-dominant hand to measure blood pressure. In addition, a 5-lead electrocar-

diagram was connected to assess heart rate and parameters of HRV. Participants were instructed to lie still and were not allowed to talk. Data was analysed with MATLAB release 2017b (The Mathworks, USA).

Statistical analysis

Data were analysed using IBM SPSS Statistics for Windows version 23.0 (SPSS Inc, Chicago, IL, USA). Data are shown as mean $\pm$ standard error of the mean (S.E.M.) Two-tailed unpaired Student's *t*-test was used to compare baseline characteristics between the two ethnicities. Two-tailed paired Student's *t*-test was used to assess the effects of the interventions within a participant and to compare effect sizes (*e.g.* delta before and after cooling, delta before and after mirabegron, and delta before and after placebo) between the three interventions within one ethnicity. Two-tailed unpaired Student's *t*-test was used to compare effect sizes between South Asians and white Caucasians. When data were not normally distributed, a non-parametric test was used. A *p*-value < 0.05 was considered statistically significant.

RESULTS

Participant characteristics

Ten healthy lean men of white Caucasian and ten of South Asian descent participated in the study. Participant characteristics are summarized in **Table 1**. South Asian and white Caucasian participants were comparable with respect to age (24.4 $\pm$ 1.0 vs. 22.9 $\pm$ 0.7 years) and BMI (22.7 $\pm$ 0.6 vs. 22.3 $\pm$ 0.3 kg/m²). However, South Asians were shorter (1.77 $\pm$ 0.01 vs. 1.86 $\pm$ 0.02 m, *p*<0.01) and tended to have a lower body weight (71.5 $\pm$ 2.4 vs. 77.7 $\pm$ 1.9 kg, *p*=0.06) compared to white Caucasians. In addition, body fat percentage was higher in South Asians (16.7 $\pm$ 1.2 vs. 12.9 $\pm$ 0.8 %, *p*<0.05) and percentage of lean body mass was lower (83.3 $\pm$ 1.2 vs. 87.1 $\pm$ 0.8 %, *p*<0.01). Fasting glucose, insulin, and lipid levels were comparable between the groups, except for LDL-C levels that were higher in South Asians compared to white Caucasians (4.3 $\pm$ 0.4 vs. 3.1 $\pm$ 0.4 mmol/L, *p*=0.05). MRI studies identified deposits of BAT in all participants and therefore, all participants could continue with mirabegron and placebo administration.

Mirabegron increases serum FFA and insulin levels in both ethnicities

Since active BAT takes up lipids and glucose from plasma, we first compared the effect of cold exposure and mirabegron on serum parameters in both ethnicities.

Cold exposure increased TC (+15%, *p*<0.05) in white Caucasians only (Figure 1A). This was mainly due to an increase in HDL-C in both ethnicities (+11%, *p*<0.01 for South Asians and +9%, *p*<0.05 for white Caucasians). Upon cooling, TG tended to increase in

South Asians (+8%, $p=0.08$) and white Caucasians (+13%, $p=0.06$) (**Figure 1B**). In addition, serum FFA increased in white Caucasians (+52%, $p<0.001$) and tended to increase in South Asians (+18%, $p=0.06$) (**Figure 1C**). Serum glucose (not shown) and insulin (**Figure 1D**) levels were not affected by cold exposure in both ethnicities. Interestingly, the increase in FFA upon cold exposure was lower in South Asians compared to white Caucasians ($p<0.05$; **Figure 1C**).

Mirabegron did not affect TC (**Figure 1A**) or TG (**Figure 1B**) nor HDL-C, LDL-C, and glucose (not shown). Serum FFA increased after mirabegron in both South Asians (+131%, $p<0.001$) and white Caucasians (+199%, $p<0.001$) to a similar extent. In both ethnicities the effect of mirabegron on FFA levels was larger than the effect of cold exposure (both $p<0.01$) (**Figure 1C**). Serum insulin levels were similarly increased upon mirabegron treatment in South Asians (+31%, $p<0.01$) and white Caucasians (+23%, $p<0.05$) (**Figure 1D**).

Placebo did not affect TC levels (**Supplementary Figure 3A**). Placebo decreased TG levels in white Caucasians only (-7%, $p<0.05$; **Supplementary Figure 3B**) and increased FFA levels in both South Asians (+55%, $p<0.01$) and white Caucasians (+48%, $p<0.05$) (**Supplementary Figure 3C**). In both ethnicities the effect of mirabegron on FFA levels was larger than the effect of placebo (both $p<0.001$; not shown). In addition, placebo decreased insulin in white Caucasians (-24%, $p<0.05$) and tended to decrease insulin in South Asians (-29%, $p=0.07$) (**Supplementary Figure 3D**). The responses to placebo did not differ between the two ethnicities.

Table 1. Participant characteristics

	White Caucasians (n=10)	South Asians (n=10)
Age (years)	22.9 (0.7)	24.4 (1.0)
Height (m)	1.86 (0.02)	1.77 (0.01) **
Weight (kg)	77.7 (1.9)	71.5 (2.4) ^b
Body mass index (kg/m ²)	22.3 (0.3)	22.7 (0.6)
Waist circumference (cm)	82.1 (1.8)	78.2 (1.6)
Hip circumference (cm)	86.7 (1.5)	86.1 (1.7)
Fat mass (%)	12.9 (0.8)	16.7 (1.2) *
Lean mass (%)	87.1 (0.8)	83.3 (1.2) *
Glucose (mmol/L)	4.5 (0.1)	4.6 (0.1)
Insulin (pg/mL)	126 (19)	203 (58)
Free fatty acids (mmol/L)	0.43 (0.05)	0.48 (0.04)
Triglycerides (mmol/L)	0.79 (0.17)	0.87 (0.23)
Total cholesterol (mmol/L)	4.8 (0.5)	6.0 (0.4) ^c
High density lipoprotein-cholesterol (mmol/L)	1.4 (0.1)	1.2 (0.9)
Low density lipoprotein-cholesterol (mmol/L)	3.1 (0.4)	4.3 (0.4) ^a

Values are presented as mean (standard error of the mean (S.E.M.)). Two-tailed unpaired student's *t*-test was used for comparison. ** $p<0.01$, * $p<0.05$, ^a $p=0.05$, ^b $p=0.06$, ^c $p=0.10$ South Asians vs. White Caucasians.

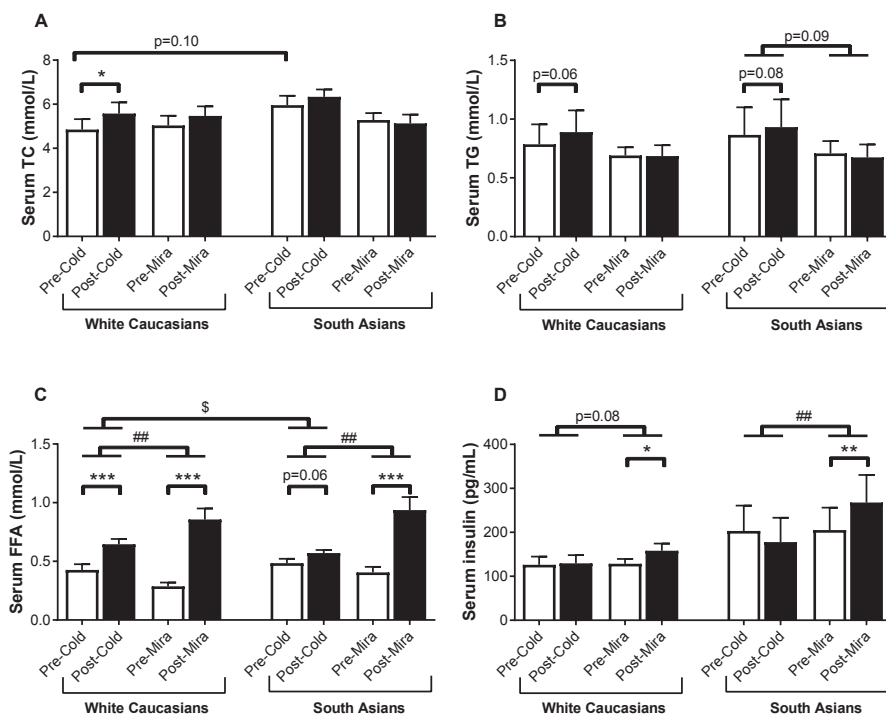


Figure 1. Effect of cold exposure and mirabegron on serum lipids and insulin in white Caucasians and South Asians Serum was collected before (white bars) and after (black bars) intervention with cold exposure or mirabegron in white Caucasians ($n=10$) and South Asians ($n=10$), and assayed for total cholesterol (TC) (A), triglycerides (TG) (B), free fatty acids (FFA) (C), and insulin (D). Data are presented as mean $\pm$ S.E.M. Two-tailed paired (within ethnicities) or unpaired (between ethnicities) Student's *t*-test was used for comparison. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ before vs. after intervention. ## $p<0.01$ effect of cooling vs. mirabegron. \$ $p<0.05$ difference between the two ethnicities. Mira, mirabegron.

Mirabegron increases REE and lipid oxidation in both ethnicities

As BAT can influence energy expenditure and substrate use, we next compared the effect of cold exposure and mirabegron on REE, lipid and glucose oxidation in South Asians and white Caucasians.

Pre-cooling REE was lower in South Asians compared to white Caucasians (1347 ± 46 vs. 1563 ± 66 kcal/day, $p<0.05$), while lipid oxidation, glucose oxidation, and respiratory quotient (RQ) were comparable (not shown). Of note, the ethnic differences in REE were no longer present after correction for lean body mass (not shown).

Cold exposure increased REE in both South Asians (+29%; $p<0.05$) and white Caucasians (+20%; $p<0.001$) (Figure 2A,B). In addition, lipid oxidation was increased in South Asians (+106%; $p<0.05$) and white Caucasians (+80%, $p<0.01$) (Figure 2C,D), in line with decreased RQ in both ethnicities (-3%, $p<0.01$ and -4%, $p<0.05$, respectively) (not shown). Glucose oxidation was not affected by cooling (not shown). The increases in REE and lipid oxidation upon cold were not different between the two ethnicities.

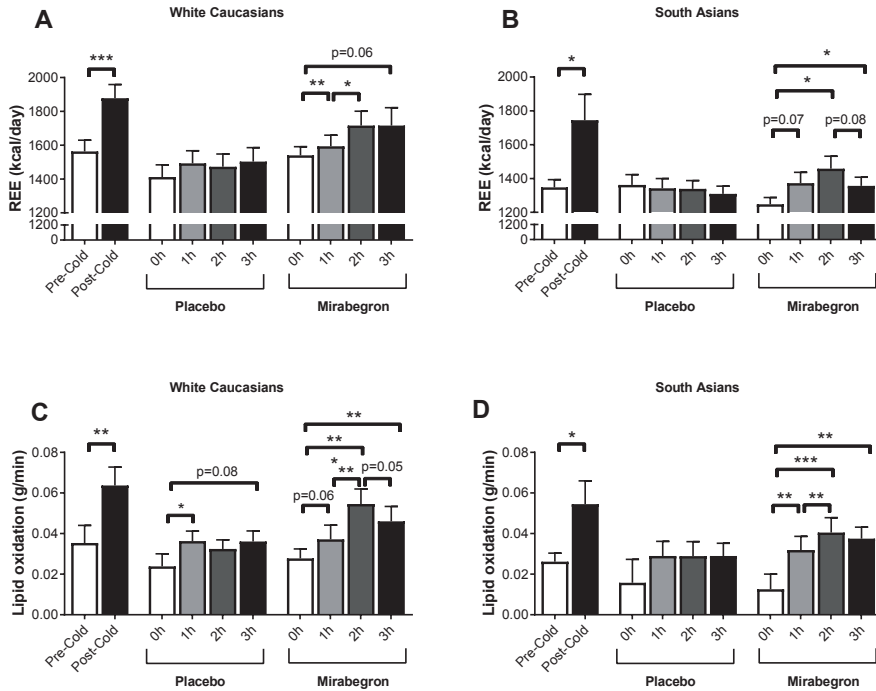


Figure 2. Effect of cold exposure, placebo, and mirabegron on energy expenditure and lipid oxidation in white Caucasians and South Asians Before (pre-Cold; white bars) and after (post-Cold; black bars) cold exposure and before (white bars), after one hour (light grey bars), after two hours (dark grey bars), and after 3 hours (black bars) of placebo or mirabegron treatment, indirect calorimetry was used to measure resting energy expenditure (REE) (**A,B**) and lipid oxidation (**C,D**) in white Caucasians (**A,C**) ($n=10$) and South Asians (**B,D**) ($n=10$). Data are presented as mean $\pm$ S.E.M. Two-tailed paired (within ethnicities) or unpaired (between ethnicities) Student's *t*-test was used for comparison. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ before vs. after intervention.

When comparing the baseline measurement with the measurement three hours after administration of mirabegron, mirabegron increased REE in South Asians (+9%, $p<0.05$; **Figure 2B**) and tended to increase in white Caucasians (+11%, $p=0.06$; **Figure 2A**). Mirabegron increased lipid oxidation in South Asians (+199%, $p<0.01$) as well as white Caucasians (+66%, $p<0.01$) (**Figure 2A,B**). Consequently, RQ decreased in South Asians (-7%, $p<0.05$) and white Caucasians (-4%, $p<0.01$) (not shown).

Placebo did not change REE (**Figure 2A,B**) or glucose oxidation in either ethnicity, but tended to increase lipid oxidation in white Caucasians only (+51%, $p=0.08$; **Figure 2C**).

The increase in REE and lipid oxidation upon mirabegron becomes even more clear when looking at the gradual change in REE and lipid oxidation after 1, 2, and 3 hours after administration of mirabegron or placebo. A clear increase was observed in both ethnicities upon mirabegron treatment, while this was absent upon placebo treatment (Figure 2A-D).

When comparing effect sizes, cold exposure increased REE to a higher extent compared to mirabegron in white Caucasians ($p < 0.05$) and tended to increase REE to a higher extent compared to mirabegron in South Asians ($p = 0.10$). The effects of cold exposure, mirabegron or placebo on lipid or glucose oxidation were not significantly different.

Mirabegron increases supraclavicular skin and core temperature in both ethnicities

The main function of BAT is heat production, and supraclavicular skin temperature positively associates with ^{18}F -FDG uptake by BAT in young healthy lean men (40). Therefore, we next compared the effects of cold exposure and mirabegron on skin and core temperature in South Asians and white Caucasians.

Pre-cooling skin temperature in the armpit (a proxy of core temperature) tended to be higher in South Asians compared to white Caucasians (35.3 ± 0.2 vs. 34.6 ± 0.3 °C, $p = 0.09$; **Figure 3A**), while supraclavicular skin temperature (**Figure 3B**), distal skin temperature, and proximal skin temperature were comparable (not shown). In addition, the difference in skin temperature between the right forearm and the right fingertip (*i.e.* estimate for vasoconstriction) tended to be lower in South Asians compared to white Caucasians (3.8 ± 0.8 vs. 5.7 ± 0.9 °C, $p = 0.08$; not shown).

Cold exposure increased core temperature in South Asians ($+1.2$ °C, $p < 0.001$) and white Caucasians ($+1.1$ °C, $p < 0.01$) (**Figure 3A**). Likewise, supraclavicular skin temperature was increased in South Asians ($+1.7$ °C, $p < 0.001$) and white Caucasians ($+1.6$ °C, $p < 0.001$) (**Figure 3B**). Furthermore, cooling decreased distal skin temperature in South Asians (-3.1 °C, $p < 0.01$) and white Caucasians (-2.4 °C, $p < 0.01$) and proximal skin temperature (-4.9 °C, $p < 0.001$ and -3.2 °C, $p < 0.001$, respectively) (not shown). The estimate for vasoconstriction was increased in both South Asians ($+67\%$, $p < 0.05$) and white Caucasians ($+51\%$, $p < 0.05$) (not shown).

Mirabegron also increased core temperature in South Asians ($+0.3$ °C, $p < 0.01$) and white Caucasians ($+0.6$ °C, $p < 0.05$) (**Figure 3A**). Furthermore, mirabegron increased supraclavicular skin temperature in both white Caucasians ($+0.4$ °C, $p < 0.05$) and South Asians ($+0.7$ °C, $p < 0.01$) (**Figure 3B**). In contrast to cold exposure, mirabegron increased rather than decreased proximal skin temperature in South Asians ($+1.4$ °C, $p < 0.01$) and white Caucasians ($+1.2$ °C, $p < 0.001$) (not shown). Mirabegron did not affect distal skin temperature and vasoconstriction (not shown).

The placebo did not change core temperature (**Supplementary Figure 4A**) or supraclavicular temperature (**Supplementary Figure 4B**), but increased proximal skin temperature in South Asians ($+1.1$ °C, $p < 0.01$) and white Caucasians ($+0.9$ °C, $p < 0.01$) (not shown).

When comparing effect sizes, the increase in supraclavicular skin temperature was larger upon cold exposure compared to mirabegron in both ethnicities ($p < 0.01$; **Figure 3B**). In South Asians, the effect of cold exposure on core temperature was larger than

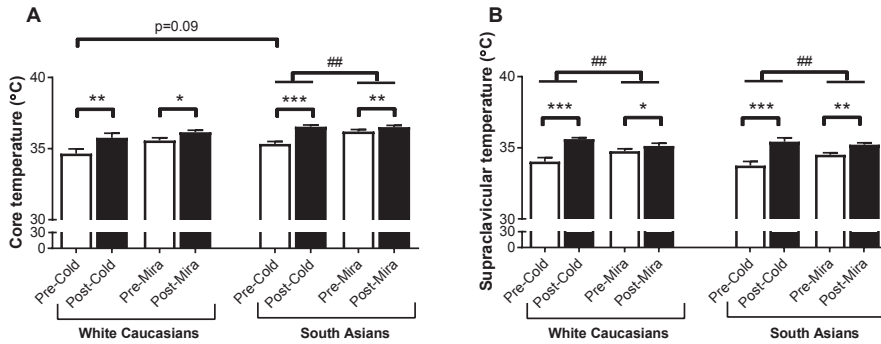


Figure 3. Effect of cold exposure and mirabegron on supraclavicular skin and core temperature in white Caucasians and South Asians Before (white bars) and after (black bars) intervention with cold exposure or mirabegron, core temperature (A) and supraclavicular skin temperature (B) were assessed in white Caucasians ($n=10$) and South Asians ($n=10$). Data are presented as mean $\pm$ S.E.M. Two-tailed paired (within ethnicities) or unpaired (between ethnicities) Student's *t*-test was used for comparison. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ before vs. after intervention. ## $p<0.01$ effect of cooling vs. mirabegron. Mira, mirabegron.

the effect of mirabegron ($p<0.01$; **Figure 3A**). The effects on supraclavicular skin and core temperature upon cold exposure or mirabegron were not different between South Asians compared to white Caucasians.

Mirabegron tends to decrease BAT fat fraction in white Caucasians

As BAT combusts intracellular lipids (20), quantification of the FF of the supraclavicular fat depot by MRI after an intervention is considered to be a read-out for its activity. Therefore, we next compared the effect of cold exposure, mirabegron and placebo on BAT volume and FF using MRI in both ethnicities.

Pre-cooling BAT volume (23.9 ± 3.7 vs. 27.9 ± 2.4 mL; **Figure 4A**) and FF (70.7 ± 1.7 vs. $69.8\pm1.3\%$; **Figure 4B**) were comparable between South Asians and white Caucasians.

Cold exposure decreased BAT volume in both South Asians (-11% , $p<0.001$) and white Caucasians (-17% , $p<0.01$) (**Figure 4A**). However, it has to be noted that after placebo BAT volume in South Asians was comparable to after cold exposure. In addition, cold exposure decreased FF in white Caucasians (-2.2% , $p<0.05$) and tended to decrease FF in South Asians (-1.0% , $p=0.08$) (**Figure 4B**).

Mirabegron tended to decrease BAT FF (-1.0% , $p=0.08$) compared to placebo treatment in white Caucasians only (**Figure 4B**), while BAT volume was unchanged (**Figure 4A**).

When comparing effect sizes, the effect of cold exposure on BAT volume tended to be larger in white Caucasians compared to South Asians ($p=0.09$) (**Figure 4A**). Furthermore, the decrease in BAT volume upon cold exposure was larger compared to the decrease between mirabegron and placebo ($p<0.01$; **Figure 4A**) in white Caucasians only. There was no difference in effect size of BAT FF upon cold exposure, mirabegron, or placebo between South Asians and white Caucasians.

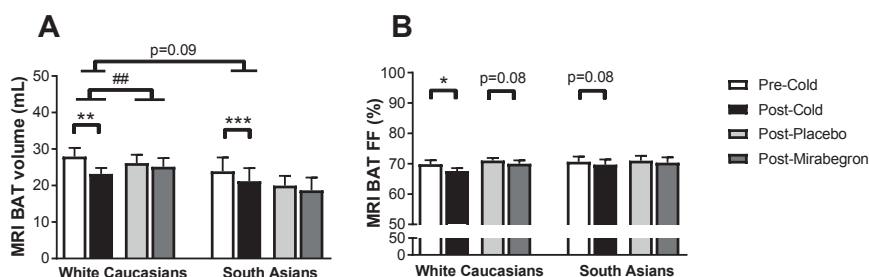


Figure 4. Effect of cold exposure and mirabegron on BAT volume and FF in white Caucasians and South Asians

Magnetic resonance imaging was used to determine brown adipose tissue (BAT) volume (**A**) and fat fraction (FF) (**B**), pre-cooling (white bars), after cold exposure (black bars), after placebo treatment (light grey bars), and after mirabegron treatment (dark grey bars) in white Caucasians ($n=9$) and South Asians ($n=10$). MRI data of one white Caucasian could not be reconstructed due to excessive movement in the scanner. A lower threshold of 50% was applied to determine fat fraction. Data are presented as mean $\pm$ S.E.M. Two-tailed paired (within ethnicities) or unpaired (between ethnicities) Student's t -test was used for comparison. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ before vs. after intervention. ## $p<0.01$ effect of cooling vs. mirabegron. \$ $p<0.05$ difference between the two ethnicities. Mira, mirabegron.

Mirabegron increases heart rate and decreases vagal components of heart rate variability

Since β_3 -ARs are also present on cardiomyocytes (41) and endothelial cells of arteries (42), we also investigated the effects of mirabegron on heart rate, blood pressure, and measures of HRV as measures of sympathetic and parasympathetic output.

Pre-cooling measurements for cardiovascular parameters were comparable between the two ethnicities (not shown).

Cold exposure decreased heart rate in white Caucasians (-2 beats/min, $p<0.01$) and tended to decrease heart rate in South Asians (-1 beat/min, $p=0.10$) (**Supplementary Table 1**). In addition, cooling increased systolic ($+9\%$, $p<0.05$) and diastolic ($+22\%$, $p<0.05$) blood pressure in South Asians only (**Supplementary Table 1**). Cooling increased RR interval in white Caucasians (RRI; $+5\%$, $p<0.01$) and tended to increase RR interval in South Asians ($+3\%$, $p=0.08$) (**Supplementary Table 1**). In South Asians, the Root Mean Square of Successive heartbeat interval Differences (rMSSD; *e.g.* estimate of vagal activity) was increased ($+44\%$, $p<0.05$; **Supplementary Table 1**). In addition, in South Asians cooling tended to increase both low frequency component (LF; *e.g.* estimate of sympathetic activity) ($+49\%$, $p=0.06$) and the high frequency component (HF; *e.g.* estimate of vagal activity) ($+89\%$, $p=0.08$) (**Supplementary Table 1**). LF/HF ratio was not affected.

Mirabegron increased heart rate in South Asians ($+10$ beats/min, $p<0.01$) and white Caucasians ($+7$ beats/min, $p<0.001$), while systolic or diastolic blood pressure were not changed (**Supplementary Table 1**). Mirabegron decreased RR interval in South Asians (-17% , $p<0.001$) and white Caucasians (-11% , $p<0.001$). Likewise, mirabegron decreased

rMSSD (*e.g.* estimate of vagal activity) in South Asians (-36%, $p<0.05$) and white Caucasians (-29%, $p<0.05$). HF (*e.g.* estimate of vagal activity) was also decreased in South Asians (-49%, $p<0.05$) and tended to decrease in white Caucasians (-48%, $p=0.07$) (**Supplementary Table 1**). LF/HF ratio was not affected.

Placebo only tended to increase heart rate (+2 beats/min, $p=0.06$) and increased LF component (*e.g.* estimate of sympathetic activity) (+53%, $p<0.05$; **Supplementary Table 1**) in South Asians but not in white Caucasians.

DISCUSSION

Activation of BAT, either indirectly via the sympathetic nervous system by cold exposure or directly by β_3 -adrenergic receptor agonism, is considered a treatment strategy to combat metabolic disease. Metabolic disease is more prevalent in South Asians compared to white Caucasians, which is possibly linked to lower sympathetic outflow. In the current study, we investigated the effect of BAT activation by cold exposure and the β_3 -adrenergic receptor agonist mirabegron on serum lipids, REE, glucose and lipid oxidation, supraclavicular skin temperature, and BAT volume and FF in healthy lean South Asians versus white Caucasians. Here we report that both mirabegron and cold exposure increase serum FFA levels, REE, lipid oxidation and supraclavicular skin temperature and tended to decrease BAT FF in both ethnicities. This indicates that pharmacological activation of BAT may be a potential strategy to combat obesity not only in white Caucasians, but also in South Asians, a population that is especially prone to develop cardiometabolic disease.

We hypothesized that the response to cold exposure and mirabegron would be different between South Asians and white Caucasians. Since South Asians have a lower FFA response upon cold exposure (18) and higher circulating endocannabinoid levels (43), we reasoned that they have lower cold-induced sympathetic outflow compared to white Caucasians. This may be the reason why cold-induced ^{18}F -Fluorodeoxyglucose uptake (^{18}F -FDG) by BAT in terms of volume is lower in South Asians (18). Since mirabegron is thought to activate BAT directly through stimulation of β_3 -AR, we thus expected a more pronounced effect of mirabegron on REE and BAT in South Asians by circumventing sympathetic activation. Indeed, although the FFA response to cold was lower in South Asians, similar responses were observed after administration of mirabegron to South Asians compared to white Caucasians. Nor was there a difference in any of the HRV parameters that indicate a difference in sympathetic output between the two ethnicities. Clearly, additional future studies are needed to clarify whether there is a true difference in sympathetic output upon cooling between South Asians and white Caucasians.

In the current study, we compared the effects of mirabegron with cold exposure in South Asians and white Caucasians. We showed that mirabegron increased FFA levels to a greater extent than cold exposure in both ethnicities. Although placebo treatment also increased FFA levels, suggesting an effect of prolonged fasting on serum FFA, the increase in FFA levels after cold and mirabegron was larger than placebo. A possible explanation for the higher effect of mirabegron on FFA levels is that mirabegron more effectively liberates FFA from white adipose tissue instead of stimulating FFA uptake or combustion (e.g. by BAT) compared to cold exposure. This would be in line with a smaller effect of mirabegron on the decrease in BAT volume compared to cold exposure, at least in white Caucasians, and a lower increase in REE upon mirabegron compared to cold exposure in both ethnicities. High FFA levels can inhibit glucose uptake and combustion, thereby elevating blood glucose and insulin levels, which eventually can result in insulin resistance and T2D (44-46). For this reason, it is desirable that FFA liberation and combustion are induced to the same extent, thereby preventing accumulation of FFA in the blood. It would be interesting to study the effect of mirabegron in combination with a treatment that further stimulates FFA combustion (e.g. induces a stronger activation of BAT), to reveal potentially beneficial effects on blood lipids in the short time frame that was used in our study. However, it might be expected that after prolonged therapy FFA liberation and combustion will be ultimately balanced.

In addition, we observed that mirabegron, in contrast to cold exposure, increased serum insulin levels. This is in line with data of Cypess et al. (22), who also found increased insulin levels upon administration of the same dose of mirabegron in healthy lean volunteers. Based on preclinical studies (47), we speculate that this rise in insulin might be a consequence of the action of mirabegron on β 3-AR on the pancreas. Stimulation of β 3-AR on blood vessels in the pancreas might induce local vasodilatation resulting in increased blood flow (47), and thus increased supply of glucose and FA to β -cells, thereby stimulating insulin release. Insulin stimulates the activity of lipoprotein lipase in adipose tissues (48). In addition, insulin increases glucose uptake by tissues due to increased translocation of glucose transporter type 4 (GLUT4) to the cell membrane (49). In this way, increased insulin levels could contribute to increased uptake of TG-derived FA and glucose from the circulation by BAT to facilitate intracellular combustion. Alternatively, the increased ^{18}F -FDG uptake by BAT might also result from vasodilation within BAT due to binding of mirabegron on β 3-AR on the endothelium of arteries or due to cross-reactivity with other β -AR subtypes (42,50).

We also observed an increase in supraclavicular skin temperature upon mirabegron treatment, which probably reflects local heat production and thus BAT activity (40). Consistent with this observation, we found that mirabegron progressively increased REE and lipid oxidation, which was not observed after placebo treatment. In addition, when assessing BAT volume and FF with MRI, we observed a trend towards decreased BAT

FF upon mirabegron treatment compared to placebo. However, the decrease in FF was only half of the effect of cold exposure on BAT FF and only clear in white Caucasians. To further investigate to what extent mirabegron activates BAT, future studies should investigate BAT activity with other imaging modalities, such as ^{11}C -acetate to investigate the oxidative capacity of the tissue, among others.

We observed that mirabegron modestly increased the heart rate, which can possibly be explained by direct effect of the drug on β_3 -AR on the myocardium (41) and/or cross-reactivity of the drug on β_1 -AR (50). Despite the effect being modest, over the long-term, an increase of a few beats per minute already increases the risk of cardiovascular morbidity and mortality (51). Heart rate is controlled by the autonomic nervous system and reflects the balance between parasympathetic and sympathetic outflow of the central nervous system (52). When assessing HRV parameters, we observed a decrease in rMSSD and HF upon mirabegron treatment, which may point towards reduced vagal activity (53). This might suggest that stimulation of the β -adrenergic receptors by mirabegron shifts the autonomic balance towards more sympathetic and less parasympathetic activity. In line with this, it has been shown that β -blockers increase, rather than decrease, rMSSD and HF (54). However, HRV can be affected by many internal and external factors and the relationship between sympathetic and parasympathic branches is complex and dynamic (53), therefore caution must be applied when interpreting HRV parameters.

A positive feature of our study is that we were able to analyse the effect of cold exposure and mirabegron on multiple parameters associated with BAT in two different ethnicities. In addition, a placebo was used to discriminate between the effects of mirabegron treatment and effects induced by prolonged fasting. One limitation of the current study is that we measured BAT FF at only one time point after cold exposure and mirabegron treatment. Since activated BAT also takes up lipids from the blood to restore intracellular lipid stores, we cannot exclude that this affected our FF measurement. This may result in an underestimation of the effect size of cold exposure and mirabegron on BAT FF. In addition, our relatively small sample size limited the statistical power. Because of the exploratory nature of the study we did not correct for multiple testing. Furthermore, we only investigated healthy lean men. Future studies should investigate if these results also apply to the general population including women.

In conclusion, we have shown that South Asians and white Caucasians have a comparable beneficial metabolic response to mirabegron and cold exposure. Moreover, both mirabegron and cold exposure increased FFA, REE, lipid oxidation, and supraclavicular skin temperature and tended to decrease BAT FF, which are suggestive for activated BAT. Future studies should be aimed at unravelling the relative effect of both treatments on true BAT activity, for instance, by using alternative read-outs for BAT activity such as glucose or lipid uptake or oxidative capacity.

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SUPPLEMENTAL APPENDIX

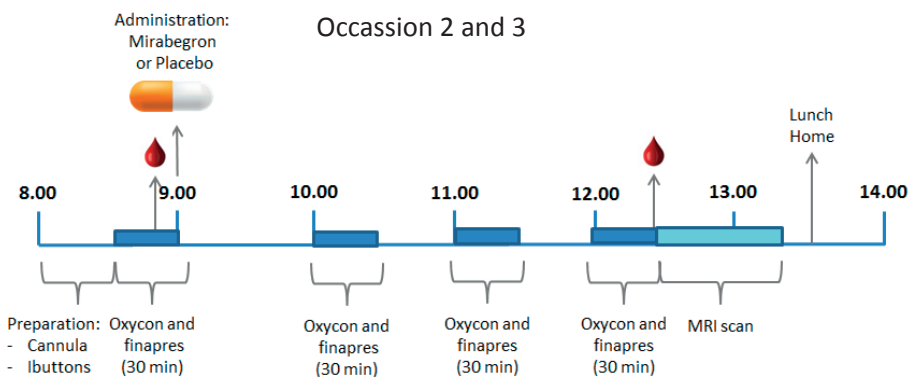
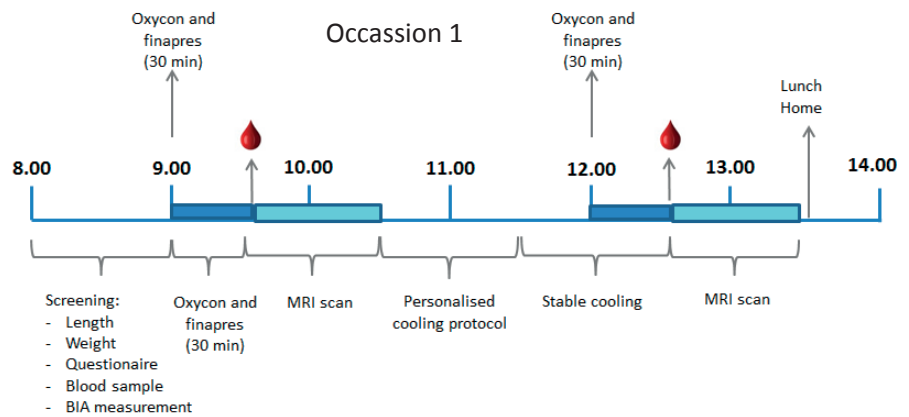
Supplementary Tables

Supplementary Table 1. Cardiovascular parameters

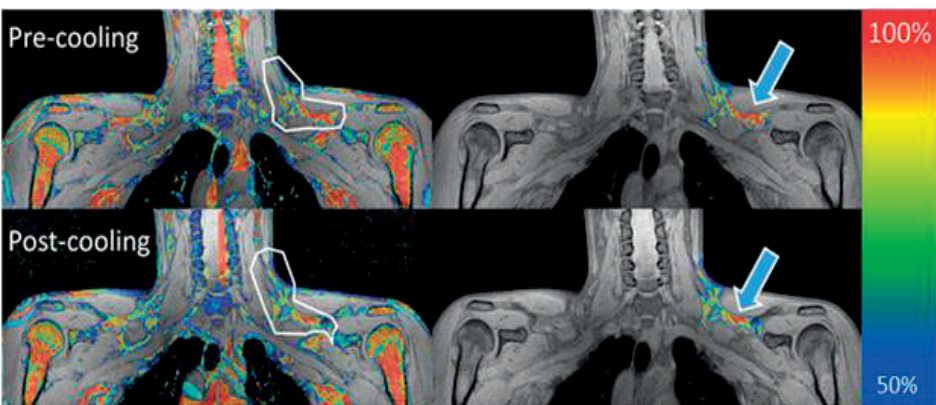
	White Caucasians (n=10)			South Asians (n=10)		
	Cooling	Placebo	Mirabegron	Cooling	Placebo	Mirabegron
Heart rate (bpm)	-2 **	0	+7 ***	-1 ^e	+2 ^a	+10 **
Diastolic BP (mmHg)	+13	+4	+6	+13 *	+4	+1
Systolic BP (mmHg)	+16	+7 *	+4	+13 *	+6	+9
Mean RRI (ms)	+49 **	+2	-108 ***	+25 ^c	-38	-152 ***
SDNN (ms)	+18	+13 ^d	-7	+26 *	+11	-17 **
rMSSD (ms)	+7	0	-20 *	+16 *	-3	-21 *
LF (ms)	+161	+172	-71	+207 ^a	+316 *	-213
HF (ms)	-46	-56	-242 ^b	+150 ^c	-11	-110 *
LF/HF ratio	+0.04	+0.27	+0.15	-0.21	+0.81	+0.56

Values are presented as delta (post- minus pre-intervention). Two-tailed paired (within ethnicities) or unpaired (between ethnicities) Student's *t*-test was used for comparison before and after intervention. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ^a $p = 0.06$, ^b $p = 0.07$, ^c $p = 0.08$, ^d $p = 0.09$, ^e $p = 0.10$ difference compared to baseline measurement. BP, blood pressure; RRI, RR interval; SDNN, Standard Deviation of Normal to Normal RR intervals; rMSSD, Root Mean Square of Successive heartbeat interval Differences; LF, low frequency; HF, high frequency.

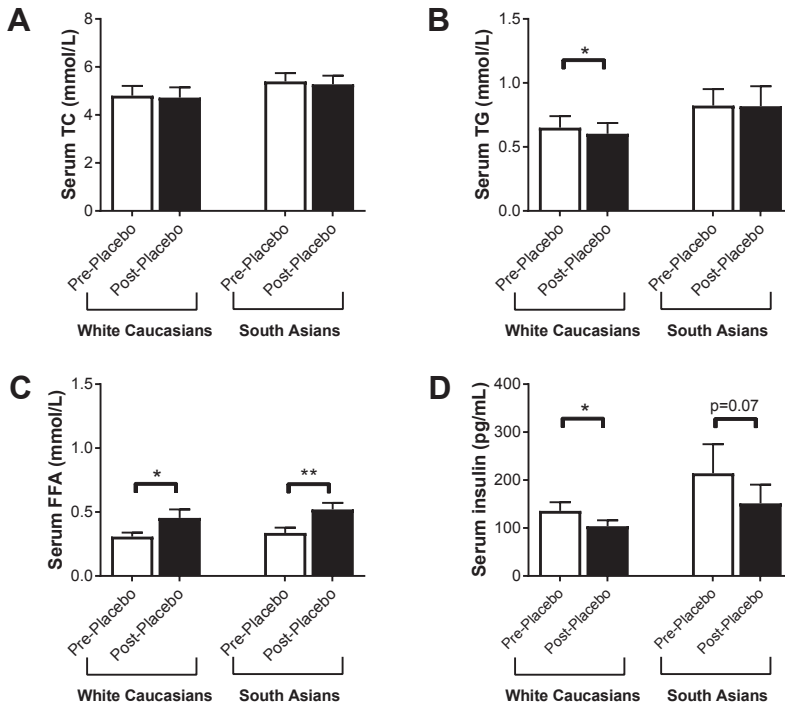
Supplementary Figures



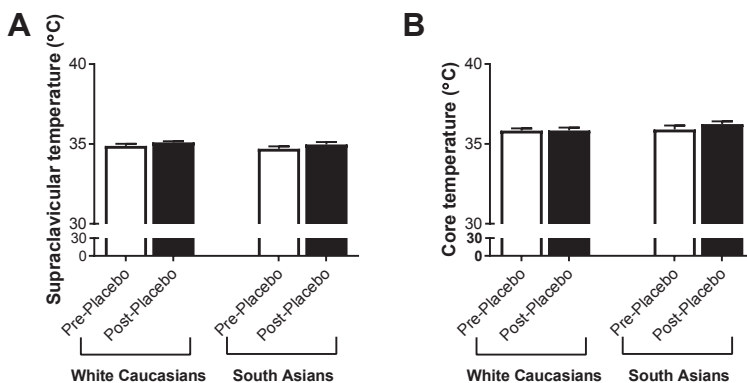
Supplemental Figure 1. Overview of the study design



Supplemental Figure 2. Region of Interest drawn on the MRI scan Example of supraclavicular ROI determination on MRI scan before and after cold exposure.



Supplemental Figure 3. Effect of placebo on serum lipids and insulin in white Caucasians and South Asians Serum was collected before (white bars) and after (black bars) intervention with placebo in white Caucasians ($n=10$) and South Asians ($n=10$) and assayed for total cholesterol (TC) (A), triglycerides (TG) (B), free fatty acids (FFA) (C), and insulin (D). Data are presented as mean $\pm$ S.E.M. Two-tailed paired (within ethnicities) or unpaired (between ethnicities) Student's t -test was used for comparison. * $p<0.05$, ** $p<0.01$ before vs. after intervention.



Supplemental Figure 4. Effect of placebo on core and supraclavicular skin temperature in white Caucasians and South Asians Before (white bars) and after (black bars) intervention with placebo, iButtons were used to measure supraclavicular skin temperature (A) and an estimation of core temperature (B) in white Caucasians ($n=10$) and South Asians ($n=10$). Data are presented as mean $\pm$ S.E.M. Two-tailed paired (within ethnicities) or unpaired (between ethnicities) Student's t -test was used for comparison.

CHAPTER 7

Effect of sitagliptin on energy metabolism and brown adipose tissue in overweight individuals with prediabetes: a randomised placebo-controlled trial

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Diabetologia (2018) in press

ABSTRACT

The aim of this study was to evaluate the effect of sitagliptin on glucose tolerance, plasma lipids, energy expenditure and metabolism of brown adipose tissue (BAT), white adipose tissue (WAT) and skeletal muscle in overweight individuals with prediabetes (impaired glucose tolerance and/or impaired fasting glucose). We performed a randomised, double-blinded, placebo-controlled trial in 30 overweight, Europid men (age 45.9 ± 6.2 years; BMI 28.8 ± 2.3 kg/m²) with prediabetes in the Leiden University Medical Center and the Alrijne Hospital between March 2015 and September 2016. 30 Participants were initially randomly allocated to receive sitagliptin (100 mg/day) ($n = 15$) or placebo ($n = 15$) for 12 weeks, using a randomisation list that was set up by an unblinded pharmacist. All people involved in the study as well as participants were blinded to group assignment. Two participants withdrew from the study prior to completion (both in the sitagliptin group) and were subsequently replaced with two new participants that were allocated to the same treatment. Before and after treatment, fasting venous blood samples and skeletal muscle biopsies were obtained, OGTT was performed and body composition, resting energy expenditure and [¹⁸F]fluorodeoxyglucose ([¹⁸F]FDG) uptake by metabolic tissues were assessed. The primary study endpoint was the effect of sitagliptin on BAT volume and activity. One participant from the sitagliptin group was excluded from analysis, due to a distribution error, leaving 29 participants for further analysis. Sitagliptin, but not placebo, lowered glucose excursion (-40% ; $p < 0.003$) during OGTT, accompanied by an improved insulinogenic index ($+38\%$; $p < 0.003$) and oral disposition index ($+44\%$; $p < 0.003$). In addition, sitagliptin lowered serum concentrations of triacylglycerol (-29%) and very large (-46%), large (-35%) and medium-sized (-24%) VLDL particles (all $p < 0.05$). Body weight, body composition and energy expenditure did not change. In skeletal muscle, sitagliptin increased mRNA expression of *PGC1 β* (also known as *PPARGC1B*) ($+117\%$; $p < 0.05$), a main controller of mitochondrial oxidative energy metabolism. Although the primary endpoint of change in BAT volume and activity was not met, sitagliptin increased [¹⁸F]FDG uptake in subcutaneous WAT (sWAT; $+53\%$; $p < 0.05$). Reported side effects were mild and transient and not necessarily related to the treatment. Twelve weeks of sitagliptin in overweight, Europid men with prediabetes improves glucose tolerance and lipid metabolism, as related to increased [¹⁸F]FDG uptake by sWAT, rather than BAT, and upregulation of the mitochondrial gene *PGC1 β* in skeletal muscle. Studies on the effect of sitagliptin on preventing or delaying the progression of prediabetes into type 2 diabetes are warranted.

INTRODUCTION

The worldwide prevalence of obesity is rapidly increasing (1). In obese individuals glucose homeostasis is frequently dysregulated, leading to a condition known as prediabetes (impaired glucose tolerance and/or impaired fasting glucose). It is estimated that up to 70% of individuals with prediabetes eventually develop type 2 diabetes mellitus (2). Therefore, delaying or preventing the development of type 2 diabetes is a potentially fruitful therapeutic strategy. In individuals with type 2 diabetes, glucose dysregulation is often accompanied by atherogenic dyslipidaemia, characterised by high triacylglycerol and LDL-cholesterol levels and low HDL-cholesterol levels (3,4). Current treatment strategies are aimed at targeting both hyperglycaemia and dyslipidaemia by combining glucose-lowering drugs with statins. However, a drug that improves both glucose and lipid profile might be of specific interest.

The dipeptidyl peptidase-4 (DPP4) inhibitor sitagliptin, which enhances the bioavailability of incretin hormones, improves both glucose tolerance and lipid metabolism in individuals with type 2 diabetes (5-7), thereby targeting both microvascular and macrovascular complications. The precise mechanism by which sitagliptin exerts these positive metabolic effects remains largely unknown. Interestingly, preclinical data with sitagliptin point towards a mechanism that involves the enhancement of energy expenditure through an increase in the activity of energy-combusting brown adipose tissue (BAT) and/or enhanced skeletal muscle respiratory capacity (8).

BAT combusts glucose and fatty acids towards heat, thereby increasing energy expenditure (9). High amounts of BAT, at least judged from the ability of BAT to take up [^{18}F]fluorodeoxyglucose ([^{18}F]FDG), are associated with lower plasma glucose levels and higher insulin sensitivity in humans (10-12) and BAT prevalence is inversely correlated with diabetic status (13). In addition, recruitment of BAT by short-term exposure to cold alleviates peripheral insulin resistance in individuals with type 2 diabetes (14). Of note, mouse studies have shown that BAT activation can also improve lipid profile and attenuate atherosclerosis development (15). Notably, in white adipose tissue (WAT) depots (16) inducible 'beige' adipocytes are present that have thermogenic capacity and can contribute to energy expenditure (reviewed in (17)).

Human skeletal muscle is an important contributor to energy expenditure and whole-body glucose metabolism. Insulin resistance of skeletal muscle is one of the earliest abnormalities that precede type 2 diabetes, and mitochondrial dysfunction has been implicated in the underlying pathogenesis (18). Improving human skeletal muscle metabolism, including mitochondrial function, is therefore another important therapeutic goal to ameliorate insulin resistance.

Although many studies have focussed on improving the metabolic profile of individuals with type 2 diabetes, less attention has been paid to identifying therapies that

improve glucose and lipid levels in prediabetic individuals. This may be of particular benefit since most of these individuals eventually proceed to overt diabetes (2,19). Therefore, the aim of this study was to evaluate the effect of sitagliptin on glucose tolerance, plasma lipids, energy expenditure, skeletal muscle metabolism and uptake of [^{18}F] FDG by BAT and WAT in overweight individuals with prediabetes.

MATERIALS AND METHODS

An extensive description of the Materials and Methods is available in the Supplementary Material and Methods.

Participants

Thirty-two overweight (BMI 25–35 kg/m²) Dutch Europid men with prediabetes, aged 35–55 years, were included. Prediabetes was defined as having a fasted serum glucose between 5.6 mmol/L and 6.9 mmol/L, according to the ADA criteria (20), and/or a plasma glucose level between 7.8 mmol/L and 11.1 mmol/L following an OGTT, according to WHO criteria for impaired glucose tolerance. Exclusion criteria were type 2 diabetes (e.g. fasted glucose >6.9 mmol/L and/or plasma glucose following OGTT >11.1 mmol/L), smoking, recent weight change, rigorous exercise, uncontrolled chronic disease or a positron emission tomography/computed tomography (PET/CT) scan within the last year. The study was approved by the Medical Ethical Committee of the Leiden University Medical Center (LUMC) and performed in accordance with the principles of the revised Declaration of Helsinki. Written informed consent was obtained from all volunteers prior to participation. Thirty participants were initially randomised. Two participants withdrew from the study prior to completion (both in the sitagliptin group—one participant because of heartburn and one participant because of joint pain) and were subsequently replaced with two new participants that were allocated to the same treatment (see **Supplementary Figure 1**). Thus, thirty participants completed the study. After completion of the study, one participant from the sitagliptin group was excluded from analyses due to a distribution error where the participant received both sitagliptin and placebo as treatment. Trial register number: NCT02294084.

Study design

Participants were enrolled in a randomised, double-blinded, placebo-controlled study and received oral administration of either sitagliptin (100 mg/day sitagliptin phosphate; Januvia; Merck Sharp and Dome, Haarlem, the Netherlands) or placebo for 12 weeks. The primary study endpoint was the effect of sitagliptin on BAT volume and activity. Secondary endpoints were the effects of sitagliptin on body weight and composition, resting energy expenditure (REE), glucose tolerance, fasting markers in blood for glucose and lipid

metabolism, [^{18}F]FDG uptake by WAT and skeletal muscle and gene expression in skeletal muscle biopsies. As a post hoc analysis, we quantified lipid and lipoprotein composition using high-throughput proton NMR metabolomics. Participants were studied at baseline and after 12 weeks of treatment. Before and after the treatment period, two measurement days took place. During the first day, body composition was measured (DEXA; iDXA; GE Healthcare, Little Chalfont, UK), followed by an individualised water-cooling protocol as described previously (21) (see Supplementary Methods). Thermoneutral and cold-exposed fasting blood samples were obtained and REE was measured by indirect calorimetry (Oxycon Pro; CareFusion, Heidelberg, Germany) (see Supplementary Methods) and skin temperature was measured (iButton; Maxim Integrated Products, San Jose, CA, USA). In addition, cold-induced [^{18}F]FDG uptake by BAT, WAT and skeletal muscle was determined by PET/CT scan (Gemini TF-64; Philips Healthcare, Best, the Netherlands) 1 h after administration of 110 MBq of [^{18}F]FDG (see Supplementary Methods). In the sitagliptin group, one participant became claustrophobic inside the PET/CT scan and could not finish this measurement. On the second day, a fasted skeletal muscle biopsy was taken from the vastus lateralis muscle (22) and then an OGTT was performed (see Supplementary Methods). All measurements took place after participants had fasted for 10 h overnight and had consumed a standardised dinner the night before. Each week during the treatment period, participants measured their blood glucose and were contacted to monitor compliance and adverse events.

Serum measurements

Commercially available enzymatic kits were used to measure serum concentrations of triacylglycerol and total cholesterol (Roche Diagnostics, Woerden, the Netherlands), NEFA (Wako Chemicals, Neuss, Germany) and glucose (Instruchemie, Delfzijl, the Netherlands). Insulin concentrations were measured using ELISA (Crystal Chem, Elk Grove Village, IL, USA). Plasma catecholamines were measured in the laboratory of Vascular Medicine (Erasmus MC, Rotterdam, the Netherlands) using standard procedure. Aspartate aminotransferase, alanine aminotransferase, γ -glutamyltransferase, HbA_{1c} and HDL-cholesterol were determined by the general hospital Laboratory of the LUMC and LDL-cholesterol was calculated using the Friedewald equation (23). Lipid and lipoprotein composition was quantified using high-throughput proton NMR metabolomics (Nightingale Health, Helsinki, Finland), as described previously (24). The following components were quantified: phospholipids, triacylglycerol, total cholesterol, non-esterified cholesterol and cholesteryl esters. The mean size for VLDL, LDL and HDL particles was calculated by weighting the corresponding subclass diameters with their particle concentrations (25). For the analysis of the OGTT, the AUC was calculated using the trapezoidal rule (26). Incremental AUC was calculated by deducting the area below the baseline value from total AUCs. Insulin sensitivity was estimated using the Matsuda index (27). The insulinogenic index (IGI; $\Delta I_{0-30}/\Delta G_{0-30}$, where I is insulin and G is glucose)

was used as a measure of early insulin secretion (28). The oral disposition index (DI_o ; $[\Delta I_{0-30}/\Delta G_{0-30}]/\text{fasting insulin}$) was used to estimate beta cell function relative to the prevailing level of insulin resistance (29).

PET/CT scan analysis

$[^{18}\text{F}]$ FDG uptake by BAT, WAT and skeletal muscle was determined from the $[^{18}\text{F}]$ FDG PET/CT scan using Fiji ImageJ 1.51d (Beth, Israel) (30) and analysed by two researchers blinded to allocation. For BAT, a personalised standardised uptake value (SUV) threshold with a tissue radiodensity between -190 and -10 Hounsfield units was used (31). For WAT, skeletal muscle and reference tissues, an SUV threshold of 0 was used and no Hounsfield units threshold was applied.

qRT-PCR analysis in skeletal muscle biopsies

Skeletal muscle biopsies were analysed for expression of genes involved in insulin signalling (*INSR*, *IRS1*), glucose metabolism (*GLUT4*), lipid metabolism (*ACACA*, *ACACB*, *ACSL1*, *CD36*, *FASN*) and mitochondrial function (*CTP1 α* , *CTP1 β* , *CTP2*, *CYCS*, *DNM1L*, *MFN2*, *OPA1*, *PPARGC1 α* [also known as *PPARGC1A*], *PPARGC1 β* [also known as *PPARGC1B*], *UCP3*), as well as *DPP4* (*DPP4*) and fibroblast growth factor (*FGF21*), using quantitative (q)PCR (Bio-Rad CFX96; Veenendaal, the Netherlands) (see **Supplementary Table 1** for primer sequences). Bio-Rad software version 3.1 (Bio-Rad Laboratories, Hercules, CA, USA) was used for analysis and quantification. Biopsy material from five participants (two from placebo and three from sitagliptin group) was insufficient for reliable mRNA analysis. Expression levels were normalised using the housekeeping gene β -actin (*ACTB*) and expressed as fold change using the $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

Power calculations were made for the primary outcome measurement of BAT activity (SUVmean), see Supplementary Materials and Methods. On the basis of previous studies (32), we anticipated a 20% increase in BAT activity after sitagliptin treatment. Assuming a bilateral alternative, we were able to detect differences of at least 20% in SUVmean, with a power of more than 80% and an α of 0.05 in a group of 30 participants. Data were analysed using SPSS Statistics (version 23.0; IBM Corporation, Armonk, NY, USA). Data are shown as mean $\pm$ SEM, unless stated otherwise. Two-tailed unpaired Student's *t* test was used to compare baseline characteristic between sitagliptin and placebo group. Mixed model analyses with treatment and occasion as fixed effects and subject-specific deviances from the mean as random effects were used to assess the effect of the treatment. If the mixed model failed to converge, a non-parametric paired test (Wilcoxon signed-rank test) was used. Statistical results are shown with adjustment for multiple testing. Bonferroni-corrected levels of significance are shown in the table and figure legends.

RESULTS

Participant characteristics and compliance

Thirty overweight, Euroid men with prediabetes completed the study, although one participant from the sitagliptin group was excluded from analyses due to a distribution error (the participant received both sitagliptin and placebo as treatment). Characteristics of the remaining participants are summarised in Table 1. All measured baseline characteristics were comparable between the two groups (**Table 1**), except for alanine aminotransferase, which was higher in the placebo group (0.73 ± 0.08 vs 0.47 ± 0.05 $\mu\text{kat/L}$; $p=0.009$ [not significant with a Bonferroni-corrected level of significance of 0.003]). A total of 83% of the participants had isolated impaired fasting glucose and 17% of the participants had combined impaired fasting glucose and impaired glucose tolerance. The daily oral administration of 100 mg sitagliptin was well tolerated and reported side effects (e.g. nasopharyngitis, heartburn and joint pains) were mild and transient and not necessarily related to the treatment. No episodes of hypoglycaemia were observed. Compliance was confirmed by counting returned tablets. In the placebo group only transient nasopharyngitis was reported.

Table 1. Participant characteristics at screening

	Placebo (n=15)	Sitagliptin (n=14)
Age (years)	47.1 (1.5)	45.3 (1.7)
Height (m)	1.83 (0.02)	1.79 (0.02)
Weight (kg)	93.7 (1.6)	95.4 (3.1)
BMI (kg/m^2)	27.8 (0.4)	29.6 (0.9)
Waist circumference (cm)	101 (2)	100 (2)
Hip circumference (cm)	101 (2)	103 (2)
Waist-to-hip ratio	1.00 (0.01)	0.98 (0.01)
Fasting glucose (mmol/L)	5.9 (0.1)	5.8 (0.1)
Fasting insulin (pmol/L)	81.3 (24.3)	54.2 (9.7)
HbA1c (mmol/L)	36.5 (0.8)	36.2 (0.9)
HbA1c (%)	5.5%	5.5%
Fasting triacylglycerol (mmol/L)	1.70 (0.31)	1.47 (0.19)
Fasting total cholesterol (mmol/L)	5.32 (0.32)	5.84 (0.24)
Aspartate aminotransferase ($\mu\text{kat/L}$)	0.52 (0.03)	0.42 (0.03)
Alanine aminotransferase ($\mu\text{kat/L}$)	0.73 (0.08)	0.47 (0.05) *
γ -Glutamyltransferase ($\mu\text{kat/L}$)	0.62 (0.08)	0.53 (0.07)

Values are presented as mean (standard error of the mean). BMI: body mass index, HbA1c: haemoglobin A1c. Two-tailed unpaired Student's *t* test was used for statistical comparison * $p=0.009$ for sitagliptin vs placebo (not significant with Bonferroni-corrected level of significance of 0.003 ($\alpha=0.05/16$)). Data are from all participants that completed the study, apart from $n=1$ in the sitagliptin group, who was excluded due to pharmacy error.

Table 2. Effect of sitagliptin on measures of glucose tolerance in overweight men with prediabetes

	Placebo (n=15)		Sitagliptin (n=14)	
	Week 0	Week 12	Week 0	Week 12
Fasting glucose (mmol/L)	6.3 (0.2)	6.1 (0.2)	5.9 (0.2)	5.9 (0.2)
Peak glucose (mmol/L)	12.2 (0.4)	12.1 (0.5)	11.0 (0.4)	9.7 (0.3) ** †
Peak glucose time (min)	52.7 (4.8)	40.7 (2.7)	44.3 (5.1)	31.4 (3.3)
AUC ₀₋₁₂₀ glucose (mmol/L x min)	1137 (52)	1088 (51)	969 (41)	819 (21) ** †
AUC ₀₋₁₂₀ incremental glucose (mmol/L x min)	387 (40)	371 (38)	276 (35)	166 (33) ** ††
Fasting insulin (pmol/L)	46.5 (6.3)	51.4 (7.0)	37.5 (6.3)	44.5 (12.5)
Peak insulin (pmol/L)	639 (111)	590 (83)	549 (76)	507 (83)
Peak insulin time (min)	61.3 (6.4)	65.3 (8.5)	46.4 (6.2)	40.7 (4.0)
AUC ₀₋₁₂₀ insulin (pmol/Lx min)	45565 (7881)	42960 (6048)	37437 (5652)	31858 (4879)
AUC ₀₋₁₂₀ incremental insulin (pmol/Lx min)	39941 (7296)	36805 (5366)	32957 (5036)	26533 (3571)
AUC ₀₋₁₂₀ glucose/ AUC ₀₋₁₂₀ insulin	0.25 (0.04)	0.25 (0.04)	0.22 (0.02)	0.21 (0.02)
Matsuda index	4.93 (0.72)	5.56 (1.16)	5.96 (0.65)	6.63 (0.73)
HOMA-IR	1.93 (0.26)	2.02 (0.28)	1.40 (0.24)	1.71 (0.51)
IGI (pmol/mmol)	77 (12)	75 (14)	100 (19)	138 (23) ** †
DI ₀	1.69 (0.21)	1.68 (0.34)	2.67 (0.30)	3.84 (0.54) ** ††

Values are presented as mean (standard error of the mean). AUC: area under the curve, DI₀: oral disposition index, HOMA-IR: homeostatic model assessment insulin resistance, IGI: insulinogenic index. Mixed model analysis was used for statistical comparison *0.003< p <0.05 and ** p <0.003 for week 0 vs week 12; †0.003< p <0.05 and †† p <0.003 for placebo vs sitagliptin. Bonferroni-corrected level of significance is 0.003 (α =0.05/15). Data are from all participants that completed the study, apart from n=1 in the sitagliptin group, who was excluded due to pharmacy error.

Sitagliptin improves glucose tolerance without changing body weight or composition

We first assessed the effects of sitagliptin on glucose metabolism. Sitagliptin did not change fasting serum glucose or insulin levels (**Table 2**). However, compared with baseline, twelve weeks of sitagliptin treatment improved glucose tolerance as demonstrated by reduced glucose excursions (AUCgluc₀₋₁₂₀ –15%, p < 0.003; **Table 2**), lower incremental glucose excursions (AUCincr₀₋₁₂₀ –40%, p < 0.003; **Table 2**; **Figure 1B,C**) and lower peak glucose levels (–12%, p < 0.003; **Table 2**) during OGTT. Incremental insulin excursions (AUCincr₀₋₁₂₀) were not significantly reduced upon sitagliptin treatment (**Table 2**; **Figure 1E,F**). Furthermore, sitagliptin improved IGI (+38%, p < 0.003; **Table 2**) and increased DI₀ (+44%, p < 0.003; **Table 2**). Placebo did not affect either glucose or insulin excursions (**Table 2** and **Figure 1A,C,D,F**). Using a mixed model, we confirmed that the sitagliptin-induced changes in peak glucose levels (p < 0.05), AUCgluc₀₋₁₂₀ (p < 0.05), AUCincr₀₋₁₂₀ (p < 0.003), IGI (p < 0.05) and DI₀ (p < 0.003) were significantly different from placebo. Sitagliptin did not affect body composition (**Supplementary Table 2**), nor did it affect proximal, distal or supraclavicular skin temperature upon cooling

(**Supplementary Table 3**). In addition, the liver enzymes aspartate aminotransferase, alanine aminotransferase and γ -glutamyltransferase were not affected by sitagliptin treatment (**Supplementary Table 4**). Of note, placebo increased alanine aminotransferase (+82%, $p < 0.01$; **Supplementary Table 4**).

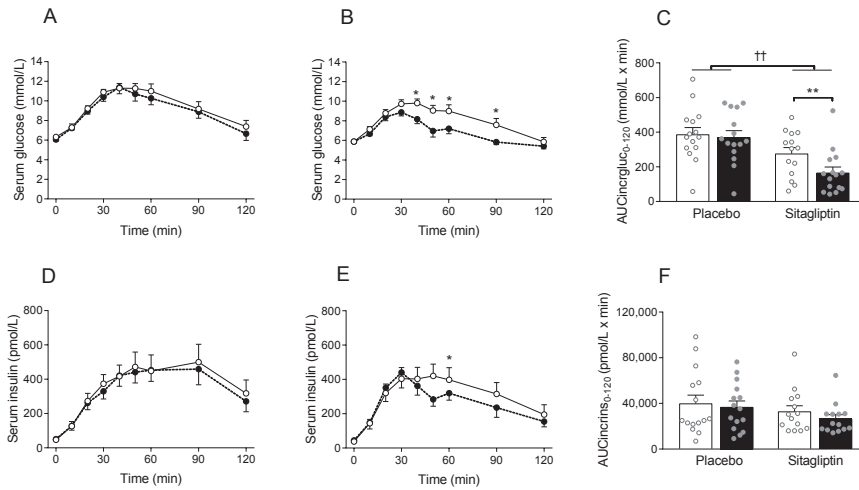


Figure 1. The effect of sitagliptin on glucose tolerance in overweight men with prediabetes. An OGTT was performed to assess glucose tolerance, before (white circles and bars) and after (black circles and bars) 12 weeks of placebo ($n=15$) or sitagliptin ($n=14$) treatment. Serum was collected before and at several time points up to 120 min after ingestion of 75 g of glucose. Glucose and insulin excursions were determined at the indicated time points after treatment with placebo (**A, D**) or sitagliptin (**B, E**). Incremental AUC (AUC_{incr}) was calculated for glucose (gluc; **C**) and insulin (ins; **F**). Data are presented as means $\pm$ SEM. Mixed model analysis was used for statistical comparison. In (**A, B, D, E**) $*0.006 < p < 0.05$ for week 0 vs week 12, not significant with Bonferroni-corrected level of significance 0.006 ($\alpha=0.05/9$). In (**C, F**) $**p < 0.01$ for week 0 vs week 12, significant with Bonferroni-corrected level of significance 0.01 ($\alpha=0.05/4$); $^{\dagger\dagger}p < 0.01$ for placebo vs sitagliptin, significant with Bonferroni-corrected level of significance 0.01 ($\alpha=0.05/4$).

Sitagliptin lowers serum triacylglycerol reflected by decreased concentrations of large and medium-sized VLDL particles

Previous studies in individuals with type 2 diabetes showed that sitagliptin improves plasma lipid levels (5,6). Therefore, we assessed whether sitagliptin also affects serum lipids and lipoprotein levels in overweight individuals with prediabetes. Sitagliptin induced a substantial reduction in serum triacylglycerol levels (-29% ; $p < 0.05$ [not significant with a Bonferroni-corrected level of significance of 0.003]; **Figure 2A**) without affecting serum total cholesterol (**Figure 2B**), total HDL-cholesterol (**Figure 2C**), total LDL-cholesterol (**Figure 2D**), NEFA levels (data not shown) or plasma catecholamines (data not shown). Placebo did not significantly affect serum triacylglycerol, total cholesterol, HDL-cholesterol or LDL-cholesterol (**Figure 2A–D**). The sitagliptin-induced reduction in triacylglycerol was not significantly different from placebo.

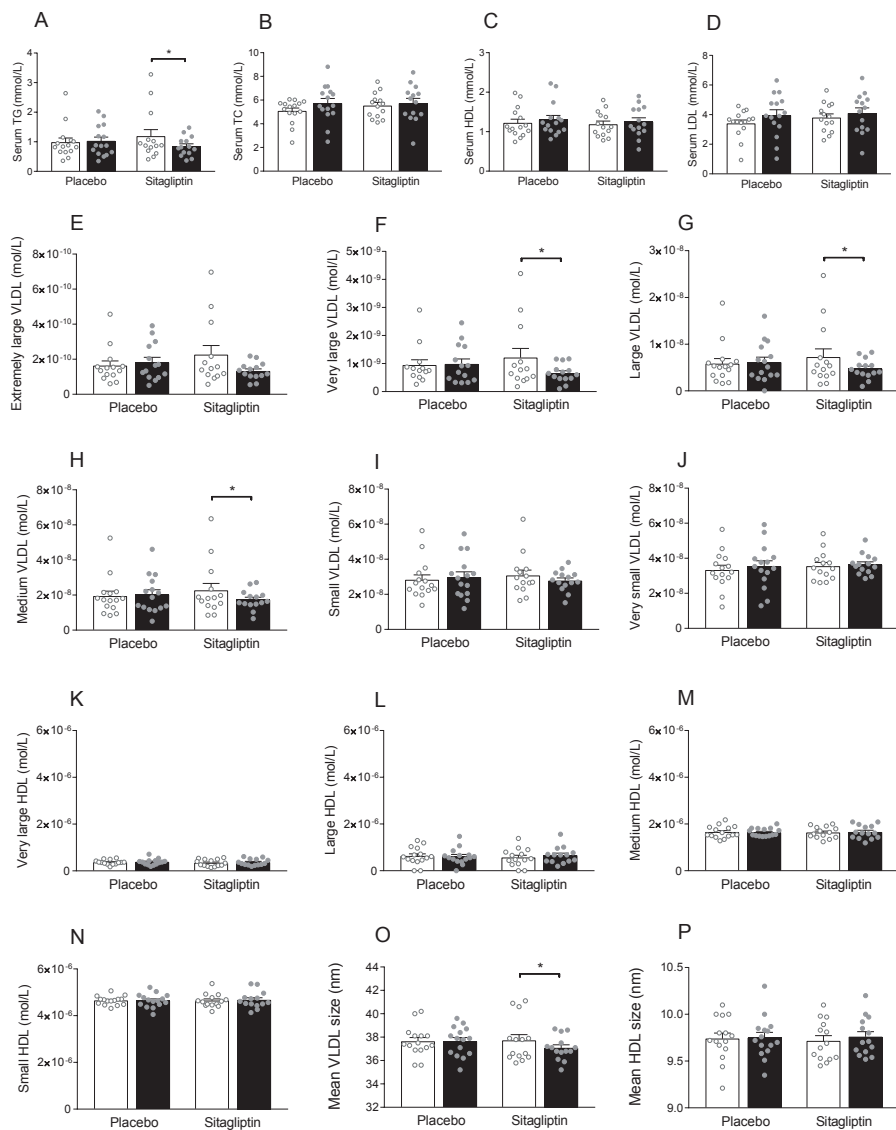


Figure 2. The effect of sitagliptin on serum lipid and VLDL and HDL particle concentration in overweight men with prediabetes. Serum was collected before (week 0, white bars) and after (week 12, black bars) treatment with placebo ($n=15$) or sitagliptin ($n=14$). Enzymatic assays were used to measure serum triacylglycerol (TG) (A) and total cholesterol (TC) (B). HDL-cholesterol was determined by the general hospital laboratory of the LUMC (C) and LDL-cholesterol was calculated using the Friedewald equation (D). NMR was used to measure serum concentrations of extremely large (E), very large (F), large (G), medium-sized (H), small (I) and very small (J) VLDL particle and very large (K), large (L), medium-sized (M) and small (N) HDL particles. In addition, mean VLDL (O) and mean HDL (P) particle size was determined. Data are presented as means $\pm$ SEM and as individual measurements. Mixed model analysis was used for statistical comparison. $*0.003 < p < 0.05$ for week 0 vs week 12, not significant with Bonferroni-corrected level of significance 0.003 ($\alpha=0.05/16$).

Since serum triacylglycerol is carried by lipoproteins, we next assessed whether the reduction in triacylglycerol induced by sitagliptin was specific for certain lipoprotein subfractions. Sitagliptin lowered serum concentrations of very large (–46%; $p < 0.05$; **Figure 2F**), large (–35%; $p < 0.05$; **Figure 2G**) and medium-sized VLDL particles (–24%; $p < 0.05$; **Figure 2H**), although these were all non-significant with a Bonferroni-corrected level of significance of 0.003. Concentrations of small (**Figure 2I**) and very small VLDL particles (**Figure 2J**) were unchanged. Overall, this resulted in a decrease in mean VLDL particle size (–1.7%; $p < 0.05$ [not significant with a Bonferroni-corrected level of significance of 0.003]; **Figure 2O**). In addition, sitagliptin did not significantly change the concentrations of very large, large, medium-sized or small HDL particle subfractions (**Figure 2K–N**) or mean HDL particle size (**Figure 2P**). Intermediate-dense lipoprotein (IDL), large, medium-sized and small LDL particle concentrations and mean LDL particle size were not affected by sitagliptin treatment (**Figure 2**). Placebo did not alter the measured lipoprotein particle concentrations or particle sizes (**Figure 2E–P**; **Supplementary Figure 2**). The sitagliptin-induced reduction in concentration of very large, large or medium-sized VLDL particles was not significantly different from placebo, nor was the sitagliptin-induced decrease in VLDL and increase in HDL mean particle size.

In line with these findings, most components of the lipoprotein subclasses in extremely large, very large, large and medium-sized VLDL particles were decreased upon sitagliptin treatment (**Supplementary Table 5**). In IDL, only non-esterified cholesterol significantly increased and in HDL particles only phospholipids in very large HDL significantly increased (**Supplementary Table 5**), although these were not significant with a Bonferroni-corrected level of significance of 0.0006. Components of the lipoprotein subclasses in LDL fractions (**Supplementary Table 5**) and fatty acid composition did not change significantly.

Sitagliptin does not affect overall resting energy expenditure or substrate preference

To assess whether sitagliptin improves glucose and lipid metabolism by altering substrate utilisation or REE, indirect calorimetry was performed. REE was similar before and after treatment with either sitagliptin or placebo, also when correcting for lean body mass (**Supplementary Table 6**). In addition, non-shivering thermogenesis, the respiratory quotient, glucose and lipid oxidation did not change upon treatment (**Supplementary Table 6**).

Sitagliptin increases [^{18}F]FDG uptake in subcutaneous WAT, but not in BAT

Next, we assessed whether sitagliptin improves glucose tolerance and lipid profile through beneficial effects on BAT, WAT or skeletal muscle. To this end, we assessed cold-induced [^{18}F]FDG uptake in these organs following PET/CT scan. Concerning the primary endpoint, we found that 12 weeks of sitagliptin treatment did not affect [^{18}F]FDG uptake in all measured BAT depots, though mean [^{18}F]FDG uptake in BAT was very

low in most participants (**Supplementary Table 7**). Furthermore, sitagliptin did not alter [^{18}F]FDG uptake in skeletal muscle or paracolic WAT. Interestingly, [^{18}F]FDG uptake in subcutaneous WAT (sWAT) was increased by sitagliptin (+53%; $p < 0.05$ [not significant with Bonferroni-corrected level of significance of 0.002]) but not placebo treatment (**Supplementary Table 7**). The sitagliptin-induced effect on [^{18}F]FDG uptake in sWAT did not significantly differ from placebo. In the placebo group, [^{18}F]FDG uptake by the trapezius muscles was increased (+15%; $p < 0.05$), as was uptake by the descending aorta (+13%; $p < 0.05$), as reference tissues (neither significant with Bonferroni-corrected level of significance of 0.002; **Supplementary Table 7**).

Sitagliptin increases PGC1 β gene expression in skeletal muscle

Next, we analysed skeletal muscle biopsies for pathways involved in mitochondrial function, glucose and lipid metabolism. Sitagliptin increased the expression of *PGC1 β* (also known as *PPARGC1B*) (+117%; $p < 0.05$ [not significant with Bonferroni-corrected level of significance of 0.002]; **Figure 3B**) in skeletal muscle. Sitagliptin also increased *DPP4* gene expression (+51%; $p < 0.05$ [not significant with Bonferroni-corrected level of significance of 0.002]; **Figure 3D**). Expression of genes involved in glucose or lipid metabolism were not significantly changed by sitagliptin (**Figure 3D**). In the placebo group, expression levels of none of the measured genes were significantly changed upon treatment (**Figure 3A,C**).

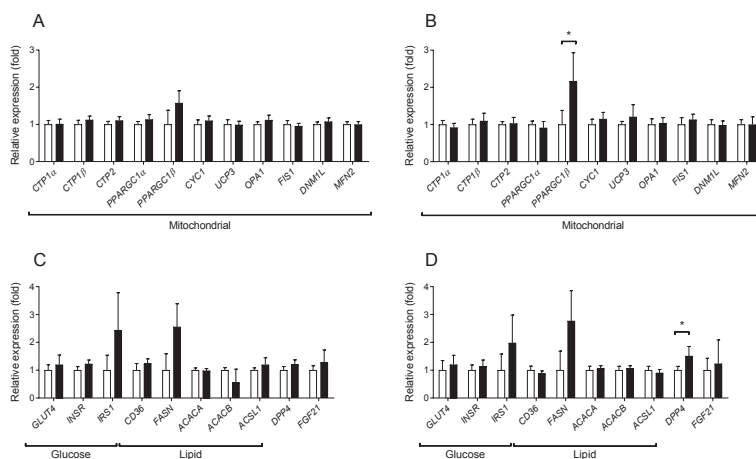


Figure 3. The effect of sitagliptin on skeletal muscle gene expression in overweight men with pre-diabetes. A fasted skeletal muscle biopsy was taken before (white bars) and after (black bars) 12 weeks of treatment with placebo ($n=13$) or sitagliptin ($n=11$). qPCR was used to determine expression of genes involved in mitochondrial function, glucose metabolism and lipid metabolism, as well as *DPP4* and *FGF21*, upon placebo (**a, c**) and sitagliptin (**b, d**) treatment. Data are presented as means $\pm$ SEM. Expression levels were normalised using the mRNA content of the housekeeping gene β -actin (*ACTB*) and expressed as fold change using the $2^{-\Delta\Delta C_t}$ method. Mixed model analysis was used for statistical comparison. * $0.002 < p < 0.05$ for week 0 vs week 12, not significant with Bonferroni-corrected level of significance 0.002 ($\alpha=0.05/21$).

DISCUSSION

The aim of the current study was to evaluate the effect of sitagliptin on glucose tolerance, plasma lipids, energy expenditure, skeletal muscle metabolism and [^{18}F]FDG uptake by BAT and WAT in overweight men with prediabetes. Here, we report that 12 weeks of sitagliptin treatment improved glucose tolerance and lipid profile in these men. These beneficial effects were accompanied by an increase in mRNA expression of *PGC1 β* , a gene encoding an inducer of mitochondrial biogenesis, in skeletal muscle. Sitagliptin did not affect [^{18}F]FDG uptake by BAT but increased [^{18}F]FDG uptake by sWAT. To our knowledge, this study is the first to provide extensive insight in the beneficial effects of sitagliptin on both glucose and lipid metabolism in overweight individuals with prediabetes, as previous studies mostly focussed on individuals with type 2 diabetes (6).

We showed that the improvement in glucose tolerance following treatment of these men with sitagliptin was not explained by changes in body composition. The improved glucose tolerance is in line with previous reports (33,34). Since we observed an improvement in IGI (i.e. measure of early insulin secretion) and DI_0 (i.e. estimation of beta cell function relative to measure of insulin resistance) during OGTT, we speculate that the improved glucose tolerance can be explained by increased early insulin secretion, in turn possibly due to improved beta cell function. In accordance with this, the DPP4 inhibitor vildagliptin (100 mg/day) improved insulin sensitivity and beta cell function in prediabetic individuals upon 6 weeks of treatment (35). Interestingly, animal studies have shown that sitagliptin can delay the progression of prediabetes into diabetes (36,37). Whether this is also the case in humans remains to be elucidated (38).

We also showed that sitagliptin lowered serum triacylglycerol levels in overweight men with prediabetes. This is in line with previous studies showing that sitagliptin reduces triacylglycerol levels in individuals with type 2 diabetes (5,39), although in those individuals total cholesterol levels were also reduced. We used NMR metabolomics to evaluate which triacylglycerol-containing lipoprotein classes were lowered. Thereby, we specifically observed a decrease in the concentration of very large, large and medium-sized VLDL particles, which may point to reduced hepatic VLDL-triacylglycerol synthesis and/or enhanced VLDL-triacylglycerol clearance. Individuals with type 2 diabetes display increased hepatic synthesis of VLDL particles, resulting in elevated VLDL-triacylglycerol (4,40), presumably due to the impaired ability of insulin to suppress VLDL synthesis. Tremblay et al (6) showed that 6 weeks of sitagliptin treatment reduces triacylglycerol levels due to reduced hepatic VLDL synthesis (6). Therefore, it is likely that sitagliptin also attenuates hepatic VLDL-triacylglycerol production in our study in prediabetic men who also have impaired insulin sensitivity. A contribution made by the increased lipolysis of VLDL-triacylglycerol by LPL-containing peripheral tissues, including skeletal muscle and BAT, cannot be ruled out, especially since enhanced glucagon-like peptide-1 (GLP-1)

receptor signalling has been shown to largely increase the uptake of triacylglycerol-derived fatty acids by BAT in mice (41). Evaluation of the potential contribution made by accelerated triacylglycerol clearance to the triacylglycerol-lowering effect of sitagliptin should be subject of future studies.

So far, the contribution of the various metabolic tissues responsible for the beneficial metabolic effects of sitagliptin has not been clarified. We investigated BAT, WAT and skeletal muscle, as those tissues are known to play an important role in energy metabolism. Despite us defining the primary endpoint of this study as the effect of sitagliptin on BAT volume and activity, we did not observe an effect of sitagliptin on [^{18}F]FDG uptake by BAT. However, sitagliptin is suggested to activate BAT in rodents, as judged from increased expression of uncoupling protein 1 gene (8). It should be realised that the read-outs of BAT activity were different in rodents compared with humans. In addition, compared with lean individuals, it is known that [^{18}F]FDG uptake in BAT is lower in obese individuals (42), in individuals with type 2 diabetes (43) and in increasing age (44), likely due to increased insulin resistance. Importantly, insulin resistance does not reduce the uptake by BAT of 14(*R,S*)-[^{18}F]fluoro-6-thia-heptadecanoic acid ([^{18}F]FTHA; a fatty acid tracer) or [^{11}C]acetate (a measure of oxidative metabolism) (43). It is feasible that our overweight men with prediabetes are insulin resistant at the level of BAT and, as a consequence, display low [^{18}F]FDG uptake in BAT. Therefore, it cannot be ruled out that in our study population [^{18}F]FDG uptake by BAT reflects insulin resistance rather than BAT metabolism. Since fatty acids rather than glucose form the main substrate for BAT thermogenesis (45,46), future studies should preferably use lipid-based PET/CT tracers such as [^{18}F]FTHA or PET/CT tracers imaging oxidative capacity, such as [^{11}C]acetate, to better reflect BAT activity, especially in insulin-resistant individuals. Interestingly, sitagliptin increased [^{18}F]FDG uptake in sWAT, possibly pointing to browning of this tissue. Indeed, enhanced GLP-1 receptor signalling induces massive browning of WAT in mice (41). Unfortunately, sWAT biopsies were not collected, so it remains unknown whether expression levels of thermogenic genes in WAT are increased upon sitagliptin treatment. Assessment of browning would be important in future human studies using DPP4 inhibitors or GLP1 agonists and would be of particular clinical relevance to overweight participants, who display large subcutaneous WAT depots.

Besides BAT, skeletal muscle is an important contributor to energy metabolism in humans. Sitagliptin increased *DPP4* expression in skeletal muscle, which seems counterintuitive since high local DPP4 levels are thought to impair insulin signalling and thereby induce/deteriorate the development of type 2 diabetes (47). The increased *DPP4* expression may be a consequence of a compensatory mechanism of increased plasma GLP-1 as a result of DPP4 inhibition, although unfortunately we were not able to measure circulating GLP-1 levels. We did show that sitagliptin increased mRNA expression of *PGC1 β* , which is in line with a previous rodent study showing that sitagliptin treatment

increases mitochondrial gene expression in skeletal muscle (8). Since *PGC1 β* modulates mitochondrial function through induction of mitochondrial biogenesis (48,49), sitagliptin may enhance skeletal muscle metabolism via this mechanism. Although we did not detect a significant effect of sitagliptin on energy expenditure, a potential contribution to whole-energy metabolism may not have been picked up by indirect calorimetry. Since skeletal muscles contribute largely to the composition of the human body, a small change in muscle respiration could have a large effect on total body metabolism.

A strength of our study is that we analysed the effect of sitagliptin on the main metabolically active organs. Moreover, we analysed multiple BAT and WAT depots by [^{18}F]FDG PET/CT. We also performed an extensive analysis of the serum lipoprotein profile using NMR metabolomics. A limitation of the current study is that we made use of the radio-tracer [^{18}F]FDG, so may have underestimated the metabolic activity of insulin-resistant tissues in individuals with prediabetes. We performed all measurements after an overnight fast. Since DPP4 inhibitors are most effective after a meal, it might be interesting to investigate the effects of sitagliptin on postprandial glucose and lipid metabolism, which might be even more pronounced. Furthermore, the relatively small sample size might have limited the statistical power. A strength and limitation is that we assessed many variables, which necessitated Bonferroni correction. The Bonferroni threshold for significance was reached only for reduction in glucose excursions by sitagliptin, the lower peak glucose levels during the OGTT and the improvement in the IGI and DI_0 . In addition, we only investigated Europid men. We chose this specific group since South Asians, another large ethnic group within the Netherlands, generally display more insulin resistance and dyslipidaemia (reviewed in (50)) as well as higher GLP-1 levels (51) compared with Europids. Combination of several ethnic groups may have increased variation within our study groups. However, future studies should investigate whether these results also apply to the general population, including women.

In conclusion, we show that 12 weeks of sitagliptin treatment improves glucose tolerance and lipid profile in overweight men with prediabetes. Those effects might be mediated by browning of sWAT and/or increased energy metabolism in skeletal muscle possibly by upregulation of *PGC1 β* . However, the precise mechanism linking DPP4 inhibition to metabolic health still remains to be elucidated. Since up to 70% of prediabetic individuals eventually develop type 2 diabetes and current lifestyle and exercise programmes are often difficult to maintain in the long term, further studies on the effect of sitagliptin in preventing or delaying the progression of prediabetes and dyslipidaemia in individuals at risk for developing type 2 diabetes are warranted.

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SUPPLEMENTAL APPENDIX

Supplementary material and methods

Participants

Thirty-two overweight (BMI 25-35 kg/m²) Dutch Euroid men with prediabetes, aged 35-55 years, were included in the study. Participants were recruited via local advertisements and underwent medical screening prior to participation, including an interview regarding medical history, physical examination, a blood chemistry test and an oral glucose tolerance test (OGTT) to exclude individuals with undiagnosed type 2 diabetes. Prediabetes was defined as having a fasted serum glucose between 5.6 mmol/L and 6.9 mmol/L, according to the American Diabetes Association (ADA) criteria (1), and/or a plasma glucose level between 7.8 mmol/L and 11.1 mmol/L following an OGTT, according to World Health Organization (WHO) criteria for impaired glucose tolerance. Exclusion criteria were type 2 diabetes (e.g. fasted glucose > 6.9 mmol/L and/or plasma glucose following OGTT > 11.1 mmol/L), smoking, recent weight change (> 3 kg within the last 3 months), rigorous exercise, use of beta-blockers, uncontrolled hypertension, hyper- or hypothyroidism, renal failure or liver dysfunction, or a positron emission tomography/computed tomography (PET/CT) scan within the last year. The study was approved by the Medical Ethical Committee of the Leiden University Medical Center and performed in accordance with the principles of the revised Declaration of Helsinki. Written informed consent was obtained from all volunteers prior to participation. Thirty participants were initially randomised. Two participants withdrew from the study prior to completion (both in the sitagliptin group; one participant because of heartburn and one participant because of joint pain) and were subsequently replaced, with two new participants, that were allocated to the same treatment. All symptoms resolved without sequelae. Thus, thirty participants completed the study. Also, one participant from the sitagliptin group was excluded from analysis, due to a distribution error where the participant received both sitagliptin and placebo as treatment. Trial register number: NCT02294084.

Study design

Participants were enrolled in a randomised, double-blinded, placebo-controlled study that was conducted in the Leiden University Medical Center (Leiden, The Netherlands) and Alrijne Hospital (Leiderdorp, The Netherlands). The primary study endpoint was the effect of sitagliptin treatment on brown adipose tissue volume and activity in overweight men with prediabetes. Secondary endpoints were the effect of sitagliptin on body weight and body composition, resting energy expenditure, fasting markers in blood for glucose and lipid metabolism, [¹⁸F]FDG uptake by white adipose tissue and

skeletal muscle and expression of genes involved in insulin signalling, glucose and lipid metabolism in skeletal muscle biopsies. As a post-hoc analysis we quantified lipid and lipoprotein composition using high-throughput proton NMR metabolomics. Participants were randomly assigned in a 1:1 ratio by the unblinded hospital pharmacist to receive oral administration of either sitagliptin (Sitagliptin phosphate, Januvia, Merck Sharp and Dome, Haarlem, The Netherlands) at a dose of 100 mg oral daily, or placebo for 12 weeks. Following initial screening, participants were studied at baseline and after 12 weeks of treatment. Before and after the treatment period there were two measurement days. During the first day, which took place at the Alrijne hospital in Leiderdorp, body composition (DEXA, iDXA, GE Healthcare, Little Chalfont, UK), skin temperature (iButton, Maxim Integrated Products, San Jose, CA, USA), thermoneutral and cold-exposed resting energy expenditure (REE; indirect calorimetry, Oxycon Pro, CareFusion, Heidelberg, Germany), and cold-induced [^{18}F]fluorodeoxyglucose ([^{18}F]FDG) uptake by BAT and other metabolic organs such as white adipose tissue (WAT) and skeletal muscle were determined by PET/CT scan (Gemini TF-64, Philips Healthcare, Best, The Netherlands). In addition, thermoneutral and cold-exposed venous blood samples were collected. On the second day at the Leiden University Medical Center, a fasting skeletal muscle biopsy was taken from the vastus lateralis muscle followed by an OGTT. All measurements took place after participants had fasted for 10h overnight and had consumed a standardised dinner the night before. In addition, participants were asked to refrain from caffeine and alcohol intake or strenuous physical activity 24 h prior to the study days. Each week during the treatment period, participants measured their blood glucose and were contacted by the investigator to monitor compliance, adverse events or signs of hypoglycemia. In addition, participants were instructed not to alter their lifestyle during the study period. The study was conducted between March 2015 and September 2016.

Indirect calorimetry, individualized cooling protocol and [^{18}F]FDG PET-CT scan

On the first measurement day an intravenous cannula was placed in antecubital vein for blood sampling during thermoneutral and mild cold conditions and injection of the [^{18}F]FDG tracer. First detailed body composition was obtained by dual-energy X-ray absorptiometry, followed by placement of 14 wireless temperature sensors at 14 prescribed ISO-defined places (2). Mean skin temperature was calculated as the average of all iButtons. Distal skin temperature was calculated as the average temperature of the hand and feet. Proximal skin temperature was defined as the average of the iButtons on the chest, abdomen, scapula and lower back. Next participants were placed in a bed in semi-supine position between two water-perfused mattresses (BlanketRol® III Sub-Zero (CSZ) Products, Cincinnati, OH, USA). As described previously (3), the protocol started with 1 h of thermoneutrality (water temperature 32°C) at which thermoneutral REE was measured for 30 minutes using indirect calorimetry and a venous blood sample was drawn. After 1

h at thermoneutrality, participants were progressively cooled until their shivering point or until they reached the minimum water temperature of 9°C. At this mild cold condition REE was measured for another 30 minutes and a second blood sample was collected. Oxygen consumption and carbon dioxide production were determined every minute. REE and respiratory quotient (RQ) were calculated and substrate utilization was assessed by calculation of lipid and glucose oxidation after correction for protein oxidation as described earlier (4). Non-shivering thermogenesis (NST) was assessed by the difference in REE during mild cooling compared to thermoneutral conditions. Subsequently, 110 MBq of [¹⁸F]FDG was injected intravenously and after 1 h of incubation, the PET/CT imaging protocol started with a low-dose CT scan (120 kV, 30 mAs), immediately followed by a PET scan (10 bed positions, 4 minutes per bed position) acquired according to EARL standards (5) to assess [¹⁸F]FDG uptake by BAT, WAT and skeletal muscle from skull to pelvis. In the sitagliptin group one participant became claustrophobic inside the PET/CT scan and could therefore not finish this measurement.

Skeletal muscle biopsy and oral glucose tolerance test

On the second measurement day, a fasted skeletal muscle biopsy was taken from the vastus lateralis muscle according to the technique of Bergström (6). Subsequently, the samples were frozen in liquid nitrogen and stored at -80° C until further analysis. After 1 h of rest, a glucose tolerance was assessed using a 75-g OGTT. A cannula was inserted in the antecubital vein for blood sampling and samples were drawn at respectively t = -10, 0, 10, 20, 30, 40, 50, 60, 90, 120 minutes after ingestion of the glucose drink. Serum was obtained, snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

Serum measurements

Commercially available enzymatic kits were used to measure serum concentrations of triacylglycerol, total cholesterol (Roche Diagnostics, Woerden, The Netherlands), NEFA (Wako Chemicals, Neuss, Germany) and glucose (Instruchemie, Delfzijl, The Netherlands). Insulin concentrations were measured using ELISA (Crystal Chem Inc., Elk Grove Village, IL, USA). The intra-assay coefficients of variability (CV) were 3.8%, 4.2%, 3.6%, 2.5% and 5.9% for triacylglycerol, total cholesterol, free fatty acids, glucose and insulin respectively. The inter-assay CVs were 5.3%, 4.0%, 7.9%, 4.7% and 7.1% for triacylglycerol, total cholesterol, free fatty acids, glucose and insulin respectively. Plasma catecholamines were measured in the laboratory of Vascular Medicine (Erasmus MC, Rotterdam, The Netherlands) using standard procedure. Aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, HbA1c and HDL-cholesterol were determined by the general hospital Laboratory of the Leiden University Medical Center and LDL-cholesterol was calculated using the Friedewald formula ($LDL; Total\ Cholesterol - HDL - (Triacylglycerol / 2.17)$) (7). In addition, lipid and lipoprotein composition

was quantified using high-throughput proton NMR metabolomics (Nightingale Health, Helsinki, Finland). This method provides simultaneous quantification of routine lipids, lipoprotein subclass profiling with lipid concentrations within 14 subclasses, fatty acid composition, and various low-molecular metabolites including amino acids, ketone bodies and gluconeogenesis-related metabolites in molar concentration units. The following components of the lipoprotein subclasses were quantified: phospholipids, triacylglycerol, total cholesterol, non-estrified cholesterol, and cholesteryl esters. The mean size for VLDL, LDL and HDL particles was calculated by weighting the corresponding subclass diameters with their particle concentrations (8). Details of the experimentation and applications of the NMR metabolomics platform have been described previously (9). Data were analysed using SoftMaxPro 5.4.1 software. For the analysis of the OGTT, the AUC was calculated using the trapezoidal rule (10). Incremental AUC was calculated by deducting the area below the baseline value from total AUCs. Insulin sensitivity was estimated using the Matsuda index (11). The insulinogenic index (IGI; $\Delta I_{0-30}/\Delta G_{0-30}$, where I is insulin and G is glucose) was used as a measure of early insulin secretion (12). The oral disposition index (DI_0 ; $[\Delta I_{0-30}/\Delta G_{0-30}]/\text{fasting insulin}$) was used to estimate beta cell function relative to the prevailing level of insulin resistance (13).

PET/CT scan analysis

$[^{18}\text{F}]$ FDG uptake by BAT, WAT and skeletal muscle was determined from the $[^{18}\text{F}]$ FDG PET/CT scan using Fiji ImageJ 1.51d (Beth Israel Deaconess Medical Center, Beth, Israel) (14) and analysed by two researchers (KJN, BMT) blinded to allocation. In the region of interest (ROI), the bilateral cervical and clavicular regions, mediastinal and paravertebral BAT areas were autocontoured using an set personalised standardised uptake value (SUVindiv) threshold with a tissue radiodensity between -190 and -10 Hounsfield units (15). SUVindiv threshold was calculated with the following formula: $1.2/\text{lean body mass (in kg)}/\text{body mass (in kg)}$, according to the latest expert panel recommendations (16). BAT metabolic volume (BMV) was measured in millilitres, BAT activity was reported in terms of SUV (the ratio of activity in kBq/mL within the ROI and the injected activity [kBq] per bodyweight [g]). Both SUV for the hottest single voxel (SUVmax) and mean SUV for all voxels within a BAT region (SUVmean) are reported. For WAT areas (subcutaneous and paracolic), skeletal muscles (sternocleidomastoid, longus colli, trapezius, deltoid, pectoralis major, psoas major, and gluteus maximus muscle) and reference tissues (liver, cerebellum and descending aorta), an SUV threshold was set at 0 and no Hounsfield units threshold was applied.

qRT-PCR analysis in skeletal muscle biopsies

Skeletal muscle biopsies were analysed for expression of genes involved in insulin signalling (*INSR*, *IRS1*), glucose metabolism (*GLUT4*), lipid metabolism (*ACACA*, *ACACB*, *ACSL1*,

CD36, FASN), and mitochondrial function (*CTP1α*, *CTP1β*, *CTP2*, *CYCS*, *DNM1L*, *MFN2*, *OPA1*, *PPARGC1α* [also known as *PPARGC1A*], *PPARGC1β* [also known as *PPARGC1B*], *UCP3*), as well as *DPP4* (*DPP4*) and Fibroblast Growth Factor (*FGF21*) using qPCR (Bio-Rad CFX96; Veenendaal, The Netherlands) (see **Supplementary Table 1** for primer sequences). Bio-Rad CFX Manager software version 3.1 (Bio-Rad Laboratories Inc, Hercules, CA, USA) was used for analysis and quantification. Biopsy material of five participants (two from placebo and three from sitagliptin group) was insufficient for reliable mRNA analysis. Expression levels were normalised using the mRNA content of the housekeeping gene β -Actin (*ACTB*) and expressed as fold change using the $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

Power calculations were made for the primary outcome measurement of BAT activity (SUVmean). On the basis of previous studies (17), we anticipated a 20% increase in BAT activity after Sitagliptin treatment. An SUVmean of 2.20 was expected in the control group and a SUVmean of 2.64 in the sitagliptin group after intervention, with a SD of 0.42. Assuming a bilateral alternative, we were able to detect differences of at least 20% in SUVmean with a power of more than 80% and an alpha of 0.05 in a group of 30 participants. Data was analysed using SPSS Statistics (version 23.0; IBM Corporation, Armonk, NY, USA). Data are shown as mean $\pm$ S.E.M, unless stated otherwise. Two-tailed unpaired Student's *t*-test was used to compare baseline characteristic between sitagliptin and placebo group. Mixed model analyses with treatment and occasion as fixed effects and subject specific deviances from the mean as random effects were used to assess the effect of the treatment. If the mixed model failed to converge a non-parametric paired test (Wilcoxon Signed-Rank Test) was used. Statistical results are shown with adjustment for multiple testing. Bonferroni corrected levels of significance are shown in the table and figure legends.

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Supplementary Tables

Supplementary Table 1. Primer sequences of forward and reverse primers for qRT-PCR

Gene	Forward primer	Reverse primer
<i>ACACA</i>	Ordered by Qiagen	Ordered by Qiagen
<i>ACACB</i>	Ordered by Qiagen	Ordered by Qiagen
<i>ACSL1</i>	Ordered by Qiagen	Ordered by Qiagen
<i>ACTB</i>	Ordered by Qiagen	Ordered by Qiagen
<i>CD36</i>	Ordered by Qiagen	Ordered by Qiagen
<i>CTP1α</i>	CCAGACGAAGAACGTGGTCA	ATCTTGCCGTGCTCAGTGAA
<i>CTP1β</i>	GGATGTTCAACACCACTCGG	TCTCCAGATCCTGAGGCTTGA
<i>CTP2</i>	CAGGCTGCCTATTCCAAACT	CCAGGGTCCCGAAATGTAGC
<i>CYCS</i>	CACTGCGGGAAGGTCTCTAC	TCAACATCTTGAGCCCCATGC
<i>DNM1L</i>	GTTGATCCAATTGGTGGCCT	CATGAACCAGTTCCACACAGC
<i>DPP4</i>	GCAGAATGTCCAGATGCCCT	GTGCTTGCAAGGTAAGTGCC
<i>FASN</i>	CTCCTTCTTCGGAGTCCACC	AGTGTGTGTTCTCGGAGTG
<i>FGF21</i>	GCAGCGGTACCTCTACACAG	GCACAGGAACCTGGATGTCT
<i>FIS1</i>	GTAAGGCATCGTGCTGCTC	ACGCCAGGTAGAAGACGTA
<i>GLUT4</i>	GGCTGGAGTCTGCTTCTGCAC	GCTGGTACATTGAACTGCAGCGA
<i>INSR</i>	GGGCAACGGCTCTTGACGG	CGGCCCATCTGGCTGCCTCTT
<i>IRS1</i>	TGTTTTTCGGAGCCTCCCTC	CCGCCACTTCTTCTCGTTCT
<i>MFN2</i>	GTGGCCCAACTCTAAGTGCC	CCAGCGTTGTTTCAGGATGA
<i>OPA1</i>	TGGACTACAGAGGATGGTGC	TGCCAGGTCTACTTTGGTCA
<i>PPARGC1α</i>	AGTGGTGCAGTGACCAATCA	CTGCTAGCAAGTTTGCTCA
<i>PPARGC1β</i>	AGCTTTGAGCAGACCTTGACA	TCGCTCTGGGTGCTTCTTTG
<i>UCP3</i>	GAAGGTCCGATTTCAGGCCA	GTAGGTACCACCTCAGCAC

Supplementary Table 2. Effect of sitagliptin treatment on body composition in overweight men with pre-diabetes

	Placebo (n=15)		Sitagliptin (n=14)	
	Week 0	Week 12	Week 0	Week 12
Total body mass (kg)	93.7 (1.6)	92.7 (1.4)	95.4 (3.1)	94.6 (2.9)
Fat mass (%)	30.1 (1.4)	29.6 (1.6)	29.7 (1.4)	29.4 (1.4)
Lean mass (%)	67.4 (1.3)	67.9 (1.5)	67.7 (1.4)	68.0 (1.4)
Bone mineral density (g/cm ³)	1.32 (0.03)	1.33 (0.03)	1.42 (0.04)	1.42 (0.04)

DEXA scanning was used to measure body composition at baseline (Week 0) and after twelve weeks (Week 12) of placebo (left, *n*=15) or sitagliptin (right, *n*=14) treatment. Data are presented as mean (standard error of the mean). Mixed model analysis was used for statistical comparison. Bonferroni corrected level of significance is 0.01 (alpha = 0.05 / 4).

Supplementary Table 3. Effect of sitagliptin treatment on skin temperature in overweight men with pre-diabetes

	Placebo (n=15)		Sitagliptin (n=14)	
	Week 0	Week 12	Week 0	Week 12
Delta mean skin temperature TN vs COLD (°C)	-5.5 (0.3)	-5.2 (0.3)	-5.6 (0.4)	-5.4 (0.3)
Delta proximal skin temperature TN vs COLD (°C)	-7.8 (0.6)	-7.6 (0.7)	-8.0 (0.8)	-7.5 (0.5)
Delta distal skin temperature TN vs COLD (°C)	-7.2 (0.7)	-6.3 (0.6)	-6.8 (0.7)	-6.0 (0.5)
Delta supraclavicular skin temperature TN vs COLD (°C)	-0.1 (0.2)	0.1 (0.1)	-0.1 (0.2)	-0.3 (0.4)

Data are presented as mean (standard error of the mean). TN; thermoneutral, before cooling. COLD; during mild cooling. Mixed model analysis was used for statistical comparison. Bonferroni corrected level of significance is 0.01 ($\alpha = 0.05 / 4$).

Supplementary Table 4. Effect of sitagliptin treatment on liver enzymes

	Placebo (n=15)		Sitagliptin (n=14)	
	Week 0	Week 12	Week 0	Week 12
Aspartate aminotransferase ($\mu\text{kat/l}$)	0.52 (0.03)	0.53 (0.03)	0.59 (0.11)	0.47 (0.04)
Alanine aminotransferase ($\mu\text{kat/l}$)	0.33 (0.03)	0.60 (0.08) **	0.38 (0.08)	0.48 (0.10)
γ -glutamyltransferase ($\mu\text{kat/l}$)	0.52 (0.06)	0.57 (0.08)	0.51 (0.08)	0.55 (0.09)

Data are presented as mean (standard error of the mean). Mixed model analysis was used for statistical comparison. ** $p < 0.01$ week 0 vs week 12, significant with Bonferroni corrected level of significance 0.01 ($\alpha = 0.05 / 3$).

Supplementary Table 5. Effect of sitagliptin on lipoprotein composition

	Placebo (n=15)		Sitagliptin (n=14)	
	Percentage	p-value	Percentage	p-value
Extremely large VLDL particles				
<i>Composition</i>				
Total Lipids	+12%	ns	-42%	<0.05
Phospholipids	+14%	ns	-47%	<0.05
Total cholesterol	+15%	ns	-48%	<0.05
Cholesterol esters	+17%	ns	-43%	0.05
Free cholesterol	+13%	ns	-54%	<0.05
Triacylglycerol	+11%	ns	-34%	<0.05
Very large VLDL particles				
<i>Composition</i>				
Total Lipids	+4%	ns	-46%	<0.05
Phospholipids	+6%	ns	-49%	<0.05
Total cholesterol	+6%	ns	-49%	<0.05
Cholesterol esters	+5%	ns	-46%	<0.05
Free cholesterol	+7%	ns	-53%	<0.05

Supplementary Table 5. Effect of sitagliptin on lipoprotein composition (continued)

	Placebo (n=15)		Sitagliptin (n=14)	
	Percentage	p-value	Percentage	p-value
Triacylglycerol	+3%	ns	-45%	<0.05
Large VLDL particles				
<i>Composition</i>				
Total Lipids	+14%	ns	-35%	<0.05
Phospholipids	+15%	ns	-35%	<0.05
Total cholesterol	+16%	ns	-35%	0.05
Cholesterol esters	+15%	ns	-30%	0.07
Free cholesterol	+17%	ns	-41%	<0.05
Triacylglycerol	+12%	ns	-34%	<0.05
Medium VLDL particles				
<i>Composition</i>				
Total Lipids	+4%	ns	-23%	<0.05
Phospholipids	+5%	ns	-22%	<0.05
Total cholesterol	+7%	ns	-20%	0.08
Cholesterol esters	+8%	ns	-16%	ns
Free cholesterol	+6%	ns	-26%	<0.05
Triacylglycerol	+4%	ns	-25%	<0.05
Small VLDL particles				
<i>Composition</i>				
Total Lipids	+6%	ns	-9%	ns
Phospholipids	+6%	ns	-7%	ns
Total cholesterol	+7%	ns	-2%	ns
Cholesterol esters	+8%	ns	+1%	ns
Free cholesterol	+6%	ns	-7%	ns
Triacylglycerol	+4%	ns	-15%	ns
Extra small VLDL particles				
<i>Composition</i>				
Total Lipids	+6%	ns	+4%	ns
Phospholipids	+6%	ns	+5%	ns
Total cholesterol	+7%	ns	+7%	ns
Cholesterol esters	+7%	ns	+6%	ns
Free cholesterol	+6%	ns	+7%	ns
Triacylglycerol	+5%	ns	-5%	ns
IDL particles				
<i>Composition</i>				
Total Lipids	+5%	ns	+4%	ns
Phospholipids	+4%	ns	+5%	0.08
Total cholesterol	+5%	ns	+6%	ns

Supplementary Table 5. Effect of sitagliptin on lipoprotein composition (continued)

	Placebo (n=15)		Sitagliptin (n=14)	
	Percentage	p-value	Percentage	p-value
Cholesterol esters	+5%	ns	+3%	ns
Free cholesterol	+4%	ns	+9%	<0.05
Triacylglycerol	+6%	ns	+2%	ns
Large LDL particles				
<i>Composition</i>				
Total Lipids	+4%	ns	+5%	ns
Phospholipids	+4%	ns	+4%	0.08
Total cholesterol	+4%	ns	+5%	ns
Cholesterol esters	+5%	ns	+4%	ns
Free cholesterol	+4%	ns	+7%	0.06
Triacylglycerol	+7%	ns	+4%	ns
Medium LDL particles				
<i>Composition</i>				
Total Lipids	+4%	ns	+4%	ns
Phospholipids	+4%	ns	+2%	ns
Total cholesterol	+4%	ns	+5%	ns
Cholesterol esters	+4%	ns	+6%	ns
Free cholesterol	+3%	ns	+4%	ns
Triacylglycerol	+6%	ns	+4%	ns
Small LDL particles				
<i>Composition</i>				
Total Lipids	+4%	ns	+4%	ns
Phospholipids	+3%	ns	+1%	ns
Total cholesterol	+4%	ns	+6%	ns
Cholesterol esters	+4%	ns	+7%	ns
Free cholesterol	+3%	ns	+5%	0.09
Triacylglycerol	+6%	ns	-5%	ns
Very large HDL particles				
<i>Composition</i>				
Total Lipids	+3%	ns	+9%	ns
Phospholipids	+1%	ns	+14%	<0.05
Total cholesterol	+4%	ns	+7%	ns
Cholesterol esters	+5%	ns	+6%	ns
Free cholesterol	+4%	ns	+9%	ns
Triacylglycerol	+12%	ns	-22%	0.09
Large HDL particles				
<i>Composition</i>				
Total Lipids	-10%	ns	+9%	ns

Supplementary Table 5. Effect of sitagliptin on lipoprotein composition (continued)

	Placebo (n=15)		Sitagliptin (n=14)	
	Percentage	p-value	Percentage	p-value
Phospholipids	-9%	ns	+8%	ns
Total cholesterol	-11%	ns	+10%	ns
Cholesterol esters	-10%	ns	+9%	ns
Free cholesterol	-10%	ns	+10%	ns
Triacylglycerol	-7%	ns	+15%	ns
Medium HDL particles				
<i>Composition</i>				
Total Lipids	-1%	ns	+2%	ns
Phospholipids	+1%	ns	+2%	ns
Total cholesterol	-1%	ns	+2%	ns
Cholesterol esters	-2%	ns	+1%	ns
Free cholesterol	-2%	ns	+7%	ns
Triacylglycerol	+5%	ns	+7%	ns
Small HDL particles				
<i>Composition</i>				
Total Lipids	-1%	ns	+1%	ns
Phospholipids	-1%	ns	-1%	ns
Total cholesterol	-1%	ns	+5%	0.09
Cholesterol esters	-1%	ns	+6%	0.06
Free cholesterol	-1%	ns	-1%	ns
Triacylglycerol	+3%	ns	-8%	ns

NMR was used to determine lipoprotein composition. Values are presented as percent increase (+) or decrease (-) from baseline (week 0). ns: not significant. Mixed model analysis was used for statistical comparison. Bonferroni corrected level of significance is 0.0006 ($\alpha = 0.05 / 84$).

Supplementary Table 6. Effect of sitagliptin on energy expenditure and substrate utilisation

	Placebo (n=15)		Sitagliptin (n=14)	
	Week 0	Week 12	Week 0	Week 12
REE (kJ/day)	4736 (138)	4858 (146)	4807 (163)	4845 (188)
REE corrected for LBM (kJ/day/kg LBM)	75.3 (1.7)	77.4 (1.3)	77.8 (1.3)	75.7 (2.1)
RQ	0.84 (0.01)	0.84 (0.01)	0.84 (0.01)	0.81 (0.02)
Lipid oxidation (g/min)	0.065 (0.005)	0.067 (0.006)	0.069 (0.018)	0.075 (0.005)
Glucose oxidation (g/min)	0.116 (0.004)	0.119 (0.004)	0.122 (0.004)	0.118 (0.006)
Non-shivering thermogenesis (%)	13 (5)	15 (5)	8 (2)	10 (4)

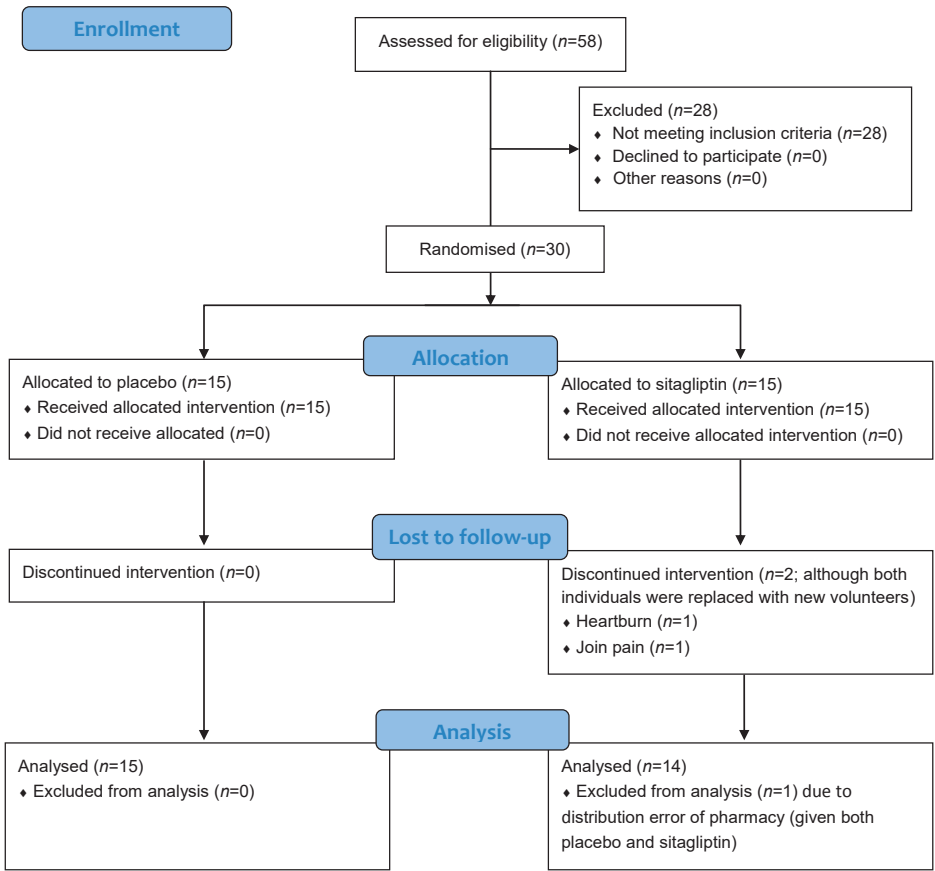
Data are presented as mean (standard error of the mean). Respiratory quotient (RQ) was calculated as $v\text{CO}_2/v\text{O}_2$ LBM: lean body mass, NST; non-shivering thermogenesis, REE: resting energy expenditure. Mixed model analysis was used for statistical comparison. Bonferroni corrected level of significance is 0.008 ($\alpha = 0.05 / 6$).

Supplementary Table 7. Effect of sitagliptin on [¹⁸F]FDG uptake in brown and white adipose tissue and skeletal muscle

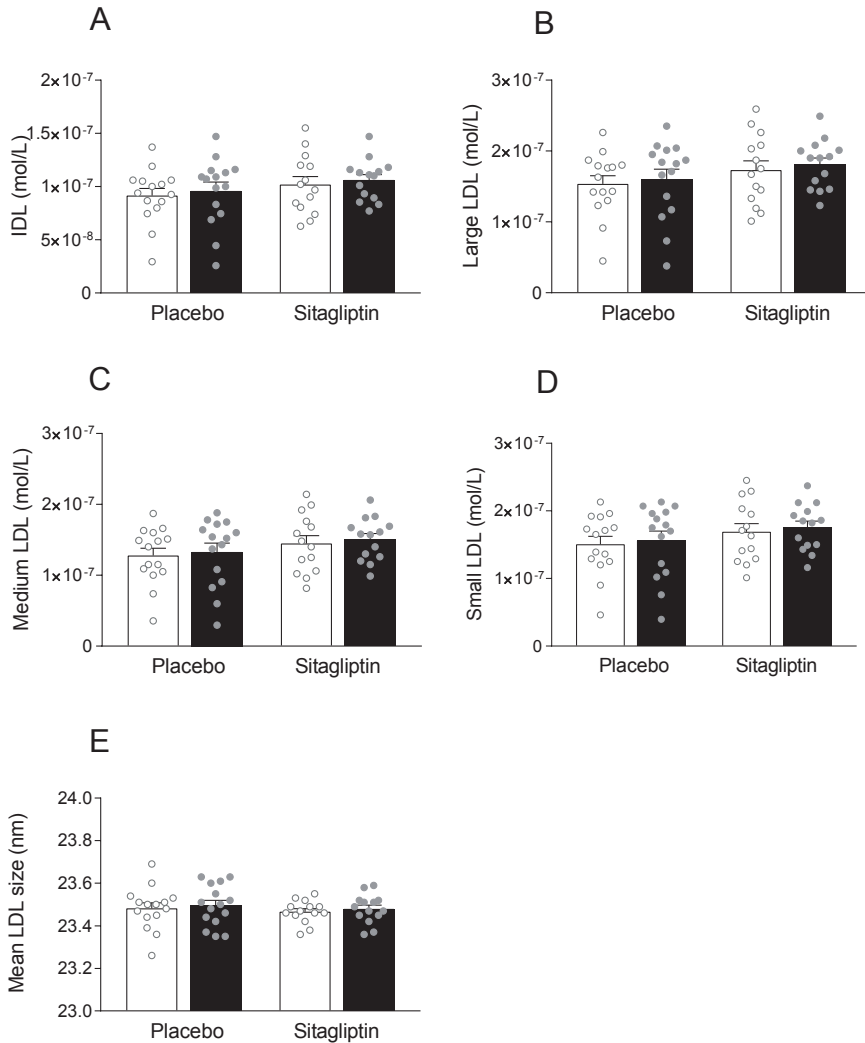
	Placebo (n=15)		Sitagliptin (n=13)	
	Week 0	Week 12	Week 0	Week 12
Brown adipose tissue depots				
<i>Classical BAT depots (cervical + supraclavicular)</i>				
Classical BMV (mL)	22.16 (8.75)	13.88 (4.67)	36.82 (13.17)	31.95 (13.09)
Classical BAT activity (SUV _{mean})	2.25 (0.11)	2.15 (0.08)	2.33 (0.14)	2.27 (0.12)
Classical BAT activity (SUV _{max})	3.94 (0.68)	2.60 (0.40)	4.78 (0.72)	4.43 (0.70)
<i>Total body BAT (cervical + supraclavicular + mediastinal + paravertebral)</i>				
Total BMV (mL)	32.85 (11.35)	25.11 (5.84)	54.25 (16.13)	47.47 (15.85)
Total BAT activity (SUV _{mean})	2.25 (0.09)	2.23 (0.08)	2.31 (0.13)	2.31 (0.12)
Total BAT activity (SUV _{max})	4.41 (0.66)	3.93 (0.38)	5.05 (0.67)	4.82 (0.62)
White adipose tissue depots (WAT)				
Subcutaneous (SUV _{mean})	0.22 (0.03)	0.26 (0.03)	0.19 (0.03)	0.29 (0.03) *
Paracolic (SUV _{mean})	0.53 (0.09)	0.59 (0.08)	0.59 (0.08)	0.58 (0.08)
Skeletal muscles				
m. Sternocleidomastoideus (SUV _{mean})	1.60 (0.20)	1.64 (0.15)	1.69 (0.33)	1.46 (0.15)
m. Longius colli (SUV _{mean})	2.20 (0.23)	2.63 (0.30)	2.33 (0.12)	2.42 (0.26)
m. Trapezius (SUV _{mean})	0.61 (0.05)	0.70 (0.03) *	0.67 (0.03)	0.67 (0.04)
m. Deltoideus (SUV _{mean})	0.55 (0.02)	0.61 (0.02)	0.63 (0.03)	0.61 (0.02)
m. Pectoralis major (SUV _{mean})	0.84 (0.05)	1.00 (0.08)	0.97 (0.18)	0.93 (0.08)
m. Psoas major (SUV _{mean})	0.96 (0.14)	1.04 (0.17)	0.76 (0.09)	0.91 (0.05)
m. Gluteus maximus (SUV _{mean})	0.56 (0.04)	0.56 (0.02)	0.56 (0.03)	0.60 (0.03)
Reference tissues				
Liver (SUV _{mean})	2.56 (0.12)	2.74 (0.08)	2.65 (0.10)	2.73 (0.09)
Cerebellum (SUV _{mean})	7.68 (0.50)	7.97 (0.36)	7.83 (0.39)	7.92 (0.31)
Descending aorta (SUV _{mean})	1.63 (0.09)	1.84 (0.07) *	1.75 (0.05)	1.78 (0.07)

Values are presented as mean (standard error of the mean). BAT: brown adipose tissue, BMV: BAT metabolic volume, SUV: standardised uptake value, WAT: white adipose tissue. Mixed model analysis was used for statistical comparison. * 0.002 < p < 0.05 week 0 vs week 12, not significant with Bonferroni corrected level of significance 0.002 (alpha = 0.05 / 18). In the Sitagliptin group one subject was claustrophobic and did not completed the PET/CT scan.

Supplementary Figures



Supplementary Figure 1. CONSORT flow diagram of the study.



Supplementary Figure 2. The effect of sitagliptin on serum IDL and LDL particle concentration in overweight men with prediabetes. Serum was collected before (open circles / white bars/ Week 0) and after (closed circles / black bars/ Week 12) 12 weeks of treatment with placebo ($n=15$) or sitagliptin ($n=14$). NMR was used to measure serum IDL (A), large- (B), medium- (C) and small-sized (D) LDL particle concentration. In addition, mean LDL particle size (E) was determined. Data are presented as mean $\pm$ S.E.M., and as individual measurements. Mixed model analysis was used for statistical comparison. Bonferroni corrected level of significance is 0.01 ($\alpha = 0.05 / 5$).

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 β 3-adrenergic receptor (β 3-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 pre-clinical 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-*d*2, 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-*d*2 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 administering 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 ANGPTL4, 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 ANGPTL3 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 β_3 -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-

tosteatosi 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 of 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**).

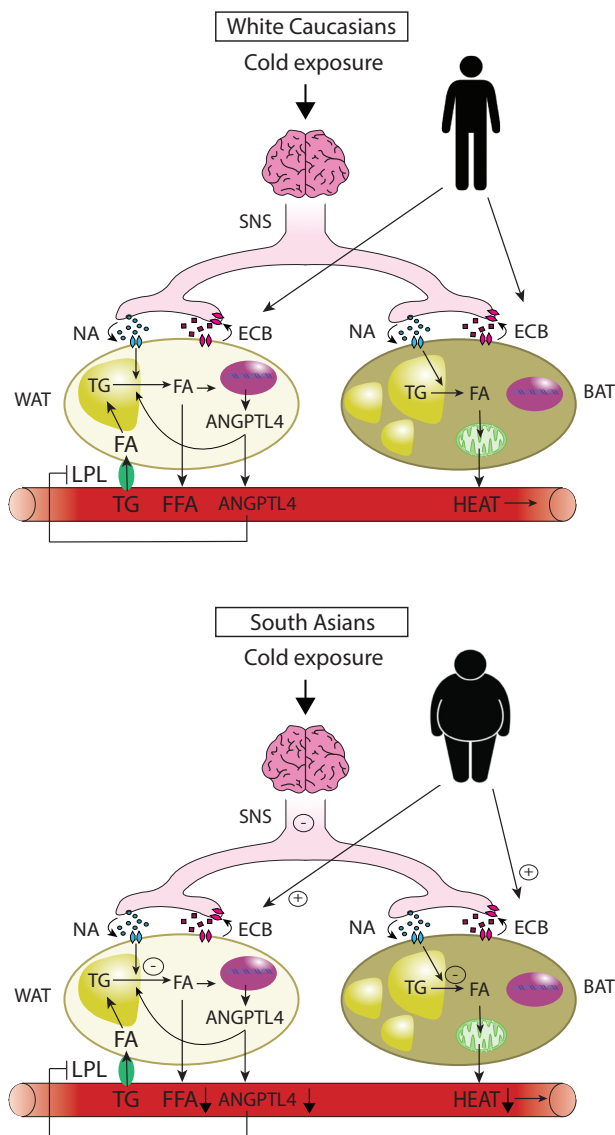


Figure 1. Hypothetical model explaining the link between body fat, endocannabinoids and sympathetic outflow Cold exposure enhances sympathetic outflow to peripheral tissues, including white adipose tissue (WAT) and brown adipose tissue (BAT). In WAT, sympathetic stimulation induces intracellular lipolysis 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 β 3-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.* β 3-AR agonism and DPP4 inhibition, which will be further discussed below.

Direct activation of BAT for the treatment of metabolic disease

Targeting the β 3-adrenergic receptor

In the past years, β 3-AR agonists gained attention as novel therapeutic tools to combat metabolic disease. Although β 3-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 β 3-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 β 3-AR as well as a less functional form of the β 3-AR which contains a missense mutation. This mutated form of the β 3-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 β 1- and β 2-adrenergic receptors. Although mirabegron is a relatively specific β 3-AR agonist, at high dose it activates the β 1- and β 2-adrenergic receptors. Interestingly, [^{18} F]FDG uptake by BAT might also partly be mediated through activation of β 1- and/or β 2- adrenergic receptors (56,57). Pre-treatment with the nonselective β 1/ β 2-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 β 2-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 β 2-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 β 3-adrenergic receptor agonists should be developed, if only to observe whether strict β 3-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 lead 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 be 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 of 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 DPP4 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, DPP4 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/m². 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/m². 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 ($\text{SUV}_{\text{individualized}} = 1.2/(\text{lean body mass}/\text{body mass})$) (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 and 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**.

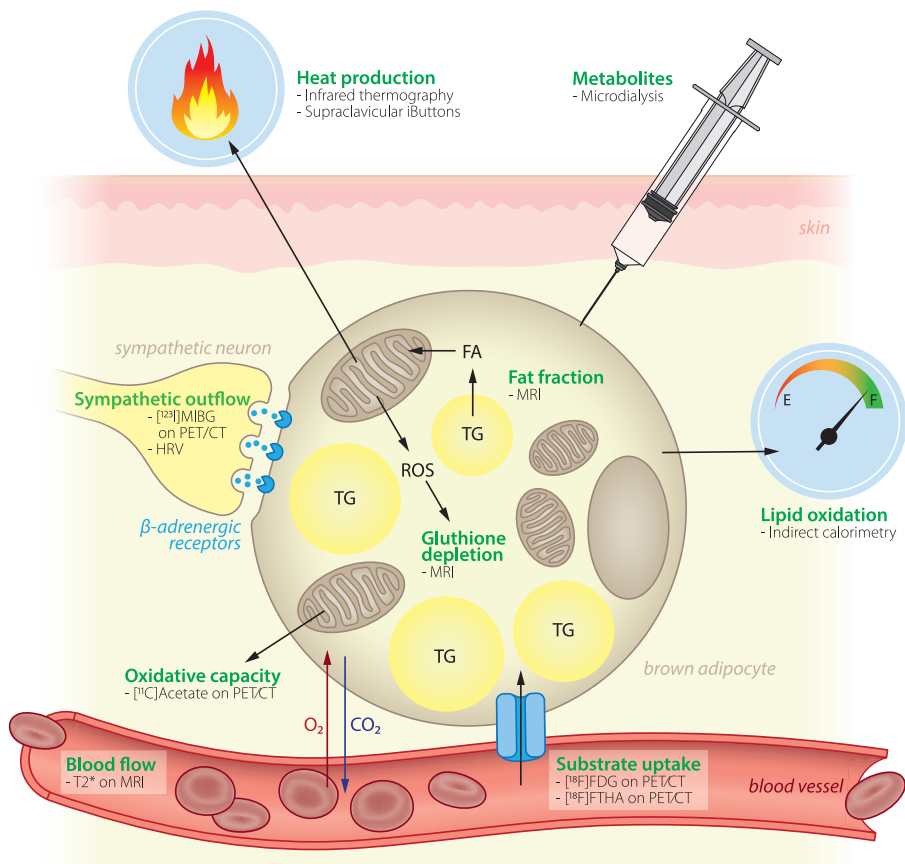


Figure 2. Direct and indirect read-outs of BAT activity. This figure summarises the currently available techniques to assess BAT activity directly and indirectly. CO₂, carbon dioxide; FA, fatty acids; [¹⁸F]FDG, [¹⁸F] fluorodeoxyglucose; [¹⁸F]FTHA, [¹⁸F]fluoro-6-thia-heptadecanoic acid; HRV, heart rate variability; [¹²³I]MIBG, [¹²³I]meta-iodobenzylguanidine; MRI, magnetic imaging resonance; O₂, 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 β 3-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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CHAPTER 9

Summary
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SUMMARY

'Diabesity', as the frequently occurring combination of obesity and diabetes is often referred to, is becoming a major global health care problem. The incidence of the obesity and of its related diseases, including type 2 diabetes (T2D) and cardiovascular diseases keep rising every year. South Asians are specifically at risk for the development of (cardio)metabolic disease, due to a combination of several known and unknown risk factors. Since effective long-term treatment strategies are currently lacking, the search for additional risk factors and development of targeted treatment strategies to combat these diseases is warranted. An attractive novel approach to combat metabolic disease seems to be activation of energy-combusting brown adipose tissue (BAT), which can result in increased energy expenditure, an improvement in glucose and lipid metabolism and possibly a reduction in the development of atherosclerosis, the main underlying cause of cardiovascular diseases. The physiological activator of BAT is cold exposure. However, modulation of BAT activation by means of cold exposure is far from optimal in humans. In this thesis, we aimed to address two key objectives: 1) unravelling the underlying mechanisms that could explain the increased predisposition for metabolic disease in the South Asian population, and 2) identifying novel treatment strategies that activate BAT and increase energy expenditure in risk populations, including South Asians and individuals with overweight and prediabetes. **Chapter 1** serves as a general introduction into the pathophysiology of obesity and related conditions such as T2D and cardiovascular diseases. In addition, the metabolic phenotype and some known risk factors for the development of metabolic disease in the South Asian population are described. Finally, the physiology of BAT is explained as well as its therapeutic potential as a target drug to combat metabolic disease.

In the first part of this thesis, we investigated different factors that may contribute to the increased predisposition for metabolic disease in South Asians compared to white Caucasians. South Asians were previously shown to have a lower resting energy expenditure (REE) as related to less energy-combusting BAT. Since over-activation of the endocannabinoid system is associated with obesity and low BAT activity, we hypothesized that South Asians have a higher endocannabinoid tone. Therefore, in **Chapter 2**, we aimed to investigate differences in circulating endocannabinoid levels between South Asians and white Caucasians. To this end, ten healthy lean white Caucasian and ten South Asian men, aged 18-28 years, were included. REE was assessed and plasma was collected for analysis of endocannabinoids and lipids. We found that, South Asians had higher plasma levels of 2-arachidonoylglycerol (2-AG), N-arachidonyl ethanolamine (AEA) and arachidonic acid (AA) compared with white Caucasians. After pooling of both ethnicities, plasma 2-AG, but not AEA, positively correlated with plasma triglycerides

and body fat percentage. Interestingly, AA negatively correlated with REE and positively with body fat percentage. Based on these data, we concluded that healthy lean South Asian men, compared to white Caucasian men, have a higher endocannabinoid tone. Given that endocannabinoids attenuate noradrenergic signalling, these data suggest that endocannabinoids may, at least in part, underlie the disadvantageous metabolic phenotype of South Asians later in life.

Since endocannabinoid tone is a reflection of local endocannabinoid regulation in metabolic organs, we next focussed on potential differences in local endocannabinoid signalling between ethnicities. Therefore, in **Chapter 3**, we aimed to investigate whether, next to circulating endocannabinoid levels, also gene expression of endocannabinoid (CB) receptors and enzymes involved in endocannabinoid synthesis and degradation in white adipose tissue (WAT) and skeletal muscle differed between middle-aged overweight, prediabetic men of South Asian and white Caucasian descent. We demonstrated that, compared to overweight prediabetic white Caucasians, overweight prediabetic South Asians had higher levels of the plasma endocannabinoids 2-linoleoyl glycerol and *N*-linoleoylethanolamine. Interestingly, in skeletal muscle of South Asians, expression of the cannabinoid receptors CB₁ and CB₂ was 10-fold lower and that of the endocannabinoid degradation enzyme FAAH2 was 5-fold lower compared to white Caucasians. Expression of genes involved in the endocannabinoid system in WAT was not different between the two ethnicities. After pooling of both ethnicities, plasma 2-AG positively correlated with plasma triglycerides and lipid oxidation. These data support our findings in chapter 2 that South Asians have a higher endocannabinoid tone compared to white Caucasians, which might possibly due to a endocannabinoid deregulation in skeletal muscle. Further studies are required to show if therapeutic interventions that target the endocannabinoid system will improve the metabolic profile of these individuals, and whether South Asians respond differently than white Caucasians.

Next, we switched our focus from the endocannabinoid system to angiopoietin-like 4 (ANGPTL4). Previously, we showed that the free fatty acid (FFA) release upon short-term cooling is lower in South Asians compared to white Caucasians. 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) and increases intracellular lipolysis. Therefore, in **Chapter 4** now also investigated the effect of short-term cooling on serum ANGPTL4 levels in healthy lean men of white Caucasian and South Asian descent. We showed that short-term cooling increased ANGPTL4 levels. The relative increase in ANGPTL4 levels upon cooling was higher in white Caucasians compared to South Asians. In addition, thermoneutral ANGPTL4 levels positively correlated with FFA levels and body fat percentage. Further-

more, ANGPTL4 negatively correlated with REE. In conclusion, our data demonstrate that short-term mild cooling increases circulating ANGPTL4 levels in healthy lean men, with a higher response in white Caucasian compared to South Asian men. As underlying mechanism we speculate that cold exposure causes intracellular lipolysis in white adipocytes to liberate FA that, via activation of peroxisome proliferator-activated receptor γ (PPAR γ), increase ANGPTL4 synthesis to limit uptake of triglyceride-derived FA by this tissue. Possibly, this sequence of events is blunted in South Asians because of reduced sympathetic signalling, which would be an interesting topic for future research.

During mild cold exposure, non-shivering thermogenesis increases to maintain core body temperature by increasing utilization of substrates, especially FA, ultimately affecting lipid-associated metabolites. In **Chapter 5**, we aimed to investigate whether mild cooling induces changes in other metabolites and whether the metabolite response differs between healthy lean white Caucasian and South Asian men. First, we showed that the effect of mild cooling on 190 measured metabolites was not different between both ethnicities. Therefore, to assess the effect of cooling on metabolites, we pooled the data. After Bonferroni correction, mild cooling significantly changed 44 of 190 metabolic parameters. Specifically, cooling increased 19 phosphatidylcholine (PC) species, only those containing very long chain FAs, and increased the total class of PC containing mono-unsaturated FAs. Furthermore, cooling increased 10 sphingomyelin species as well as the amino acids glutamine, glycine and histidine, and decreased short-chain (C3 and C4) acylcarnitines. In conclusion, mild cooling elicits substantial effects on serum metabolites in healthy males, irrespective of white Caucasian or South Asian ethnicity. Future studies should focus on the physiological relevance of this change in metabolome.

In the second part of this thesis, we focussed on two promising pharmacological strategies to activate BAT and investigated these strategies in risk populations (e.g. South Asians and individuals with overweight and prediabetes). In **Chapter 6**, we aimed to study the effect of the β 3-adrenergic receptor agonist mirabegron on REE and BAT in healthy lean South Asian and white Caucasian men. To this end, we performed a randomized, double-blinded, cross-over study consisting of three different interventions; short-term individualized cooling protocol, placebo (once p.o.) and mirabegron (200 mg once p.o.) treatment. We showed that both mirabegron and cold exposure increased FFA, REE, lipid oxidation and supraclavicular skin temperature, and tended to decrease BAT FF, which might be suggestive for activated BAT. We did not observe clear differences in metabolic response between the two ethnicities. Based on these data we concluded that mirabegron increases REE and activates BAT in both ethnicities similarly. However, additional studies should be aimed at unravelling the relative effect of both treatments

on true BAT activity, for instance by using alternative read-outs for BAT activity such as glucose or lipid uptake, or oxidative capacity.

Next to a β 3-adrenergic receptor agonist, dipeptidyl peptidase-4 (DPP4) inhibitors might also be a promising class of drugs to investigate in the context of BAT activation. In **Chapter 7**, we assessed the effect of the DPP4 inhibitor sitagliptin on energy metabolism and BAT in overweight prediabetic men. Currently, there is only little attention for identification of therapies that improve glucose and lipid metabolism in these individuals. This may, however, be highly relevant since most of these individuals eventually proceed to overt diabetes. Therefore, the aim of this study was to evaluate the effect of sitagliptin on glucose tolerance, plasma lipids, energy expenditure and metabolism of BAT, WAT and skeletal muscle. To this end, thirty overweight, prediabetic white Caucasian men received sitagliptin (100 mg/day) (n=15) or placebo (n=15) for twelve weeks. We demonstrated that sitagliptin, but not placebo, lowered glucose excursion during OGTT, accompanied by an improved insulinogenic index and oral disposition index. In addition, sitagliptin lowered serum triglycerides, and very large-, large- and medium-sized very-low-density lipoproteins. In skeletal muscle, sitagliptin increased mRNA expression of *PGC1 β* , a main controller of mitochondrial oxidative energy metabolism. Furthermore, sitagliptin increased [^{18} F]FDG uptake in subcutaneous WAT but not in BAT or muscle. From this study, we concluded that twelve weeks of sitagliptin in overweight, prediabetic white Caucasian men improves glucose tolerance and lipid metabolism, as related to increased [^{18} F]FDG uptake by subcutaneous WAT rather than BAT and upregulation of the mitochondrial gene *PGC1 β* in skeletal muscle.

Finally, in **Chapter 8** we evaluated the results of the studies described in this thesis and discussed their implications for future research. In this final chapter, we highlighted some novel factors that might, at least in part, contribute to the increased risk of the South Asian population for developing metabolic disease, and speculated about the underlying mechanisms. Furthermore, we discussed novel treatment strategies that might activate BAT, thereby modulating REE, lipid and glucose metabolism and thus potentially serve as a novel treatment strategy to combat metabolic disease. In addition, challenges in current research into BAT will be discussed as well as novel techniques to assess BAT activity in human. The clinical value of BAT activation in common clinical practice and its implications for the treatment for obesity, T2D and cardiovascular diseases in humans probably will become clear in the forthcoming years.

NEDERLANDSE SAMENVATTING

Ernstig overgewicht, ofwel obesitas, is momenteel wereldwijd één van de grootste gezondheidsproblemen. Obesitas ontstaat door overmatige opslag van suiker en vet als 'triglyceriden' in vetweefsel. Men spreekt van obesitas als de *body mass index* (BMI), berekend als het gewicht gedeeld door de lengte in het kwadraat, hoger is dan 30 kg/m². Sinds 1980 is het aantal mensen met obesitas verdubbeld tot maar liefst 12% van de wereldbevolking in 2015 (604 miljoen mensen). Naar verwachting zal dit aantal de komende jaren blijven stijgen. Obesitas gaat vaak gepaard met een situatie waarin organen niet meer gevoelig zijn voor het hormoon insuline (zogenaamde 'insulineresistentie'), wat leidt tot suikerziekte ('diabetes'), een conditie waarin het bloedsuiker verhoogd is. Insulineresistentie leidt ook tot ophoping van triglyceriden en cholesterol in het bloed (zogenaamde 'dyslipidemie'), waardoor obesitas vaak samengaat met slagaderverkalking ('atherosclerose'), de belangrijkste oorzaak van hart- en vaatziekten.

De huidige behandelstrategieën voor obesitas zoals calorische beperking en bewegen zijn vaak niet effectief op de lange termijn met uitzondering van sommige operaties die tot doel hebben om gewicht te verminderen ('bariatrische chirurgie'), zoals maagverkleining of een zogenaamde 'bypass'. Daarnaast verschilt de incidentie van obesitas per bevolkingsgroep. Zo heeft de Hindoestaanse populatie een verhoogd risico op obesitas en gerelateerde ziekten waaronder diabetes en hart- en vaatziekten ten opzichte van West-Europeanen. Waarom Hindoestanen hier een verhoogd risico op hebben is niet volledig bekend. Door de ontstaansmechanismen van obesitas in de diverse etnische groepen beter te begrijpen kunnen er specifieke behandelstrategieën ontwikkeld worden in de strijd tegen obesitas en gerelateerde ziekten.

In het lichaam zijn twee typen vetcellen te onderscheiden: witte en bruine vetcellen. Witte vetcellen zorgen voor de opslag van triglyceriden terwijl bruine vetcellen juist triglyceriden kunnen verbranden voor de productie van warmte ('thermogenese'). Lange tijd werd gedacht dat alleen pasgeborenen over bruin vet beschikken voor het in stand houden van de lichaamstemperatuur. Echter, in 2009 werd ontdekt dat volwassen ook nog bruin vet hebben (ongeveer 200-300 gram), en dat dit door blootstelling aan kou wordt geactiveerd wat resulteert in een toename in het energieverbruik. Daarom wordt bruin vet momenteel beschouwd als een interessant aangrijpingspunt om obesitas en gerelateerde ziekten te behandelen. Bij blootstelling aan kou wordt in de hersenen een signaal opgewekt dat via het stimuleren van zenuwen bruine vetcellen aanzet tot warmteproductie. Belangrijk hierbij is dat door zenuwuiteinden het hormoon noradrenaline wordt uitgescheiden dat vervolgens bindt aan zogenaamde β 3-adrenerge receptoren op de bruine vetcellen. Alhoewel bruin vet dus geactiveerd kan worden door kou, is blootstelling aan kou geen prettige behandelstrategie om de hoeveelheid lichaamsvet

in mensen te doen verminderen. Wereldwijd zijn onderzoekers dan ook naarstig op zoek naar andere manieren om bruin vet te activeren, bijvoorbeeld door geneesmiddelen.

De studies waarvan de resultaten in dit proefschrift zijn beschreven, hadden tot doel om 1) nieuwe verklaringen te vinden waarom Hindoestanen een verhoogd risico hebben op de ontwikkeling van obesitas en obesitas-gerelateerde ziekten, en 2) nieuwe behandelstrategieën te onderzoeken om bruin vet te activeren en het energieverbruik kunnen verhogen in bevolkingsgroepen met een verhoogd risico op obesitas en gerelateerde ziekten, waaronder Hindoestanen en mensen met overgewicht en een voorstadium van diabetes ('prediabetes'). **Hoofdstuk 1** fungeert als een algemene inleiding waarin de ontstaanswijze van obesitas, diabetes en hart- en vaatziekten wordt uitgelegd. Daarnaast worden het metabole fenotype en de reeds bekende risicofactoren voor de ontwikkeling van obesitas en gerelateerde ziekten van de Hindoestaanse bevolking beschreven. Tenslotte wordt de werking van bruin vet uitgelegd alsmede de mogelijke rol van bruin vet in de behandeling van obesitas en gerelateerde ziekten.

In het eerste deel van dit proefschrift onderzochten we verschillende factoren die mogelijk het verhoogde risico op het ontstaan van obesitas en obesitas-gerelateerde ziekten in Hindoestanen mede verklaren. Het was al bekend dat hoge bloedconcentraties van endocannabinoïden, lichaamseigen stoffen die de noradrenaline signalering kunnen remmen, geassocieerd zijn met obesitas en met een lage activiteit van bruin vet. Tevens is uit eerder onderzoek gebleken dat Hindoestanen een lager energieverbruik in rust ('ruststofwisseling') en minder bruin vet hebben dan West-Europeanen. Op basis van deze gegevens vormden wij de hypothese dat Hindoestanen hogere plasmaconcentraties van endocannabinoïden hebben dan West-Europeanen, wat we vervolgens onderzochten in **Hoofdstuk 2**. Hiertoe includeerden wij tien jonge, gezonde, slanke Hindoestaanse mannen tussen de 18 en 28 jaar en tien West-Europese mannen van dezelfde leeftijd en BMI. Het energieverbruik werd gemeten en bloedplasma werd verzameld om endocannabinoïden en lipiden te meten. We toonden aan dat Hindoestanen, vergeleken met West-Europeanen, hogere plasmaniveaus hadden van de endocannabinoïden 2-arachidonoylglycerol (2-AG), N-arachidonylethanolamine (AEA) en arachidonzuur. Tevens bleek er een positief verband te bestaan tussen plasma 2-AG niveaus en plasma triglyceridenniveaus en tussen plasma 2-AG niveaus en het lichaamsvetpercentage. Daarnaast was er een negatief verband tussen het plasmaniveau van arachidonzuur en het energieverbruik en een positief verband tussen het plasmaniveau van arachidonzuur en het lichaamsvetpercentage. Aangezien endocannabinoïden de noradrenalinesignalering remmen zouden de verhoogde plasma endocannabinoid levels mogelijk kunnen bijdragen aan het ontstaan van het ongunstige metabole fenotype van Hindoestanen op latere leeftijd.

Omdat endocannabinoïden in verschillende organen worden geproduceerd en vervolgens aan het bloed worden afgegeven, vroegen wij ons af in welke mate de diverse metabole organen bijdragen aan het plasmaniveau van endocannabinoïden. Om die reden hebben wij in **Hoofdstuk 3**, naast endocannabinoïdenniveaus in het plasma, ook de regulering van endocannabinoïden in wit vet en spierweefsel bestudeerd. Tien Hindoestaanse mannen tussen de 35 en 50 jaar met overgewicht en prediabetes en tien West-Europese mannen van dezelfde leeftijd en BMI, werden geïnccludeerd in deze studie. Wederom werd het energieverbruik gemeten en bloedplasma verzameld om endocannabinoïden en lipiden te meten. Daarnaast werden er bipten genomen van wit vet en spierweefsel om genexpressie van de endocannabinoïdreceptoren en de expressie van genen die betrokken zijn bij de productie en afbraak van endocannabinoïden te meten. Wij toonden aan dat Hindoestanen hogere niveaus hadden van de endocannabinoïden 2-linoleoyl glycerol en *N*-linoleoylethanolamine in bloedplasma vergeleken met West-Europeanen. Daarnaast was de genexpressie van de endocannabinoïdreceptoren CB₁ en CB₂ in spierweefsel veel lager in Hindoestanen vergeleken met West-Europeanen. Ook was de genexpressie van het enzym FAAH2 dat betrokken is bij de afbraak van endocannabinoïden veel lager in Hindoestanen. In wit vet was er geen verschil in de genexpressie van de endocannabinoïdreceptoren of de expressie van genen die betrokken zijn bij de synthese en afbraak van endocannabinoïden tussen de etniciteiten. Tevens bleek er een positief verband te bestaan tussen 2-AG niveaus in het bloedplasma en zowel triglyceridenniveaus als de vetverbranding. Deze resultaten ondersteunen onze bevindingen in Hoofdstuk 2, namelijk dat Hindoestanen hogere endocannabinoïdenconcentraties in het bloedplasma hebben dan West-Europeanen. Mogelijk kan dit verklaard worden door een verlaagde afbraak van endocannabinoïden in de spier. Aanvullende studies zijn nodig om te onderzoeken of Hindoestanen mogelijk baat hebben bij behandelingen die het endocannabinoïdensysteem beïnvloeden.

Vervolgens verplaatsten wij onze focus van het endocannabinoïdensysteem naar angiopoietine-achtige eiwitten, specifiek het angiopoietine-achtig eiwit 4 (ANGPTL4). Eerder onderzoek heeft laten zien dat blootstelling aan kou de vrije vetzuurniveaus in het bloedplasma minder doet stijgen in Hindoestanen vergeleken met West-Europeanen. Vrije vetzuren kunnen peroxisoomproliferatorgeactiveerde receptoren activeren die betrokken zijn bij de synthese van ANGPTL4. ANGPTL4 remt de lipolytische activiteit van lipoproteïne lipase, en daarmee de opname van vrije vetzuren door organen vanuit triglyceriden uit het bloed. In **Hoofdstuk 4** bestudeerden wij het effect van kortdurende blootstelling aan milde kou op het serumniveau van ANGPTL4 in jonge, gezonde Hindoestaanse en West-Europese mannen. Wij toonden aan dat ANGPTL4 niveaus stegen na blootstelling aan milde kou. De toename in ANGPTL4 was minder groot in Hindoestanen vergeleken met West-Europeanen. Daarnaast was er een positief verband tussen thermoneutrale ANGPTL4 niveaus en vrije vetzuurniveaus in het serum en met

het lichaamsvetpercentage. ANGPTL4 niveaus lieten een negatief verband zien met het energieverbruik. Als onderliggend mechanisme speculeren wij dat blootstelling aan kou intracellulaire lipolyse in wit vet stimuleert, wat resulteert in vrijmaking van vetzuren en ten gevolge daarvan een verhoging van vrije vetzuren in het bloed. Intracellulair kunnen vrije vetzuren via peroxisoomproliferatorgeactiveerde receptoren de aanmaak van ANGPTL4 stimuleren dat vervolgens de opname van vrije vetzuren vanuit triglyceriden vanuit het bloed remt. De efficiëntie van dit proces is lager in Hindoestanen, mogelijk door verminderde sympathische signalering.

Tijdens blootstelling aan kou neemt de warmteproductie toe om de lichaamstemperatuur op peil te houden. Hierbij worden vetten verbrand, wat resulteert in de vorming van afbraakproducten ('metabolieten'). In **Hoofdstuk 5** onderzochten wij hoe kortdurende milde blootstelling aan kou 190 metabolieten in het bloed beïnvloedt en of deze respons verschilt tussen Hindoestanen en West-Europeanen. Hoewel milde kou effecten had op meerdere metabolieten, was de respons niet verschillend tussen de twee etniciteiten. Na het samenvoegen van de data van beide etniciteiten, konden wij aantonen dat blootstelling aan kou 44 metabolieten beïnvloedde. Met name de fosphatidylcholines met lange vetzuurketens en sphingomyelines namen toe na kou. Daarnaast verhoogde kou glutamine, glycine en histidine en verlaagde het de korte-keten acylcarnitines (C2 en C4). Kortdurende blootstelling aan milde kou induceert dus substantiële veranderingen in de serum metabolieten maar deze effecten zijn onafhankelijk van de vergeleken etniciteiten. Toekomstig onderzoek zal moeten uitwijzen wat de fysiologische relevantie is van deze veranderingen in niveaus van metabolieten in het serum in respons op kou.

In het tweede deel van dit proefschrift bestudeerden wij twee veelbelovende farmacologische strategieën om bruin vet te activeren in Hindoestanen en mensen van West-Europese afkomst met overgewicht en prediabetes, beiden risicopopulaties voor het ontwikkelen van diabetes en hart- en vaatziekten. In **Hoofdstuk 6** onderzochten wij het effect van mirabegron, een specifieke agonist van de β_3 -adrenerge receptor die in de fysiologische situatie door stimulatie van het centraal zenuwstelsel wordt geactiveerd, op het energieverbruik en bruin vet in 20 jonge, gezonde Hindoestaanse en West-Europese mannen. Zij participeerden in een gerandomiseerde, dubbelblinde, cross-over studie die bestond uit drie verschillende interventies; kortdurende milde kou, een enkele dosis mirabegron (200 mg) en een placebo behandeling. Wij toonden aan dat zowel blootstelling aan kou als mirabegron resulteerden in een toename van vrije vetzuren in het bloed, het energieverbruik, de vetverbranding en de supraclaviculaire huidtemperatuur. Daarnaast leken beide strategieën het percentage vet in bruin vet te verlagen. Aangezien actief bruin vet zijn intracellulaire vetten verbrandt, suggereert de afname van de intracellulair vetfractie activatie van bruin vet. Er was echter geen verschil in de metabole respons tussen de ethniciteiten. Aanvullend onderzoek moet

uitwijzen of kou en mirabegron ook de vetopname en oxidatieve capaciteit van bruin vet verhogen.

Remmers van dipeptidylpeptidase-4 (DPP4), die de beschikbaarheid van het hormoon glucagonachtige peptide-1 (GLP-1) verhogen, activeren bruin vet in muizen. Om te onderzoeken of DPP4 remming ook bruin vet activeert in mensen bestudeerden wij in **Hoofdstuk 7** het effect van de DPP4 remmer sitagliptine op de bruin vet activiteit in West-Europese mannen met overgewicht en prediabetes. Daarnaast onderzochten wij ook het effect van sitagliptine op de suiker- en vetstofwisseling en de spierstofwisseling in deze populatie. Dertig West-Europese mannen met overgewicht en prediabetes participeerden in een dubbelblinde, gerandomiseerde, placebo-gecontroleerde studie waarin zij gedurende 12 weken sitagliptine (100 mg/dag) ofwel placebo toegediend kregen. Voor en na de 12-weekse behandeling werden er bloedmonsters afgenomen voor bepaling van suiker, insuline en lipiden en werden het energieverbruik en de suiker- en vetverbranding gemeten. Ook werd de opname van een suikerlabel door bruin vet, wit vet en spier gemeten door middel van een PET-CT scan, en werd een orale glucose tolerantie test verricht en een spierbiopsie afgenomen. Wij toonden aan dat sitagliptine, ten opzichte van placebo, de gevoeligheid voor suiker verbeterde. Daarnaast verlaagde sitagliptine de triglyceridenniveaus in plasma. In de spierbiopsies verhoogde sitagliptine de expressie van *PGC1 β* , een gen dat betrokken is bij de verbranding in de mitochondriën. Daarnaast verhoogde sitagliptine de opname van het suikerlabel in wit vet, maar niet in bruin vet en spier. Hieruit concludeerden wij dat sitagliptine een gunstig effect heeft op de suiker- en vetstofwisseling in mannen met overgewicht en prediabetes en dat deze effecten mechanistisch mogelijk verklaard kunnen worden door ‘verbruining’ van het witte vet en/of een verbeterde oxidatieve capaciteit van de mitochondriën in de spier.

Tot slot werden de belangrijkste resultaten van de studies die beschreven zijn in dit proefschrift geëvalueerd in **Hoofdstuk 8**. Tevens werden de implicaties voor toekomstige onderzoeken besproken. Enkele nieuwe factoren worden benadrukt die deels kunnen bijdragen aan het verhoogde risico op obesitas en obesitas-gerelateerde ziekten in de Hindoestaanse populatie en er wordt gespeculeerd over onderliggende mechanismes. Ook komen er nieuwe mogelijke behandelstrategieën aan bod die bruin vet kunnen activeren en daarmee ook het energieverbruik en de suiker- en vetstofwisseling kunnen moduleren om daarmee obesitas en obesitas-gerelateerde ziekten te behandelen. Tot slot worden enkele methodologische uitdagingen in het huidige onderzoek naar bruin vet besproken, alsmede enkele nieuwe uitleesmaten voor de activiteit van bruin vet in mensen. Wat de klinische waarde is van bruin vetactivatie in de kliniek voor de behandeling van obesitas, type 2 diabetes en hart- en vaatziekten zal waarschijnlijk duidelijk worden in de komende jaren.

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CURRICULUM VITAE

Kimberly Jessica Nahon werd geboren op 31 oktober 1987 te Voorburg. Zij groeide op in Zoetermeer met haar ouders, zus Joya en broer Dennis. Haar VWO diploma behaalde zij in 2006 aan het Alfrink College te Zoetermeer. Vervolgens begon zij aan de bachelor Biomedische Wetenschappen aan de Universiteit van Leiden.

Tijdens haar bachelorstage deed zij onderzoek naar Ewing en Osteosarcoom onder leiding van dr. Dagmar Berghuis en dr. Emmeline Buddingh binnen het Immunologisch laboratorium van de Kindergeneeskunde. In 2009 behaalde zij haar bachelordiploma Biomedische Wetenschappen. In datzelfde jaar nam zij deel aan het schakeltraject 'Journey into Medicine' om toegelaten te worden tot de bachelor Geneeskunde. In 2011 behaalde zij tevens haar bachelordiploma voor Geneeskunde, waarna zij in de daarop volgende jaren de master Biomedische wetenschappen en Geneeskunde combineerde.

In het kader van de master Biomedische wetenschappen deed zij twee onderzoeksstages: eerst participeerde zij in klinisch onderzoek naar de interactie tussen alcohol en een nieuw medicament in het Center for Human Drug Research (CHDR) te Leiden, onder supervisie van Erik te Beek en prof. dr. Joop van Gerven. Vervolgens deed zij preklinisch onderzoek naar het effect van salsalaat op de activiteit van bruin vet binnen de afdeling Interne Geneeskunde van het Leids Universitair Medisch Centrum onder supervisie van dr. Andrea van Dam, dr. Mariëtte Boon en prof. dr. Patrick Rensen. Voor haar onderzoek verricht tijdens deze stage ontving zij in 2015 de Hippocrates Studiefonds Award. In het kader van de master Geneeskunde liep zij een deel van haar coschappen in het buitenland. Het coschap Kindergeneeskunde deed zij in Suriname en het coschap Oogheelkunde in Nepal. Haar semi-artsstage deed zij op de afdeling Kindergeneeskunde van het Alrijne ziekenhuis te Leiderdorp. In augustus 2014 behaalde zij haar master diploma voor Geneeskunde en Biomedische wetenschappen (*cum laude*).

In september 2014 keerde zij terug naar de afdeling Interne Geneeskunde van het Leids Universitair Medisch Centrum waar zij, onder supervisie van prof. dr. Patrick Rensen, dr. Mariëtte Boon en dr. Ingrid Jazet, startte met haar promotieonderzoek. In 2014 won zij de prof. dr. J. Terpstra Young Investigator Award van de Nederlandse Vereniging voor Diabetes Onderzoek (NVDO) voor beste onderzoeksvoorstel op het gebied van Diabetes. Daarnaast ontving zij in 2015 een reisstipendium van de Nederlandse Vereniging voor Farmacologie (NVF) voor het bijwonen van een congres in Snowbird (Amerika). In 2016 ontving zij een Young Investigator Fellowship Award van de European Atherosclerosis Society (EAS). Het promotieonderzoek, waarvan de resultaten zijn beschreven in dit proefschrift, werd afgerond in juli 2018. In augustus van dat jaar startte Kimberly als arts-niet-in-opleiding (ANIOS) op de afdeling Kindergeneeskunde in het Alrijne ziekenhuis te Leiderdorp.

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